
# 16th EAFP INTERNATIONAL CONFERENCE ON DISEASES OF FISH AND SHELLFISH

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# BOOK OF ABSTRACTS

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# BOOK OF ABSTRACTS

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**Conference keynote**

# HISTOPATHOLOGY: A KEY INTEGRATIVE TOOL FOR AQUATIC ANIMAL DISEASES

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Gross pathology can be frustratingly similar, and difficult for inexperienced personal to distinguish between different pathological conditions, notably where overlapping infections may be present, challenging the histopathologist to differentiate between ‘dying of’ from ‘dying with’ a given agent. The examination of stained tissue sections remains a gold standard for pathological investigations in human and veterinary medicine and no less important in wild fisheries and aquaculture. The significant technological advances particularly the advent of molecular biology has contributed to our understanding of specific proteins, viruses and enzymes in tissues. However, to make ‘biological sense’ of these data histopathology is essential and integral to all aspects of fish health whether it be for emerging diseases, diagnostic investigations or for pure research. In all cases judgment based on case-history observations, research findings, familiarity with the literature, experience, diagnostic test results help reach an accurate interpretation or result. Curiosity and an open mind combined with thorough knowledge and experience will remain an important attitude for the fish pathologist.

KN-2

# FISH VIROLOGY: MECHANISMS AND EVOLUTION OF VIRULENCE

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Unlikely in animal husbandry viral infections in aquaculture often tend to develop towards increased severity. Understanding virus evolution towards increased pathogenicity in the aquaculture environment is a requirement for being able to taking appropriate actions to prevent it.
Some of the recent studies concerning virulence mechanism will be presented and discussed with focus on VHSV, ISAV and IPNV. For these viruses only very few nucleotide mutations, inserts or deletions suffice to completely change the pathogenicity pattern in various species. Some changes involve the adhesion and entrance into to the fish and cells while others influence the virus replication and release. A review on some of the fascinating in vitro and in vivo studies recently conducted will be given.

Priority should be given to studies that

a. Demonstrate pathogen mutagenesis, e.g by including historical field samples in experimental trials and genetic analyses
b. Identify virulence markers and traits.
c. Assess factors that trigger increases in pathogen virulence
d. Assess risk of pathogen mutagenesis towards increased pathogenicity
e. Assess risk of presence of low pathogenic strains for disease development in aquaculture
f. Establish thresholds for pathogen discrimination
g. Design management strategies for preventing virulence evolution

When the understanding of virulence mechanisms have improved appropriate actions can include specific vaccination strategies, treatment strategies and operational procedures which take into consideration the risk of virulence evolution under aquaculture conditions.

# DEVELOPING VACCINES IN THE ERA OF GENOMICS

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Vaccines have a significant impact on human and animal health. Vaccinology in the era of genomics is taking advantage of new technologies to tackle diseases for which vaccine development has so far been unsuccessful. The availability of complete genome sequences of a pathogen provides access to its entire antigenic repertoire. Genomics has induced a shift in vaccine development towards sequence-based 'Reverse Vaccinology' approaches, which use high-throughput in silico screening of the entire genome of a pathogen that enable rapid targeted identification of novel vaccine antigens. Furthermore, the increasing availability of genome sequences has led to the development and application of additional technologies to vaccine discovery, including comparative genomics, transcriptomics, proteomics, structural biology and immunomics. Vaccine candidates identified from a pathogen's genome or proteome can then be expressed as recombinant proteins and tested in appropriate in vitro or in vivo models to assess immunogenicity and protection. The process of reverse vaccinology has been applied to several human pathogens, including serogroup B *Neisseria meningitidis, Streptococcus agalactiae, Streptococcus pyogenes, Streptococcus pneumoniae* and pathogenic *Escherichia coli,* and has provided scores of new candidate antigens for preclinical and clinical investigation. These powerful technologies are now expected to accelerate the identification of candidates also for the development of vaccines against animal pathogens.

# MAKING THE CASE FOR DISEASE FREEDOM – WHAT CAN EPIDEMIOLOGY CONTRIBUTE?

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A central tenet of EU aquatic animal health legislation and international standards is that susceptible species can only be traded live between areas of equal health status (or from a higher to lower status) for listed diseases. Thus demonstrating disease freedom is crucial to maintaining a high health status. Epidemiology provides the scientific underpinning to the design and analysis of surveillance to demonstrate freedom. Methods, implemented in free software (e.g. FreeCalc), to calculate sample sizes for structured surveys now take account of the minimum detectable prevalence and test characteristics, leading to improved design and interpretation of surveillance to demonstrate freedom. Scenario tree modelling (STM) goes further by allowing non-structured data sources to be used and for sampling to focus on high risk regions or farms, i.e. risk based surveillance. These methods enabled a move from input (e.g. 30 samples from every farm) to output based standards (e.g. demonstrate freedom with 95% confidence that the pathogen is not present above 2%).

Recent decisions in Europe on disease freedom illustrate epidemiology's wide role, over and above the technical challenges of surveillance. In the UK a decision not to attempt eradication of koi herpes virus was in part based on mathematical modelling of the spread of disease under different scenarios that quantified the likelihood of success, and time-scale, of an eradication programme. No EU Member State has made a case to demonstrate freedom from white spot syndrome virus, despite an absence of outbreaks. This is discussed alongside consideration of how import risk analysis (IRA) supports decisions about mitigation measures to maintain freedom. The EU ceased listing epizootic ulcerative necrosis as an exotic disease in part due to the likely costs of necessary trade restrictions (the decision was also informed by risk assessments of the likelihood and consequences of introduction).

Epidemiology provides many of the solutions to the technical questions that arise when making the case for disease freedom. However, governments must balance the potentially competing interests of all stakeholders, including consumers. To this end socio-economic analysis, informed by epidemiological data, of the costs and benefits of disease freedom is also crucial.

# ORAL PRESENTATIONS

# VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV) GENOTYPE IVB IS A POTENTIAL THREAT TO WILD FRESH WATER FISH IN JAPAN

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In 2003, viral hemorrhagic septicemia virus (VHSV) genotype IVb invaded the Great Lakes basin of North America and caused mass mortalities in several wild fish species throughout the watershed. In order to analyze the risk of further spread of this new genotype to other watersheds or countries, basic microbiological information such as pathogenicity studies is essential. In this study seven indigenous fresh water fish species in Japan were experimentally infected with VHSV genotype IVb (MI03 isolate) by immersion ($10^{5.2}TCID_{50}$/mL/1h). In experiment #1 the cumulative mortality in bluegill (*Lepomis macrochirus*), which was used as positive control, Japanese fluvial sculpin (*Cottus pollux*) and iwana (*Salvelinus leucomaenis pluvius*) was 50%, 80% and 0%, respectively. Experiment #2 included Japanese fluvial sculpin (*Cottus pollux*), Japanese rice fish (*Oryzias latipes*) and yoshinobori (*Rhinogobius sp.*) where cumulative mortalities of 100%, 100% and 10%, respectively, were observed. No mortality was observed in honmoroko (*Gnathopogon caerulescens*), akaza (*Liobagrus reini*) and Japanese striped loach (*Cobitis biwae*). The waste water from both experimental infections was disinfected by treatment with 50 ppm sodium hypochlorite for 10 min followed by two times exposure to UV radiation. Clinical signs in most dead bluegill were skin haemorrhages, and exophthalmia, while in Japanese rice fish, haemorrhages in every part of the body, distended abdomen and exophthalmia were observed from all dead fish. All dead fish were examined for VHSV by RT-PCR using pooled samples of kidney, spleen, and brain tissues. Virus was isolated from all dead fish. In addition, at 50 days post-exposure we were still able to identify virus in the brain of some surviving bluegills. These results revealed that if VHSV IVb were introduced to Japan it could become a threat to wild fresh water fish species, and that some surviving fish could become carriers of the virus. Japanese rice fish are often used as experimental fish in line with zebra fish and as they are highly susceptible to VHSV IVb infection by immersion exposure, this species may be useful as a model for studies of viral immunity and pathogenesis.

# LOW PATHOGENIC INFECTIOUS SALMON ANEMIA VIRUS (ISAV) *IN VIVO*: A COMPARATIVE GENOMIC STUDY

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Since the initial identification of ISAV in Norway in 1984, viral evolution and selective pressure, combined with improved detection have revealed an interesting and challenging ISAV portrait: the presence of essentially avirulent strains, such as the HPR0 variant, as well as highly virulent strains, such as the HPR4 variants, in addition to many additional strains with varying degrees of virulence.
In eastern Canada, outbreaks of ISAV were reported in 2012, and the avirulent HPR0 strain is detected regularly. It is interesting that the HPR0 variant of ISAV has not caused typical outbreaks of ISAV like the HPR4 variant. Still, many believe that spontaneous mutations/deletions of the HPR0 genome could lead to virulent forms of ISAV.
ISA is a systemic disease of Atlantic salmon, affecting primarily the blood and the vasculature resulting in variable organ manifestations. Head kidney and blood are the best choices for diagnostic of the disease during outbreaks, but for detection of all stages, including the detection of viruses in carriers, the examination of gills is often recommend. We recently observed that fish exposed to a low path ISAV had detectable viral RNA in their kidney 18 months past exposure. In contrast, our own experience suggests that fish surviving a high path ISAV return to a negative state rapidly after the end of mortalities. Further, we believe that low path ISAV can be transmitted horizontally and create a state similar to what is referred as 'herd immunity', whereas a proportion of fish were exposed and are thus naturally immune to ISAV infections, thus reducing the risk of an outbreak caused by a virulent ISAV.
The differences between low and high path ISAV are still largely unknown.
The objectives of this project are (1) to measure the viral load in various organs e.g. gills, blood, head kidney, of fish exposed to low path and high path ISAV, using quantitative real-time reverse transcriptase-polymerase chain reaction (RT-qPCR), focusing on the detection of carrier fish (low levels of ISAV); (2) to analyze gene responses in gills, in order to compare fish responses to high and low path ISAV, and responses of immune Atlantic salmon by comparison to naïve Atlantic salmon, using microarrays; (3) to evaluate the infectious potential of surviving fish, i.e. carrier fish, using *in vivo* challenges. Preliminary results on mortality rates, disease resistance after secondary exposure, and immune response will be discussed.

O-003

# HIGH VIRULENCE DIFFERENCES BETWEEN PHYLOGENETICALLY DISTINCT ISOLATES OF THE FISH RHABDOVIRUS VHSV IS NOT ASSOCIATED WITH VARIABILITY OF THE SURFACE GLYCOPROTEIN G NOR THE NONVIRION PROTEIN NV

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Viral haemorrhagic septicaemia virus (VHSV) is an important viral pathogen in European rainbow trout farming. Isolates from wild marine fish and fresh water trout farms show highly different virulence profiles: isolates from marine fish species cause little or no mortality in rainbow trout following experimental waterborne challenge, while challenge with rainbow trout isolates results in high levels of mortality. Phylogenetic analyses have revealed that the highly virulent trout isolates from freshwater farms have evolved from VHSV isolates from marine fish host species during the past 60 years. Recent isolates from rainbow trout reared in marine zones show intermediate virulence. The present study aimed at identification of molecular virulence markers which could be used to classify VHSV isolates according to their ability to cause disease in rainbow trout. By a reverse genetics approach using a VHSV-related novirhabdovirus (the infectious hematopoietic necrosis virus (IHNV)), four chimeric IHNV-VHSV recombinant viruses were generated. These chimeric viruses included substitution of the IHNV glyco- (G) or nonstructural- (Nv) protein with their counterparts from either a trout derived or a marine VHSV strain. Comparative challenge experiments, in rainbow trout fingerlings revealed similar levels of cumulative mortality induced by the recombinant (r)IHNV-VHSV chimeric viruses regardless of whether the G- or Nv genes originated from VHSV isolated from a marine fish species or from rainbow trout. Interestingly, recombinant IHNV gained higher virulence following substitution of the G gene with those of the VHSV strains, while the opposite was the case following substitution of the Nv genes. The results suggest that differences in the G and NV genes between trout-virulent and avirulent isolates of VHSV are not by themselves the explanation for the different abilities of the isolates to cause disease.

# GENETIC DIVERSITY OF *ANGUILLID RHABDOVIRUS* USING N, P AND G GENES

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Wild freshwater eels populations have dramatically declined in the last five decades in Europe, likely because of several anthropogenic factors. In addition to this infectious, diseases may play a role. In particular, the prevalence of viruses is suspected to be high, but their virulence, geographic circulation and genetic diversity is poorly documented, despite a long-standing international trade in eels. Indeed, the first rhabdovirus from eels was discovered in Japan in 1974, in a shipment of American elvers from Cuba, and designated Eel Virus American (EVA). The second virus was isolated in a shipment from France to Japan in 1976, and initially named Eel Virus European X (EVEX). A recent proposal is to consider EVA and EVEX as isolates of a unique species named Anguillid rhabdovirus (Stone, pers. comm.) and three complete or nearly-complete genome sequences were published recently exhibiting 98% of nucleic acid identity (Galinier *et al.*, 2012). The aim of the present study is to obtain more genetic information on Anguillid rhabdovirus isolates from Europe. The complete N, P, and G genes of 21 viruses originating from France, Denmark, and Germany, over a time period of 24 years were examined. Globally, the diversity between isolates was low for the three genes studied (95-100 % DNA identity) despite the different origins of the genotypes. Interestingly, the phosphoprotein exhibited a slightly higher variability compared to N and G. Moreover, 35% of the mutations in P were non synonymous, when N and G exhibited levels of only 5% and 10%, respectively. More isolates will be studied in the future, both to improve the design of diagnostic tools and to provide insights into the circulation of Anguillid rhabdovirus genotypes in Europe.

**Reference**

Galinier R. et al. (2012) complete genomic sequence and taxonomic position of eel virus European X (EVEX), a rhabdovirus of European eel. Virus Res 166:1-12.

O-005

# CHANGES IN N-GLYCOSYLATION AFFECTS PATHOGENICITY OF VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS (VHSV)

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Viral infection with the important fish rhabdovirus viral haemorrhagic septicaemia virus (VHSV) is dependent on attachment to the host cell via the surface exposed viral glycoprotein (G). The G protein of VHSV strain DK-3592b is N-glycosylated at four asparagine (Asn) residues that anchor biantennary complex-type oligosaccharides structures. To analyze the importance of N-glycosylation on the G protein for virus infectivity and virulence, six recombinant VHSV virus variants with mutated N-glycosylation sites were generated. The variants include a variable number (one to four) substitutions of the four glycosylated Asn residues with glutamines (Gln). All glyco variants were able to replicate in cell culture. The expected reduction of N-glycosylation of the G protein was confirmed by Western blotting. Reactivity with monoclonal antibodies (MAbs) recognizing a conformational and an O-glycan dependent epitope, respectively, confirmed that the G protein of all glyco variants was properly folded as well as O-glycosylated. Time course studies of replication in cell cultures and immersion challenge of rainbow trout fingerlings revealed that reduction of N-glycosylation reduced replication rate and pathogenicity of the virus. However, the effect was highly dependent on which of the four N-glycosylation sites that had been eliminated. While a variant lacking all four N-glycans was completely avirulent, a single N-glycosylation at the second Asn (N) site allowed the virus to retain intermediate pathogenicity. Plaque neutralisation tests with MAbs as well as sera obtained from immunized fish suggested that N-glycans are not significantly involved in neutralization epitopes.

# FUNCTIONAL FEEDS AFFECT GENE EXPRESSION PROFILES AND MORPHOLOGY OF THE GILLS OF ATLANTIC SALMON REARED AT DIFFERENT TEMPERATURES

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Effective immune function in fish is, in part, influenced by their nutritional intake, as shown for humans and terrestrial livestock. Thus, fish feed companies are interested in developing functional feeds to boost immune function and support key physiological processes of the fish with a view to maximising fish welfare and minimising the impact of stress and disease. To evaluate the usefulness of a number of feeds in terms of immune enhancement at different environmental temperatures, a study was performed using three groups of Atlantic salmon, held in duplicate tanks at two different temperatures (4 $^{o}$C and 12 $^{o}$C). Fish were fed with three different diets (A, B and D) including a standard commercial diet (A) as a control. After 12 weeks of feeding, fish were sampled to measure gene expression of immunoglobulin M (IgM) and immunoglobulin T (IgT) and morphometric changes in gills.
The relative gene expression (GenEx software) of IgM in all three dietary groups was higher (*i.e.* significantly higher $p \leq 0.05$ in diet D) at the higher temperature of 12 °C, which agrees with previous published findings. In contrast, relative gene expression of IgT was significantly differentially expressed among dietary groups. Fish fed the control A and diet B had similar patterns of IgM gene expression. Conversely, fish fed diet D had significantly higher expression of IgT genes at 4°C. It is known that the immune defences of salmon are not optimal at lower temperatures, although innate immunity does seems to be less compromised compared to adaptive immunity at this temperature. Diet D appears to be a good diet for feeding fish at both high and low temperatures, with significantly higher mucosal antibody responses obtained at the low temperature and higher humoral adaptive responses (IgM) at higher temperatures.
In parallel to the gene analysis, morphometric assessment of gills sampled from experimental fish was also performed, using a gill image analysis tool. This allowed quantification and assessment of the morphology, pathology and plasticity of gills in this trial. The combination of gene expression and morphometric analyses can help to evaluate the effects of different nutritional regimes by providing an overview of pathophysiological responses of gills when subjected to a range of stressors.

O-007

# FEEDING OF B-GLUCAN INCREASES THE DIVERSITY OF THE INTESTINAL MICROFLORA OF CARP (*CYPRINUS CARPIO*)

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β-glucan is an immunomodulant that is used in fish nutrition for health improvement. However, the value of feeding β-glucan could not be proofed till now. Therefore we examined the effect of dietary β-glucan on the bacterial community and diversity in the gut of common carp (*Cyprinus carpio*). We also examined responses of the intestinal microflora to an oral application of *Aeromonas hydrophila* in carp fed with or without ß-glucan supplementation. Carp received feed supplemented with 1% MacroGard®, a commercially available β-1,3/1,6-glucan, the control group received feed without ß-glucan. All fish were fed for 14 days at 1% of their body weight per day. For oral application of *A. hydrophila* carp were intubated with $10^9$ colony forming units (CFU) of *A. hydrophila* in PBS, or with $10^9$ CFU of *A. hydrophila* in PBS containing 1% MacroGard. The control groups were intubated with PBS containing 1% MacroGard or only with PBS. Samples were taken 12 hours to 7 days after application. Separated parts of the gut were analysed by classical microbiological techniques as well as Denaturing Gradient Gel Electrophoresis.
We could detect a significantly higher bacterial diversity in the gut after feeding with MacroGard. In all control groups the percentage of *A. hydrophila* in the microflora was in mean 44%. Twelve hours after application of bacteria, the highest amount of *A. hydrophila* could be found in carp fed without β-Glucan and the lowest amount was isolated from carp fed with 1% β-Glucan without oral application of Macrogard. Over the period of 7 days in all groups that were intubated with bacteria, the percentage of *A. hydrophila* decreased. This reduction was seen especially in carp fed with 1% β-Glucan and intubated with MacroGard (10% after 7 days) and in carp fed without β-Glucan (22% after 7 days). In carp fed with 1% β-Glucan that were not orally intubated with MacroGard, the percentage of *A. hydrophila* decreased less (39% after 7 days).
Our results suggest that feeding of β-glucan results in a more diverse and therefore more stable intestinal community that could help to prevent the invasion and establishment of pathogenic microorganisms.

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# THE EFFECTS OF DIETARY SOY PROTEIN CONCENTRATE (SPC) LEVELS ON GROWTH, COMPOSITION AND IMMUNE FUNCTION OF ATLANTIC SALMON (*SALMO SALAR* L.) PARR VACCINATED WITH A COMMERCIAL *AEROMONAS SALMONICIDA* VACCINE

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Soy protein concentrate (SPC) as an alternative to fish meal (FM) is considered a premium protein source of great potential because of its competitive price and high nutritional properties. It can substitute high levels of FM in Atlantic salmon post-smolt diets without causing negative effects on growth and intestinal integrity. In the current trial four experimental diets, containing 35, 50, 65 or 80 % of the dietary protein from SPC, were fed to Atlantic salmon (*Salmo salar* L.) parr. Growth and innate immunity were assessed after 97 days of feeding, then the fish were vaccinated with a commercial *Aeromonas salmonicida* vaccine to determine any dietary effects on fish immune response 7 and 34 days post-vaccination. Evaluation of the health status of the fish was performed by measuring basic haematology (haematocrit, total white blood cells and differential leucocyte counts) and several other immune responses (i.e. plasma lysozyme, anti-protease and alternative complement activity, plasma protein, total and *Aeromonas salmonicida* specific immunoglobulin M levels, and oxygen radical production by head kidney macrophages). Carcass and bone proximate composition, phosphorus and mineral analysis were assessed at the end of the trial. It was shown that the immune response of experimental fish did not appear to be size dependent since the linear decline of fish growth and total mineral content, with increasing dietary SPC inclusion levels, was not followed by a concomitant decrease in their immune response. Decreased ash levels were attributed to the linear decrease of Ca2+, Mg2+ and Mn2+ with increasing SPC inclusion in the diets. Moreover, feeding Atlantic salmon parr on diets containing up to 65 % dietary protein from SPC enhanced salient components of innate immunity such as plasma alternative complement and lysozyme activity while also plasma total IgM levels; with the diet containing 50 % protein from SPC giving the best performance both in terms of growth, carcass and bone composition of the fish and immune response. Diets containing 80 % of dietary protein from SPC were still able to promote some non-specific immune responses, but to a lesser degree than diets containing 50 and 65 % protein from SPC, however plasma total IgM levels in these fish were found at lower levels than in fish fed the control diet. This study reveals the need for increased supplementation of certain minerals in diets with increased protein levels from SPC. Furthermore the immunostimulatory effects of medium to high dietary SPC inclusion on Atlantic salmon parr requires further investigation, and the best way to investigate this would be through performing disease challenges.

O-009

# A REVIEW OF CLINICAL STUDIES ON THE HEALTH EFFECTS OF FUNCTIONAL FEEDS FOR SALMONIDS

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There is a striking abundance (~90) of review papers covering the health effects of fish diets, in particular dealing with probiotics and various immunostimulants including beta-glucans, while papers containing original results from salmonids are only some 150 in total. The vast majority of these papers report indirect measures of health, predominantly immune parameters such non-specific immune responses, immune cell activity, antibody levels, or expression of various immune genes. Beneficial clinical effects are documented for some of the ingredient groups (cell wall polysaccharides including beta-glucans, nucleotides and vitamins/carotenoids through a very limited number of controlled challenge trials with Atlantic salmon or rainbow trout. However, support for these observations from field trials appears absent, with the exception of one single study involving beta-glucan administration to Atlantic salmon.

Various categories of probiotics (lactic acid bacteria, aeromonads/pseudomonads, micrococci/enterococci) have shown beneficial clinical effects in rainbow trout challenge trials, but not in Atlantic salmon. Despite the widespread use of proprietary brands of functional feed ingredients or formulas, there are only a dozen papers in total, addressing their clinical effects.

Administration of feed to farmed salmonids must take place in groups, and cannot be measured or estimated per individual without exceptional efforts. As a consequence, the group in a pen or tank collapses into one single unit of concern for statistical purposes. Furthermore, infectious diseases spread between individuals in a group, and hence the criterion of independence between individuals is typically broken. In experimental and natural disease situations alike, specific statistical techniques are needed in order to address this situation adequately.

Based upon the discussion we bring up in this report, we suggest that documentation of clinical effects from functional fish feeds should comprise:

1. Solid support for relevant disease-specific benefits in experimental studies.
2. Clear prevention or modification effects shown in a controlled disease challenge setup.
3. Final documentation and quantification of clinical effects from large-scale field trials/ cohort studies.

It is strongly recommended to employ various multivariable regression techniques in order to adjust for group effects or other design issues when analysing data from studies on the health effect of nutritional interventions.

# TISSUE DISTRIBUTION AND FIELD EVALUATION OF CAPRYLIC ACID AGAINST NATURAL INFECTIONS OF *SPARICOTYLE CHRYSOPHRII* IN CAGE-REARED GILTHEAD SEA BREAM *SPARUS AURATA*

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In-feed administered caprylic acid (CA) was evaluated against the monogenean *Sparicotyle chrysophrii* in gilthead sea bream, *Sparus aurata*. Kinetic experiments were performed to select the appropriate CA dosing for the field trials. A dosing of 200 mg/kg fish showed a superior profile compared to 100 mg/kg fish, in terms of CA concentration in plasma, gills and skin. CA was delivered for 60 d in two *S. chrysophrii*-invaded farming units. At the commence of the experiment in the first farm, prevalence (44-47%) and mean intensity (1.1-1.5 adults) of *S. chrysophrii* were low but both showed an increasing pattern while the experiment was progressing. At the final sampling, mean intensity was significantly reduced in fish treated with caprylic acid (6 ± 2.6 vs 14.1 ± 3.1) while growth remained unaffected. A higher infestation with *S. chrysophrii* was already established at the initiation of therapy in the second farm. Prevalence was 100% in both groups and intensity reached values up to 17 adults. Both parameters remained at the same levels 30 and 60 d after the start of the trial. Growth and parasitic intensity between experimental groups were not significantly affected by caprylic acid treatment during the 2-month medication period. It is concluded that a dietary dosing of 200 mg caprylic acid/kg for 60 d can significantly affect *S. chrysophrii* intensity in cage-reared gilthead sea bream when treatment is implemented at the first disease stages. It is thus recommended that the progress of the disease should be continuously monitored in the farmed population during the production cycle and administration of caprylic acid should be initiated prior to the development of the infection.

# IMMUNE STATUS AND GROWTH OF GUPPIES (*POECILIA RETICULATA*) FED WITH DIFFERENT COMMERCIAL FISH FEEDS

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Good food quality is a prerequisite for the rearing of fish in recirculating aquaculture systems in order to ensure optimal growth, health and water quality. In this study we examined growth rate and health status of guppies (*Poecilia reticulata*) fed with commercial fish feeds that varied in concentrations of protein, fat and additives *i.e.* pigments and algae. Feeds tested included: Ocean Nutrition (O.N.) Breeder and O.N. Community Pellets (products of Ocean Nutrition, Newark, Canada) and MeM, MeM Ornamental, MeM Premium and BernAqua Experimental feed (EF) (products of BernAqua, InVivo, Hagelberg, Belgium). Feeding began at hatch and continued for 56 days. During the first 2 weeks fish were also partially fed with Artemia. Results revealed that feeding with MeM and MeM Premium displayed significantly higher growth rates (427 g and 417 g respectively). Interestingly reduced protein/ fat ratio correlated with the increase in fish growth rate. Deformity was significantly higher in the EF-fed fish, with levels reaching over 12% as compared to 3% or less in all other diets. Histological analysis revealed accumulation of liver glycogen and/or lipid in most of the fish with no differences between treatments. Fat accumulation in the abdomen was most pronounced in the EF and MeM-fed fish. Muscle dystrophy was observed in all treatment groups in *c.* 50% of the fish except for the MeM Ornamental group. Lysosyme levels did not differ significantly between the treatment groups. At termination of the experiment fish were challenged with the protozoan parasite *Tetrahymena* sp. (Pimenta Leibowitz & Zilberg, 2009) at an LD50 dose. The highest mortality occurred in the group fed with EF (87%), significantly higher than the MeM ornamental-fed fish which displayed the lowest mortality (58%). Mortality rates in the other groups ranged between 69- 76%. Increased susceptibility to *Tetrahymena* infection appeared to be correlated with elevated dietary protein concentrations. Based on these results, feeding with MeM Ornamental resulted in uniform-sized fish, with no muscle dystrophy and negligible deformity, as well as the greatest resistance to parasitic infection, however growth rate, although high, was not the highest of the tested diets.

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# DEVELOPMENT AND VALIDATION OF A SEMI-AUTOMATED CLASSIFICATION SYSTEM, INVOLVING ADVANCED IMAGE ANALYSIS, FOR ASSESSMENT OF THE EFFECTS OF DIETARY COMPONENTS ON INTESTINAL MORPHOLOGY OF ATLANTIC SALMON

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Virtual histology, the process of assessing digital images of histological slides is gaining momentum as an approach to supplement traditional histological evaluation methodologies. At the same time, digital image acquisition systems are becoming increasingly commonplace in laboratories, allowing image analysis methods to be employed to complement and extend traditional histological assessment.

Image analysis of digitised histological sections provides a practical means for quantifiable assessment of structural and functional changes in tissues, being both objective and reproducible. This study focused on the development of a rapid, practical analytical methodology based on advanced image analysis that was able to measure and characterise a range of features of the intestinal histology of Atlantic salmon in a quantitative manner.

The performance of the developed system was validated through direct comparison with previously described semi-quantitative scoring of intestinal samples from fish fed four experimental diets with differential inclusion of anti-nutritional factors. The generation of quantitative data, comprising 22 distinct variables, allowed multivariate statistical approaches to be conducted. The outcomes of these analyses showed significant correlation to those generated by well established, but highly laborious and specialist methods for gut health assessment. Moreover, the new analyses could be demonstrated to be both accurate and reproducible.

Overall, the results of this study show that the novel assessment system developed can help histopathologists to quantify and interpret the effects of dietary modulation on intestinal structure and function in Atlantic salmon.

O-013

# DISTRIBUTION OF THE PARASITE *BONAMIA OSTREAE* INSIDE ITS HOST, THE FLAT OYSTER *OSTREA EDULIS*

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The protozoan *Bonamia ostreae* is a parasite of flat oysters *Ostrea edulis*. It mainly infects haemocytes. The parasite cycle is partly elucidated, transmission of the parasite being direct from infected to naïve oysters. However, the roots of entrance and release of the parasite from the oyster are not exactly known. In order to investigate the distribution of the parasite within its host, two complementary approaches were followed: the first one consisted in using *in situ* hybridization to test naïve oysters after contact experiments with infected oysters. The second approach consisted in evaluating the parasite load by Real Time quantitative PCR in different organs of flat oysters collected from natural beds known to be infected.

In total 94 in 408 challenged oysters showed specific labeling by *in situ* hybridization. Among these positive oysters, 63%, 29% and 8% respectively displayed low, moderate and highly infection levels. Oysters lightly infected generally showed positive signal in gills whereas the parasite was detected in most of the organs in highly infected oysters.

Real Time PCR allowed detecting and estimating parasite load in different organs of the oysters. Gills, adductor muscle and labial palps were more often found infected than mantle, gonad and digestive gland. Parasite load was found higher in mantle compared to other organs.

Altogether these results suggest that the parasite enters through the gills. Once they have passed the epithelium barrier, they are internalized within haemocytes which contribute to spread the parasite in all the organs. Both *in situ* hybridization and Real Time PCR confirm that gills are organs of interest for the diagnostic of *Bonamia ostreae*.

# TYPE 1 OSTREID HERPESVIRUS (OSHV-1) VARIANTS IN JAPAN

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Ostreid herpesvirus 1 (OsHV-1) µVar is a newly reported variant of OsHV-1 that is suspected of being the causative agent of acute mass mortality events of Pacific oysters during summers in Europe since 2008. OsHV-1 µVar differed from reference OsHV-1 by nucleotide mutations in the C2/C6 fragment including ORF4, and in the IA2/IA1 fragment including ORF42/43 (Segarra *et al*., 2010). Japan is one of the major producers of Pacific oyster. Our previous study indicated that, 23 types of OsHV-1 variant, showing 96% to 99% similarity to the reference OsHV-1, were obtained from Pacific oyster, *Crassostrea gigas*, kumamoto oyster, *C. sikamea* and suminoe oyster, *C. ariakensis* collected in 2007 and from Pacific oyster in 2011 in Japan (Shimahara *et al*., 2012). Although 18 variants among the 23 obtained possessed a microsatellite deletion unique to OsHV-1 µVar in France (GenBank accession no. HQ842610) in C2/C6, the nucleotide sequence was not identical to the OsHV-1 µVar. In this study, further surveillance of OsHV-1 variants was conducted for Pacific oysters in 2012. Nine hundreds of Spat, or juveniles of Pacific oysters collected in the 4 main oyster producing areas were used for specimens. DNA was extracted with Maxwell 16 Tissue DNA Purification Kit (Promega), and PCR was performed using the primer pairs C2 and C6 (Renault and Arzul 2001). PCR products were amplified from 40 out of 900 oysters, and 13 different nucleotide sequences, showing 96% to 99% similarity to the reference OsHV-1, were obtained. Although 11 sequences among the 13 obtained possessed a microsatellite deletion unique to OsHV-1 µVar, all PCR products contained two conserved nucleotides that were shared with the reference OsHV-1 and not with OsHV-1 µVar in France (HQ842610). Here, we found variable types of OsHV-1 in oysters in Japan, but their nucleotide sequences were not identical to that of OsHV-1 µVar in France (HQ842610). Further epidemiological studies are currently in progress in Japan to collect more information.

# DIVERSITY AND PHYLOGENETIC RELATIONSHIPS OF OSHV-1 SAMPLES FROM DIFFERENT GEOGRAPHIC ORIGINS

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Ostreid herpesvirus 1 (OsHV-1) is a DNA virus belonging to the *Malacoherpesviridae* family from the Herpesvirales order. This virus has been associated with mortality outbreaks in the Pacific cupped oyster, *Crassostrea gigas*, since 1991. However, since 2008, a significant increase in the occurrence, intensity and geographic distribution of these outbreaks has been reported. This augmentation has been related to the detection of OsHV-1µVar, a variant described on the basis of specific polymorphisms in ORF4 and ORFs 42-43. In particular, ORF4 of OsHV-1µvar is affected by a deletion at a microsatellite site that does not exist in the « reference » genome of OsHV-1, sequenced prior to 2008.

Based on variations occurring at ORF4, other variants have been defined, raising interest in the characterization of the genetic variability among OsHV-1 specimens. Recent studies have shown that the analysis of the sequences of three particular regions (ORF4, ORFs35-36-37-38 and ORFs42-43) - both independently and as concatemerized units- made it possible to define different sub-groups within known «genotypes ». This principle was applied to an initial set of samples collected between 1993 and 2010 mainly in France but also in other countries.

Since the publication of these results, the sequence compilation was enriched with samples collected in 2010-2012 in France, Ireland, the Netherlands, Spain, Tunisia, Korea, Japan, USA, Mexico, Brazil, Ireland, and New Zealand. Partial sequence data was generated and analyzed following the previously described methodology. Results show that:

- Sub-groups based on the geographic origin of the samples tend to be consistent over several sampling periods
- Virus samples collected during mortality outbreaks demonstrated some diversity,
- The presence of the characteristic deletion at the microsatellite area in ORF4 is not sufficient to classify a given sample as OsHV-1µVar

The data generated from a broader set of samples has allowed for a better understanding of the genetic diversity of OsHV-1 samples from different geographic origins. Moreover, the obtained data suggest preliminary hypotheses concerning the distribution of OsHV-1.

# EVALUATION OF THE IMPACT OF THE INFECTION WITH OSTREID HERPESVIRUS-1 (OSHV-1) µVAR IN THE ON-GROWING OF PACIFIC OYSTERS *CRASSOSTREA GIGAS* IN GALICIA (NW SPAIN)

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The on-growing of batches of Pacific oyster *Crassostrea gigas* spat was monitored in two raft areas in ría de Arousa (Galicia, NW Spain), to evaluate the impact of the ostreid herpesvirus-1 (OsHV-1) µVar. A *C. gigas* spat batch (A) imported from France was deployed in 03/05/2012, with 10.4 mm in mean height. Two *C. gigas* spat batches produced in a Spanish hatchery were also used, one batch (B, 8,9 mm) was transferred directly from hatchery to raft, while the other batch (C, 23,9 mm) had been pre-grown in a floating nursery before deployment in raft. Additionally, a batch of flat oyster *Ostrea edulis* spat (D, 26.1 mm) was transferred to raft. The batches B, C and D were deployed in 11/07/2012. Monthly sampling was performed to estimate mortality and to diagnose OsHV-1 by PCR, according to the procedure in the Commission Regulation (UE) 175/2010.
No case of OsHV-1 infection was detected in batch A before deployment and 27 days later 97% oysters were positive for OsHV-1 µVar; 10% and 96% of the oysters of batches B and C, respectively, were positive for OsHV-1 µVar before deployment. Cumulative mortality of batch A reached 80% in both rafts after 3 months; since then mortality was almost null. In batch B, 97% mortality was recorded 22 days after deployment. Mortality was lower in batch C (oldest), reaching 47% in cumulative mortality after 3 months and then becoming insignificant. The percentage of positive OsHV-1 µVar cases trend to decrease after an early maximum in *C. gigas* batches as infected spat died. No case of OsHV-1 was detected in the *O. edulis* batch before deployment but more than 50% positive cases for OsHV-1 µVar were detected through on-growing; however, cumulative mortality after 7 months was just 3%.
Conclusions: OsHV-1 µVar caused high Pacific oyster spat mortality; the younger the spat the higher the mortality. Using OsHV1-free spat does not guarantee high survival when on-growing takes place in a OsHV-1 µVar affected area, because infection is acquired shortly. Flat oyster spat is tolerant to OsHV-1 µVar but its role as reservoir should not be despised.

O-017

# EVERY COCKLE FOR ITSELF: VIEWING THE SURFACING PHENOMENON FROM A MICROCOSM APPROACH

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Since Snieszko, (1974) proposed that disease develops following a close interaction between host - parasite and environment, the view has been held that external drivers can influence parasite development within the host. To build on this a site-specific aetiology for surfacing in cockles is proposed in the present study. Mass surfacing and mortality events have been reported in the last 20 years over geographically distinct regions in the commercially and ecologically important common cockle, *Cerastoderma edule*. Across its range the bivalve is known to provide a habitat for 16 species of digenean trematodes, in addition to copepods, gregarines and various turbellaria and cilates. Various aetiologies have been proposed for surfacing in this infaunal bivalve including the pathological condition disseminated neoplasia, haplosporidia and digenean trematodes but the effects and impacts of a number of parasites, all interacting and affecting the physiology of an individual, along with external drivers of disease development in a cockle together have not been considered fully by histological and molecular means. The present study attempted to explore the occurrence of these surfacing events at two quite different sites in Ireland. Prevalence and intensity of any parasites or pathological conditions were then considered in the context of local conditions like site, temperature/season, and position on or in the sediment, in addition to aspects of each cockle itself (sex, gonadal maturity, number of growth rings). Results suggest that the surfacing aetiology is site specific, with surfacing at one site (Bannow Bay) being driven by a complexity of parasite infections and season being a key component, while attaining a threshold age of three years seems to be the most significant component at the second site resulting in a threshold infection load leading to disease and death in these cockles

# COLLAPSE OF THE COCKLE *CERASTODERMA EDULE* FISHERY IN RÍA DE AROUSA (GALICIA, NW SPAIN) DUE TO MARTEILIOSIS

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Cockle *Cerastoderma edule* fishery is the most important shellfishery in Galicia (NW Spain), in biomass terms. One of the most productive cockle beds of the region is located in Lombos do Ulla, in ría de Arousa. Monitoring of population dynamics in this bed started in 2002 to assist shellfishery management; it is based on surveys performed twice each year, involving estimation of cockle density, cockle biomass and population size/age structure. Additionally, monthly sampling to estimate cockle mortality and to assess the health status through histopathology plus monitoring of salinity and temperature started in 2007. A huge increase of mortality rate was detected in April 2012, reaching 90%, followed by 100% in May 2012, leading to disappearance of live cockles. The only new circumstance with regard to previous years that could justify the huge mortality was an infection caused by a *Marteilia*-like protist, which was first detected in February 2012 and reached 100% prevalence in April 2012. This protist had never been detected in Galician cockles. Highly abundant sporulation stages of this protist occurred in the epithelium of digestive diverticula, causing severe cockle emaciation. Morphological features of this protist, observed both with light and transmission electron microscopy, resembled *Marteilia* sp. infecting unburied cockles in France (Comps et al. 1975). Morphological differences with *Marteilia refringens* infecting mussels in ría de Arousa involved the number of spores per sporont (6 in the cockle parasite and 4 in the mussel one) and the electron-dense granules occurring in the sporont cytoplasm (smaller and much more numerous in the cockle parasite). SSU rDNA, ITS and IGS sequences showed close homology with those reported from *Marteilia* sp. infecting cockles in Catalonia (NE Spain) (Carrasco et al. 2012). Morphological and molecular data support the cockle parasite is a different species from *M. refringens*, within the *Marteilia* genus. Sampling of the other main cockle beds in ría de Arousa was performed in June 2012; cockle marteiliosis was highly prevalent throughout this ría and cockle fishery collapsed. The fact that marteiliosis affected juvenile cockles and the lack of recruitment since spring 2012 presage an uncertain future.

O-019

# PATHOGEN OCCURRENCE IN THE MOLLUSK BIVALVE *LITHOPHAGA LITHOPHAGA* SAMPLED FROM THE BIZERTA BAY (NORTH TUNISIA)

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Bivalves are known as a good bioindicators of pollution because of their large filtering power and their ability to accumulate different kinds of pollutant and pathogen in their bodies. In Tunisia, despite the shellfish field development, it stills suffering from several problems that can be associated to pollutants and / or parasites that may have a bad effect on the grow-out sites. The present study was done with a view to characterize the health status of the Tunisian date mussel *Lithophaga lithophaga* which is endolithic specie belonging to the IUCN Red List (International Union for Conservation of Nature) and considered among the threatened or endangered species in the Mediterranean Sea. This mytilid with a high organoleptic quality was collected monthly between September 2002 and September 2003 from the Bizerta Bay. Examination of the whole soft part and histological gonad sections showed the existence of some Protozoa mainly represented by *Perkinsus* sp. and *Bonamia* sp. and some species of Trematoda. The identification of these parasites and the calculation of some epidemiological indices allowed us to have an idea on the health status of *L. lithophaga* and their effects on the biology of this species. In addition, macroscopic and microscopic observations with bacteriological analyses were carried out seasonally from 2004 to 2006 on samples collected from the same site. Results showed the presence of burrowing annelids and sipunculids living inside galleries on the internal valve surface of *L. lithophaga* which caused a serious shell morphological disturbance affecting the commercial quality of this species. Bacteriological analyses revealed important accumulation of heterotrophic mesophyllic bacteria particularly in summer and autumn ($3.10^7$ and $2.10^8$ CFU $ml^{-1}$, respectively) while pathogen *Vibrionaceaes* appeared in high concentrations during all the study period.

# STUDIES OF THE TYPE I INTERFERON RESPONSE TO CYPRINID HERPESVIRUS 3 INFECTION IN COMMON CARP REVEALS VIRAL ANTI-IFN ACTIVITY IN FIBROBLASTS

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A Cyprinid herpesvirus 3 (CyHV-3) infection in common carp (*Cyprinus carpio* L.) can induce a severe systemic disease with a rapid spreading of the virus through multiple organs and a fast onset of mortality in up to 100% of infected fish. During the first phase of the infection, type I interferons have been proven to be essential in the activation of the innate response against viral pathogens although very little is known about this response to alloherpesviruses in fish.
The aim of the presented work was to analyse whether type I IFN responses can limit the spread of CyHV-3 *in vivo*. Secondly the interaction of the virus with type I IFN was explored in a series of *in vitro* studies.
Upon infection, two divergent lines of carp presented a 20% difference in survival. IFN transcription was up-regulated in a virus dependent manner in skin and head kidney, however it did not seem to limit virus spreading. Carp from the more susceptible line harboured a higher virus load and showed a significantly higher interferon response. Subsequent *in vitro* studies using poly I:C as a type I IFN stimulator in fibroblastic cells showed that an activation of this response can limit the titer and spreading of the virus in cell culture. However when comparing the responses in fibroblasts (CCB) and head kidney leukocytes (HKL), CyHV-3 induced fundamentally different type I IFN response patterns. While CCB cells allowed replication of CyHV-3 and were lacking an IFN activation, leukocytes reacted with a strong activation of the IFN system and allowed virus replication only at a low level. The lack of an IFN type I response of CCBs to CyHV-3 was a clear indicator of anti-IFN actions of the virus. Studies on cycloheximide or UV inactivated virus showed that the nature of this mechanism most likely is not based on a *de novo* production of proteins.
In conclusion: CyHV-3 virus is capable of blocking the type I IFN response in fibroblastic cells which appear to be virus replication sites. This can result in an uncontrolled spread of the virus throughout the organism leading to mortality.

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O-021

# PROTECTION OF ATLANTIC SALMON AGAINST INFECTIOUS SALMON ANEMIA VIRUS (ISAV) INFECTION BY INTRAMUSCULAR INJECTION (i.m.) OF IFNc EXPRESSION PLASMID

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DNA-vaccination experiments have shown strong protection against several virus diseases in fish demonstrating that fish muscle cells take up plasmids and express encoded viral genes that are under the control of an eukaryotic promoter. This inspired us to test if i.m. injection of plasmids expressing type I interferon (IFN) might provide protection of Atlantic salmon against virus infection. Salmon has three type I IFN subtypes, IFNa, IFNc and IFNb, which all induce an antiviral state in cell lines by inducing antiviral genes such as Mx, ISG15 and viperin. In this work we have injected salmon i.m. with plasmids encoding IFNa, IFNb or IFNc under the control of a CMV promoter or with a control plasmid and measured expression of antiviral genes in organs and studied protection against ISAV infection. While all three IFN plasmids induced Mx expression in the muscle at the injection site, only IFNb and IFNc plasmids induced Mx expression in head kidney 1 week after injection. Injection of IFNc plasmid induced antiviral genes (Mx, viperin, ISG15 and ISG58) and receptors for virus RNA (RIG-I, TLR3 and TLR7) in head kidney throughout the 8 week experimental period. Immunoblotting showed increased Mx protein expression in liver with time. Finally, challenge of the fish with ISAV by i.p. injection or cohabitation infection 7 to 9 weeks later showed strong protection of the IFNc plasmid injected, but no protection of the IFNa and IFNb plasmid injected fish. These data suggests that i.m. injection of the IFNc expression plasmid offers a new method of protecting Atlantic salmon against virus infection.

# IFITS: FIRST DESCRIPTION OF A NEW FAMILY OF INTERFERON INDUCED GENES IN FISH

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Interferon (IFN) and IFN-stimulated genes (ISGs) are the main effectors of the innate response against virus. In fish, although IFN-like activities have been characterized, the classification of those virus-induced IFN genes was controversial since they were more diverse than previously thought.
In fish, the knowledge of the antiviral properties of ISGs is limited to a few intensively studied being the Mx one of the most studied until now.
We described for the first time in fish the complete repertoire of a new family of IFN-stimulated genes whose structural feature is the presence of tetratricopeptide repeats (TRP domains), called IFIT family. We proposed a nomenclature based on structural and phylogenetic analyses and analyzed their involvement on the interferon cascades or antiviral activities. To further explore the antiviral properties of these ISGs, *in vivo* and *in vitro* experiments were conducted in zebrafish (*Danio rerio*) after treatment with different recombinant IFNΦ and also after viral infections.

O-023

# CLONING AND IDENTIFICATION OF A NOVEL GENE IL8-2 IN JAPANESE FLOUNDER *PARALICHTHYS OLIVACEUS*

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Interleukin 8 (IL-8), is the first known chemokine, that plays a vital role in the pro-inflammatory phase. In the past decades, IL-8 has been identified in several kinds of fishes beside mammals, avian and amphibians. Recent research revealed the presence of different types of chemokines in cyprinid species. In our study, we found a novel IL-8 (IL8-2) from Japanese flounder EST database. The transcript contains an open reading frame of 291 nucleotides encoding 97 amino acid residues, with a 20 amino acid signal peptide. Similar to the previously sequenced Japanese flounder IL-8, the IL8-2 amino acid sequence lacks the typical ELR motif upstream of the first pair of cysteines, where TPR is present. However, the comparison of amino acid sequences between Japanese flounder IL8-2 and IL-8 revealed only 37.5% identity and 55.4% similarity. IL8-2 expression was detected in various tissues by real-time quantitative PCR. Highest transcript expression was observed in kidney followed by spleen, while lower transcript expression was observed in liver, gill, brain, skin and muscle. Infection experiment by immersion showed no significant increase in kidney IL8-2 expression from day 0 to day 6 post infection by either *S.iniae* or *E.tarda.* On the other hand, a significant increase was observed in day 3 and day 6 post VHSV infection. In addition, PolyI:C treatment significantly increase IL8-2 expression in PBL.

# MOLECULAR CLONING AND FUNCTIONAL EFFECTS OF JAPANESE FLOUNDER (*PARALICHTHYS OLIVACEUS*) INTERLEUKIN 12

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In this study, inteleukin-12 (IL-12) that is one of key cytokines that plays important roles in the regulation of adaptive immunity was examined in Japanese flounder. IL-12 is produced early in the immune response by monocyte, macrophage and dendritic cells in mammals. It induces the production of interferon-γ (IFN-γ) from T cell and natural killer cell, and enhances cytolytic function of cytotoxic T cell. IL-12 is a heterodimeric molecule composed of two covalent subunits, p35 and p40. The Japanese flounder IL-12 (JFIL-12) cDNA was amplified by RACE-PCR using primers design from EST sequences obtained by the next generation sequencer. The expression in various organs and time course expressions during *Edwardsiella tarda*, *Streptococcus iniae* and viral hemorrhagic septicemia (VHSV) infection were quantified by RT-PCR. The coding region of IL-12p35, IL-12p40a and IL-12p40b were 609, 1071 and 948 nucleotides (nt) encoding 203, 357 and 316 amino acids, respectively. Phylogenetic analyses confirmed the two different JF p40 subunits which both were homologues to p40 of other fish species. JFIL-12p35 and IL-12p40a expressions were detected in all tissues examined. On the other hand, JFIL-12p40b transcripts were detected in the gill, head-kidney, trunk kidney, heart and spleen. The JFIL-12p35 and JFIL-12p40a transcripts were slightly increased at day 1 post *E. tarda* infection and then decreased at day 3. The JFIL-12p40b expression was highly up regulated at day 6 after *E. tarda* infection. Only JFIL-12p40b were highly up regulated at day 1 to 6 after *S.iniae* infection. We are presently investigating the adjuvant efficiency of JFIL-12 by detected the expressions of IL-12, IL-2 and IFN-**γ** after the expression plasmid injection.

O-025

# ISOLATION OF *YERSINIA RUCKERI* STRAIN H01 FROM FARM RAISED AMUR STURGEON, *ACIPENSER SCHRENCKII*, IN CHINA

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*Yersinia ruckeri* is the causative agent of enteric redmouth disease or yersiniosis, which affects salmonids and several other species of fish. However, there are no reports on the characteristics and pathogenicity of *Y. ruckeri* isolated from farm-raised Amur sturgeon, *Acipenser schrenckii*. Here, we isolated and characterized *Y. ruckeri* strain H01 from the diseased Amur sturgeon in China. The phenotypic and genotypic characteristics of *Y. ruckeri* were observed and its virulence was tested by examining experimentally infected sturgeons. Examination of the flagellar morphology of *Y. ruckeri* by transmission electron microscopy showed 5-8 peritrichous flagella located on the cell body. Actively dividing cells with an obvious cell membrane were approximately 0.64 μm in diameter and between 1.7 and 2.5 μm in length. LD50 value was determined to be $7.2\times10^6$ CFU and *Y. ruckeri* could be re-isolated from the liver and kidneys of infected sturgeon. Antimicrobial susceptibility tests showed that H01 was susceptible to 10 antimicrobial agents. Part of the *16S rRNA* sequences (563 bp) was amplified and sequenced to study the genotypic characterization in *Y. ruckeri* (GenBank accession number JQ657818). The phylogenetic tree revealed H01 was clustered together with *Y. ruckeri* strains. Together, this study describes the isolation, characterization and phenotypic/genotypic analysis of a *Y. ruckeri* strain isolated from farm-raised Amur sturgeon. The results discovered may provide some theoretical basis for the prevention and control of yersiniosis in Amur sturgeon.

# EPITHELIOCYSTIS IN WILD FISH POPULATIONS: TEMPERATURE DEPENDANCE AND SPECIES VARIATION

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Epitheliocystis is an emerging bacterial disease affecting many fish species around the world. Infections of the gills and skin of fresh and salt water fish lead to intracellular inclusions which differ greatly in their size and form, a reflection of a wide diversity in the infectious agents. These are mainly members of the Phylum *Chlamydiae*, but recent studies showed that other agents can also be involved, like betaproteobacteria in seawater farmed Atlantic salmon (*Salmo salar*). Two species have been identified so far in salmonids, namely '*Candidatus* Piscichlamydia salmonis' and '*Candidatus* Clavochlamydia salmonicola'. The latter is the closest relative to the *Chlamydiaceae*, traditional terrestrial members of this bacterial phylum, whereas the former is the most ancient member of the phylum. Both have been observed infecting the same fish, and we have observed temperature dependence of infections with disease mainly present during summer months. In cultured fish, mortality is attributed to epithelial cell proliferation and increase of mucus production around heavily infected gills. Fish become lethargic and show respiratory distress. The environmental origins of these bacteria are unknown. To help close this knowledge gap, we have begun a wide ranging study of Swiss rivers to establish the distribution in brown trout *(Salmo trutta).* A total of 74 river sites were sampled by electrofishing and 1484 brown trout young-of-the-year collected. Gills are being analyzed by histopathology, electron microscopy and molecularly by PCR and sequencing to establish the identities of the epitheliocystis agents involved. On the basis of histopathology, 208 epitheliocystis positive cases representing 14% of the population sampled in Swiss rivers could be identified, with at least three different morphologies, indicating a diverse group of agents. These studies are continuing.

O-027

# FRANCISELLOSIS IN ORNAMENTAL FISH – A THREAT TO NATIVE FISH SPECIES?

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Infections with fish pathogen strains of the genus *Francisella* are recognised as a serious threat in aquaculture. Outbreaks of the disease have been described in a variety of fish such as Atlantic cod, *Gadus morhua*, tilapia, *Oreochromis sp*. Atlantic salmon, *Salmo salar* and even molluscs. In one case, molecular genetic investigation and in situ hybridisation of archived, formalin-fixed, paraffin–embedded tissues revealed *F. noatunensis* susp. *orientalis* as the causative agent of granulomatous disease in ornamental African cichlids.
Until now, however, the pathogen had not been described in connection with cinical disease outbreaks in ornamental fish.
We found that an infection with *F. noatunensis* subsp. *orientalis* was the cause of granulomatous disease in a breeding facility of ornamental Malawi cichlides. Sequencing and Blast alignment of the 16S rDNA showed a 99 % level of identity with *F. noatunensis* subsp. *orientalis.*
In this presentation we will discuss the results of cultivation of our isolate and susceptibility studies on the native Austrian fish species common carp, *Cyprinus carpio*. In order to conduct this susceptibility experiment, fish were challenged intraperitoneally and by immersion with serial dilutions of the *Francisella* strain. Water temperature was 25°C, a temperature easily reached during summer in many European countries.
The aim of the study was to evaluate the risk of transmission of *F. noatunensis* susp. *orientalis* from tropical ornamental fish to native freshwater fish species.

# DETECTION OF CARP EDEMA-LIKE VIRUS DURING DISEASE OUTBREAKS IN KOI AND COMMON CARP (*CYPRINUS CARPIO)* IN THE UK

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The pox virus that is the disease agent of koi sleepy disease (KSD), also known as carp edema virus (CEV), has only been reported to occur in Japan. Losses from CEV are seen in spring and autumn, over a temperature range of 15 – 25°C, and mortalities can reach 80%. Gross clinical signs include lethargy, enophthalmia and skin erosion. The gills may show hyperplasia and necrosis very similar to that seen in fish affected by koi herpesvirus (KHV) disease. CEV was originally described in Japan in the 1970's as a viral oedema of juvenile carp and the virus was shown to cause severe damage to gill lamellae, leading to hypoxia and lethargy. In older carp the lethargy manifests as sleepy behaviour, where the fish lie on the bottom of the pond and eventually die of anoxia. In Japan outbreaks of KSD are managed or prevented by holding the fish in 0.5% salt water following any stress events such as grading or transportation.
At the Cefas laboratory a CEV-like virus was first detected by PCR in imported koi, showing signs of KSD, in 2009 and again in 2011. A further detection was then made from KSD-affected koi in a hobbyist's pond in June 2012. To improve reliability of detection an alternative nested PCR assay was developed based on the partial sequence data (T Miyazaki unpublished) for CEV. Using this assay the amplification products from these detections all shared a 97.5 – 98.4% nucleotide identity with the original Japanese CEV.
More significantly, earlier in March 2012, a CEV-like virus was detected for the first time in common carp, displaying KSD-signs, obtained from a cluster of fishery sites in south- east England. Then, in late November 2012, CEV-like virus was again detected at a fishery site in the English Midlands. The detections from the fishery carp shared a 98.2% identity with each other and only a 93.3 – 95.6 % identity with the original Japanese CEV and the other CEV-like detections in koi. The Midlands site reported a further outbreak of disease in February 2013 and the collection of a number of live, disease-affected carp from the site allowed more extensive virology investigations to be carried out. The results of the investigations at Cefas will be reported and the consequences of the practice of managing koi sleepy disease for disease spread and control discussed.

O-029

# *NUCLEOSPORA CYCLOPTERI*, A NOVEL INTRANUCLEAR MICROSPORIDIAN PARASITE CAUSING SEVERE PATHOLOGY IN WILD ICELANDIC LUMPFISH

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Lumpfish, *Cyclopterus lumpus*, are distributed throughout the North Atlantic and are a commercially important species historically targeted during a coastal spring fishery as their eggs are valuable and used as a caviar substitute. More recently, lumpfish have been shown to be effective in removing parasitic copepods from captive salmon, and they are currently being assessed for use as cleaner fish for the biological control of salmon lice in commercial Atlantic salmon farms. However, captive lumpfish kept in rearing facilities are susceptible to infections which have led to high mortalities, and during recent Icelandic lumpfish landings, extensive renal pathologies have been observed in some fish.
Lumpfish from the Icelandic coast were dissected and used in histological and molecular studies to determine the causative agent of the kidney enlargement. Lumpfish, with various clinical signs, were observed at 12 of the 43 sites sampled around Iceland. From a total of 77 fish examined, 18 had clear clinical signs, the most prominent of which was an extensive enlargement and pallor of the kidneys. The histopathology of the most severely affected fish consisted of extensive degeneration and necrosis of kidney tubules and vacuolar degeneration of the haematopoietic tissue. Intranuclear microsporidians were detected in all organs examined in fish with prominent clinical signs and most organs of apparently healthy fish using a combination of nested PCR and histological examination. One or multiple uniformly oval shaped spores were observed in the nucleus of affected lymphocytes and lymphocyte precursor cells. DNA sequencing provided a ribosomal DNA sequence that was strongly supported in phylogenetic analyses in a clade containing other microsporidian parasites from the Enterocytozoonidae, showing highest similarity to the intranuclear microsporidian *Nucleospora salmonis*.
Intranuclear microsporidian infections are common in wild caught lumpfish from around the Icelandic coast. Infections can cause severe clinical signs and extensive histopathological changes, but are also present, at lower levels, in fish that do not show clinical signs. Due to the importance of wild lumpfish fisheries in northern Atlantic countries, and their potential use as cleaner fish in Atlantic salmon farms, it is vital to evaluate this pathogen and its potential impact upon wild spawning stocks and captive fish.

# SKIN LESIONS AND MEAT RESIDUE OF BELUGA (*HUSO HUSO*) FED DIETS CONTAINING AFLATOXIN $B_1$

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*Huso huso* is one of the most important native sturgeon species in Caspian Sea. The purpose of this investigation is studying skin lesions and meat residue in Bluga after feeding different levels of dietary AFB1.These factors are important in fresh fish marketability and public health. Some 180 fish weighted 120 ± 10gr with stocking density of 12 fish/tank , fed different levels of AFB1(0,25,50,75 & 100ppb/kg of diets) under controlled conditions for 15 weeks. Skin lesions evaluated through clinical observations and meat residues determined by HPLC, monthly. With regard to toxin concentration and time of exposure to AFB1 in experimental fish, different degree of skin lesions(simple hemorrhage to progressive wounds)were observed in different parts of body specially in vent, caudal peduncle, fins, and head "Yellow sores" on head region are considerable and led to deterioration of appearance. Meat accumulation of AFB1 in different treatments is not so considerable but is significantly different with control fishes ($P<0.01$). Different levels of degenerative pathological changes in liver sections, emphasizes the mentioned results. Omitting dietary AFB1 in experimental fish approximately led to meat clearance from AFB1 residues after one month.

Key words: Huso huso, $AFB_1$, Skin lesions, Meat residues, Public health.

O-031

# ARE SALMONIDS REALLY SUSCEPTIBLE TO INFECTION WITH THE KOI HERPESVIRUS (KHV)?

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Due to unexpected and sudden outbreaks of koi herpesvirus (KHV) disease (KHVD) in common carp or koi (*Cyprinus carpio*) only, many working groups started to work to identify possible carriers or vectors which may transmit infectious KHV. KHVD is only observed in carp or koi but in some cases other fish are involved in mortality as well, e.g. tench (*Tinca tinca*) or roach (*Rutilus rutilus*). Other fish around carp pond with KHVD history were found to be KHV infected but always clinically healthy. In a wild fish project finished in 2011, KHV was also detected in rainbow trout (*Oncorhynchus mykiss*). To clarify this, rainbow trout (n=40) were immersed with KHV for 2 hours at 18°C water temperature. After that rainbow trout were kept at 15 and 20°C (n=20), respectively. Lethal and non-lethal sampling was carried out on day 0 (before immersion), day 7, day 14 and day 28 post infection (dpi). On 8 dpi, carp (n=5), tested negative for KHV, carp pox virus and spring viremia of carp virus by PCR or RT-PCR, were cohabitated with rainbow trout at 15 and 20°C, respectively. The rainbow trout were stressed the day before by netting. Within 14 days one carp died with KHVD symptoms (enophthalmus, round patches on the skin) in the aquarium with 20°C water temperature. KHV was detected by different PCRs. No clinical signs were found in carp in aquarium with 15°C water temperature. On 28$^{th}$ dpi (rainbow trout) and 21$^{st}$ day post cohabitation (dpcoh), from all surviving fish samples were collected non-lethally (serum, leukocytes, swabs from gill, skin and after) and lethally (gills, spleen, kidney).
While no KHV was detected in all samples from o dpi or o dpcoh (rainbow trout and carp), on 7$^{th}$ dpi samples (serum, leukocytes, swabs, organ tissues) from rainbow trout were positive tested by PCR. No reaction was observed with sera by serum neutralization assay (SNT) or antibody ELISA (ELISA). On 28$^{th}$ dpi, KHV was detected in organ tissues and indirectly in sera obtained from rainbow trout kept at 15°C by ELISA and SNT. Rainbow trout kept at 20°C water temperature were positive by PCR and by ELISA but not by SNT. In most of the surviving carp KHV was detected by PCR, SNT and ELISA.

# AN OUTBREAK OF VIRAL HAEMORRHAGIC SEPTICAEMIA (VHS) IN WRASSE COHABITING WITH ATLANTIC SALMON IN THE SHETLAND ISLES, SCOTLAND

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Viral haemorrhagic septicaemia (VHS) is an infectious disease of farmed and wild fish and has an extensive host range in both freshwater and marine environments. The causative virus (VHSV) is an enveloped negative-strand RNA virus belonging to the genus *Novirhabdovirus*, within the *Rhabdoviridae* family.
In December 2012, a wrasse population consisting of Ballan (*Labrus bergylta*), Corkwing (*Crenilabrus melops*), Cuckoo (*Labrus mixus*), Goldsinny (*Ctenolabrus rupestris*) and Rockcock (*Centrolabrus exoletus*), held at a seawater hatchery in the Shetland Isles, experienced a mortality event. Approximately 10,000 wrasse were being held at the facility on behalf of an Atlantic salmon (*Salmo salar* L.) aquaculture company for use as a means of biological control as part of their sea lice management strategy.
Diagnostic samples were sent to a third party and reported as VHSV positive by real-time reverse transcriptase PCR (qRT-PCR). Fish Health Inspectors from Marine Scotland Science were immediately informed and movement restrictions were applied on the suspect site, the site that supplied the wrasse and 16 A. salmon sites stocked with wrasse that were cohorts to those at the suspect site. Statutory sampling of the aforementioned sites was conducted as required by the Aquatic Animal Health (Scotland) Regulations 2009, in accordance with EU Council Directive 2006/88/EC; 30 fish test from the suspect site, 150 fish sample taken from all susceptible species stocked on the supplying site and 15 wrasse per site from the 16 sites containing cohorts to the suspect site. The 16 sites were initially considered as one population and with an increased sampling regime being implemented if VHSV were detected.
The site of initial suspicion was confirmed VHSV positive by virus isolation followed by ELISA (30/30) and by qRT-PCR (27/30). The supplying site screened negative and all Scottish mainland and Western Isles sites (11/16 sites) stocked with wrasse siblings also tested negative. However, the additional 5 sites, all located within the Shetland Isles, screened VHSV positive. Further testing of the 11 sites on the Scottish mainland and Western Isles at the 150 wrasse level produced negative results while 150 cohabiting A. salmon from each of the 5 positive sites in Shetland were screened for VHSV and produced negative results. Nucleic acid sequencing of the N- and G-genes was conducted and all isolates were > 99-99.8% similar at the nucleotide level and phylogenetic analysis determined that they belong to Genotype III. This suggests that the infection is uniquely connected to Shetland.

# THE CYPRINID HERPESVIRUS-3 (CYHV-3) USES LIPID RAFTS AS A MODE OF ENTRY INTO CARP CELLS

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The Cyprinus herpesvirus-3 (CyHV-3) is a member of the new *Alloherpesviridae* virus family in the *Herpesvirales* order. CyHV-3 has been implicated in a large number of disease outbreaks in common carp (*Cyprinus carpio L*.) which can cause up to 100% mortality. Some members of the *Herpesvirales* order have been shown to utilise lipid rafts as a mode of entry into cells. These lipid rafts have been identified in fish and are described as cell membrane microdomains enriched in cholesterol, sphingomyelin and certain types of protein. Lipid rafts are essential for signalling, trafficking, nutrient uptake, and they have also been implicated in virus entry and exit from a cell. The aim of this study was to investigate if an aquatic herpesvirus also utilises cholesterol-rich lipid rafts as a mode of entry into CCB cells. Firstly, a method was established facilitating the isolation and lipid analysis of cell membrane lipid raft microdomains, and the purified CyHV-3. Interestingly, the results showed that CyHV-3 and CCB cell lipid rafts contained similar lipid profiles, which suggested that during the budding step of the virus's cycle, the lipid envelope may have been acquired from lipid rafts, and therefore indicating that lipid rafts are required at some stage of the replication cycle. Secondly, the role of cholesterol and lipid rafts in virus entry was ascertained. For this, plasma membrane cholesterol was depleted from carp CCB cells with methyl-β-cyclodextrin (MβCD) in order to remove lipid rafts. The addition of MβCD was able to reduce the cholesterol content by 70% for at least 2 hours post incubation. Treated and non-treated cells were infected with CyHV-3 and the infection parameters were evaluated using RT-qPCR and immunocytochemistry. RT-qPCR results showed a significant decrease in the expression of CyHV-3 associated genes 48 and 120 hours post infection. However, cells depleted of cholesterol and then later replenished showed no change in gene expression levels. Similar data was also obtained using immunocytochemistry at 4 and 7 days post infection. The results show that CyHV-3 requires lipid rafts to enter cells and that lipid raft mediated virus entry is therefore conserved amongst herpes viruses.

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# PANCREAS DISEASE BATH CHALLENGE IN ATLANTIC SALMON FRY

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Salmon pancreas disease (SPD) is one of the most significant diseases affecting marine salmon farming in Europe. This study describes development of a bath challenge model with SPD virus in Atlantic salmon at the fry stage (average weight of 1.2g). Two salmonid alphavirus (SAV) genogroups were used: SAV 1 and SAV 5 at two viral doses: $10^6$ and $10^5$ $TCID_{50}$/ml for SAV 1 and $10^4$ and $10^3$ for SAV 5 in duplicate tanks. Tanks of salmon fry challenged with either genogroup showed very low cumulative percent mortalities of 1.2% or less. Virus was recovered from the majority of mortalities (24 of 30 deaths) which took place between 11 to 34 days post challenge. Titres of virus recovered from the mortalities ranged from $10^2$ to $10^7$ $TCID_{50}$/ml. Histopathological changes consistent with pancreas disease pathology were observed in moribund fish sacrificed and analysed.

Fry were sampled at 3, 5 and 7.5 weeks post challenge from one of the higher dose replicate tanks for each isolate tested. Between 15 and 30 fish on each sampling date were processed for virus isolation by inoculation onto CHSE-214 cells followed by specific RT-PCR confirmation of cells showing cytophatic effect. Between 15 and 20 fish of each sample were also assessed for histopathology and localisation of virus by *in situ* hybridisation. We observed a high prevelance of infection in fry in the absence of clinical signs and a general decrease in the proportion of positive fish within the samples as time post challenge increased. For SAV1, 80% of sampled fish were virus positive at 3 weeks post challenge reducing to 60% at 5 weeks and 13.3% for at 7.5 weeks post challenge.

The presence and localisation of viral genome was confirmed in sampled fish by *in situ* hybridisation (ISH) Presence was most easily identified in skeletal muscle. Heart showed a low level of labelling. Pancreas was difficult to study by ISH due to the infected fish showing dramatic pathology and loss of pancreatic tissues. Liver showed strong labelling but with high background in negative controls and strong labelling was also detected in the lamina propria of the intestine.

O-035

# INFECTION OF *ARTEMIA* SP. BY LYMPHOCYSTIS DISEASE VIRUS (LCDV)

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Genera in the family *Iridoviridae* are traditionally divided into two groups (probably representing subfamilies), mainly based on host range and level of genomic methylation. Members of the genera *Iridovirus* and *Chloriridovirus* infect invertebrates (e.g., insects and crustaceans). In contrast, members of the *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus* genera infect cold-blooded vertebrates such as fish, amphibians, and reptiles. The Lymphocystis disease virus (LCDV), which belongs to the genus *Lymphocystivirus*, is the causative agent of lymphocystis disease, a well-known pathology that affects more than 140 species of teleost fish from marine, estuarine and freshwater environments, with a worldwide geographical distribution.
The brine shrimp *Artemia* sp. is essential in the dietary regimen of larval stages of fish and crustaceans in aquaculture practice. *Artemia* nauplii have been considered as possible vectors for the introduction of different microbial pathogens into fish and shrimp rearing systems, including some viral pathogens. Recently, we have demonstrated that infective LCDV persists along *Artemia* life cycle after bath challenge, being LCVD-positive nauplii a possible vehicle of viral introduction in fish hatcheries.
In the present work, different developmental stages of *Artemia* sp. (metanauplius, juvenile and adult) were experimentally infected with LCDV by immersion. Results of viral quantification (both by qPCR and cell-culture viral titer determination) and expression showed that LCDV establishes a productive infection in *Artemia*, at least under experimental conditions, extending the host range of this virus to crustaceans.

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# DEVELOPMENT OF ALTERNATIVE METHODS FOR THE *IN VITRO* STUDY OF FISH HEART DISEASES: 3D SELF CONTRACTING CARDIOMYOCYTE AGGREGATES (SCCS)

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Infectious diseases are a major constraint to the aquaculture industry and several viral agents have been found to target the fish heart. Currently, *in vitro* research is based on non-cardiac tissues models, which can reflect some *in vivo* events but not necessarily those occurring at a higher degree of cell organization, i.e. the tissue and organ levels. Cellular models are increasingly used both in the human and veterinary fields. For example, human cardiomyocytes have been generated *in vitro* based on stem cell differentiation and recently, by applying a similar approach, autonomously beating cardiomyocytes generated from rainbow trout larvae have been proposed as a model for human pharmacological testing. The current study focuses on the development of primary cultures of self contracting cardyomyocytes (SCCs) from Atlantic salmon embryos for its particular application in the study of fish cardiac diseases. Results show that it is possible to optimise the generation of SCCs to achieve numbers required for trials and monitoring tools applicable to their small size (~up to 400µm) were developed to assess infection, including histological sectioning, immunostaining and qPCR. Initial infections using different isolates of heart targeting virus showed SCCs can be infected and that replication occurs, while the kinetics of response can also be followed.

O-037

# VERTICAL TRANSMISSION OF THE *TETRACAPSULOIDES BRYOSALMONAE* (MYXOZOA), THE CAUSATIVE AGENT OF PROLIFERATIVE KIDNEY DISEASE

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*Fredericella sultana* (Bryozoa) is the most studied and well known intermediate host for the *Tetracapsuloides bryosalmonae* (Myxozoan), the causative agent of the proliferative kidney disease in salmonids fish. *F. sultana* reproduces mainly asexually through the production of dormant stages named statoblast. Reproduction through colony fission was found to have a significant role in the transmission of the infection from infected individuals to the new developing one. In the present study, statoblasts collected from field samples of *F. sultana* colonies were tested positive by PCR for the presence of *T. bryosalmonae*. Statoblasts were also collected from laboratory *T. bryosalmonae* infected colonies and kept in our laboratory to hatch and produce new *F. sultana* colonies. These new colonies were cohabitated with specific pathogenic free (SPF) brown trout (*Salmo trutta*). Cohabitated brown trout were tested by PCR, which confirmed the transmission of the *T. bryosalmonae* from the bryozoan colonies to the SPF fish. Our results demonstrate that the parasite can be vertically transmitted through the statoblasts. This is the first report to detect the presence of the parasite *T. bryosalmonae* as cryptic stage in the dormant stage of the intermediate host.

# THE LIFE CYCLE OF THREE *MYXOBOLUS* SPP. AND TWO *THELOHANELLUS* SPP. (MYXOZOA) FROM FISHES OF LAKE BALATON AND KIS BALATON RESERVOIR

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Myxosporean parasites have dual developmental cycles. Their actinospore stages develop in oligochaetes, but the myxospores in fishes. After morphological analysis we have examined 18S rDNA sequences of actinospores isolated from oligochaetes of Lake Balaton and Kis-Balaton Reservoir and compared them with sequences of known myxospore stages.In between 2010 to 2012, we studied the natural infection of the oligochaetes, *Branchiura sowerbyi* Beddard, 1892, *Isochaetides michaelseni* Lastockin, 1936 and *Nais* sp. Müller, 1774. Eleven actinosporean stages (4 triactinomyxon-type, 5 aurantiactinomyxon-type, 1 echinactinomyxon-type and 1 raabeia-type) were found.
Previous studies (Molnar et al., 2009) revealed the occurrence, and followed the intrapiscine infection of *Myxobolus erythrophthalmi* Molnár, Eszterbauer, Marton, Cech, & Székely, 2009 from *Scardinius erytrophthalmus* Linnaeus, 1758 and *Myxbobolus shaharomae* Molnár, Eszterbauer, Marton, Cech & Székely, 2009 from *Alburnus alburnus* Linnaeus, 1758 in the renal interstitium, liver, testes and lamina propria of the intestinal fold of the fishes. In 2010 occurrences of three new *Myxobolus* species were reported from *Rutilus rutilus* Linnaeus, 1758 of Hungarian lakes and rivers (Molnar et al., 2010). Of these species *Myxobolus fundamentalis* Molnár, Eszterbauer, Marton & Székely, 2010 were collected from the connective tissue in the gill arch of the *R. rutilus*.
Our molecular data showed that three actinospores (Triactinomyxon-type 1, 2, 3) had 100% similarity to the *M. erythrophthalmi*, *M. shaharomae* and *M. fundamentalis* myxospores. In addition, partial sequence of Aurantiactinomyxon-type 1 isolated from a *Nais* sp., corresponded to the *Thelohanellus nikolskii* Akhmerov, 1955 showing 99.8% similarity. Another complete sequence analysis of Aurantiactinomyxon-type 2 collected from *B. sowerbyi* showed 99.4% match with *Thelohanellus kitauei* Egusa & Nakajima, 1981, though the myxospores of *T. kitauei* has not been recorded in Hungary hitherto.

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O-039

# COMPARATIVE SUSCEPTIBILITY OF BROWN TROUT (*SALMO TRUTTA*) AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) TO *TETRACAPSULOIDES BRYOSALMONAE* (MYXOZOA)

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*Tetracapsuloides bryosalmonae* is an enigmatic myxozoan parasite which causes proliferative kidney disease in salmonids. This parasite is found in Europe and North America and can lead to severe losses in trout farms and the associated economic impacts of this disease make it an important factor for aquaculture. Brown trout, *Salmo trutta* and rainbow trout, *Oncorhynchus mykiss* are an important cultured fish species of cold water aquaculture. In this study, difference in susceptibility to the *T. bryosalmonae* between brown trout and rainbow trout was examined chronologically. Specific pathogen free (SPF) brown trout and rainbow trout (mean length 5.5 ±0.5 cm, mean weight 2.3±0.5 gm) were exposed to mature spores of *T. bryosalmonae* at 18 $^{0}$C for 24 hours and sampled chronologically at 6, 8, 10, 12, 14 and 17 weeks post exposure (wpe). Additionally, *T. bryosalmonae* infected brown trout and rainbow trout were cohabitated with SPF bryozoa, *Fredericella sultana*. Parasite load was quantified in kidneys of both fish species by quantitative real-time PCR and parasite stages were examined in kidney, liver and spleen tissues by immunohistochemistry. Parasite load was higher in kidneys of infected brown trout compared to kidneys of infected rainbow trout at all time points except at 6 wpe. The number of the interstitial extrasporogonic parasite stages in the kidneys of brown trout was low and high in the kidneys of rainbow trout. High numbers of intra-luminal stages (sporogonic stages) were detected in the kidneys of brown trout, while, in rainbow trout, sporogonic stages were not seen during the investigation period. Additionally, extrasporogenic stages were low in the spleen and liver of brown trout and higher in rainbow trout. *F. sultana* colonies cohabitated with infected brown trout showed *T. bryosalmonae* stages, however, no visible sign of *T. bryosalmonae* stages were seen in *F. sultana* colonies cohabitated with infected rainbow trout. The results show remarkable difference in the development and infection progress of *T. bryosalmonae* in the host species, brown trout and rainbow trout.

# PROJECT IDASMYX, INFECTION DYNAMIC OF AQUACULTURE SEABASS AND SEABREAM BY MYXOZOA

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The project IDASMyxintend to study the infection dynamic of aquaculture European seabass and seabream by Myxozoa that are known to cause serious growing delays, with great economic losses. There are in Portugal at least 4 species reported (*Sphaerospora dicentrachi*, *Ceratomyxa labracis*, *C. diplodae* and *Myxobilatus* sp.) so far, which are quite frequent and their life cycle is unknown. For seabream we do not have systematic parasitological surveys in Portugal and none of the recorded Myxozoa species abroad has known life cycle. The new approach of this project is to determine the life cycle of Myxozoa, whose knowledge will allow in a near future to propose measures to control those infections. We intend under this project to characterize the life cycle of the species of Myxozoa known in seabass and seabream: adding important information on their ultrastructure; performing the molecular characterization of the forms actinosporean and myxosporean in order to match them and, that way, close their life cycles, and to provide an efficient tool of diagnose for each parasitosis; and also to describe their actinosporean stages in Annelida and test some infection conditions. In first place we will sample seabass and seabream, and other fishes, and annelids in aquaculture that produces fish in a semi-intensive way, in Algarve, in order to obtain infected tissues by species of Myxozoa and thus to study its ultrastructure and perform its molecular characterization. At the same time we will do experimental infections of Annelida, with myxosporean spores, where the infection relationship with the host sex and size will be evaluated. At last, we will select the sequence data from the species found either in the form of actinospore or myxospore and compare them with other sequenced available in the Genbank and thus evaluate the life cycle of some species.
A general overview of the first year results of the project will be presented.

O-041

# THE DISTRIBUTION OF *PARVICAPSULA PSEUDOBRANCHICOLA* IN WILD SALMONIDS IN NORWAY

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The myxozoan *Parvicapsula pseudobranchicola*, the causative agent of parvicapsulosis in Atlantic salmon (*Salmo salar*) in Norway, was first described from diseased seawater farmed salmonids in 2002. Since then, numerous cases of parvicapsulosis have been diagnosed each year and the disease represents a significant cause of losses in the Norwegian salmon farming industry. Analyses of wild and farmed salmonids show that the parasite is present both in the North and South of Norway. However, disease outbreaks are almost completely restricted to the northernmost regions. The reason for this is unknown, but among possible causes are: i) strain variation in parasite virulence, ii) differences in abundance of the invertebrate alternate host or iii) delayed, more intense release of infective stages in the sea in the north. The invertebrate host is not known, but is likely a polychaete. *Parvicapsula pseudobranchicola* is so far known to infect Atlantic salmon, sea trout (*Salmo trutta*), Arctic charr (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*) (PCR positive hosts), but pseudobranch infections with myxospores are only known from salmon and rainbow trout and the role of wild salmonids in the life cycle of the parasite is poorly known.
In order to get a more detailed knowledge on the distribution and prevalence of *P. pseudobranchicola*, we analysed samples from Atlantic salmon, sea trout and Arctic charr from a selection of populations along the entire coast of Norway in addition to samples from Russia and Denmark. Samples were analysed by real-time PCR and positive results were also confirmed by sequencing. The results from these analyses as well as a general update on parvicapsulosis will be presented.

# NEW SPECIES OF *UNICAPSULA* INFECTING ESOPHAGUS OF *SIGANUS SUTOR* CAUGHT OFF THE SOUTHERN WATERS OMANI COASTS

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As far as we are aware there are no studies on protozoan and myxozoan parasites from marine fishes of the Arabian Peninsula. During investigations on the parasite fauna of Siganids from the Coasts of the Sultanate of Oman (Sea of Oman and Arabian Sea), a myxozoan parasite belonging to the genus *Unicapsula* Davis, 1924 was revealed. Spore of this genus are unsual in that they have only one polar capsule (Lester, 1982). So far, this genus *Unicapsula* includes 8 species that were mostly reported from the gills of *Polydactilus quadrifilis* from Senegal (Dibakate et al., 1999), from the skeleton muscles of *Lithognathus morimirus* and *Spicara smaris* from the Mediterranean (Alama-Bermejo et al., 2009). The species discussed in this presentation is the first to be found infecting the esophagus lining of their host. Preliminary morphological results suggest that this species could be a new addition to the genus. Due to the economical importance of Siganids in the region and to their high potential for mariculture industry and owing to its vital site of infection, this parasite could be a potential threat to the future mariculture industry of the Sultanate of Oman. Light microscopy and ultrastructure microscopy results will be discussed and results of molecular analysis will be revealed.

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O-043

# MYXOZOAN INFECTIONS IN TUNISIAN MARINE FISHES

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Several myxosporean species are causing significant losses of fish in Mediterranean countries. In Tunisia, with the increase of fish culture in marine net-cages, knowledge of myxosporean species is imperative.
In this work, an overview of our research on myxozoan infections is presented. The parasitological surveys of marine fishes belonging to different families (Mugilidae, Labridae, Sparidae, Soleidae, Serranidae, Merluccidae, Carangidae, Trachinidae, Scorpaenidae…) revealed the presence of histozoic and cœlozoic myxosporean species.
From Mugilid fishes, 6 *Myxobolus* spp. were identified, and two of them were described as new species. Recently, additional myxosporeans have been found by Bahri et al. (2010) from Labrid fishes and by Yemmen et al. (2012 & 2013) from Soleid fishes. They described 1 *Henneguya*, 1 *Ceratomyxa*, and 1 *Zschokkella* species. These new species were compared morphologically and molecularly with previously described myxosporean species using optic and scanning electron microscopy as well as 18S rDNA sequencing. The potential damage that these species might cause in the organs of the fish was investigated on histological sections and by TEM.
In our study molecular phylogenetic data supported traditional taxonomic approaches for classifying members of the Myxozoa (Lom and Arthur 1989) while providing additional insights into the relationships between freshwater and marine *Myxobolus* spp. Molecular data have also revealed that species of *Henneguya* probably arose from ancestral *Myxobolus* several times during myxosporean evolution, and both marine and freshwater species of these genera form a polyphyletic clade. Moreover, our phylogenetic results prove again that the caudal appendages might not represent a valid character for distinguishing *Myxobolus* and *Henneguya*.
Recently, about 15 *Ceratomyxa* species were identified infecting the gallbladders of fish from Tunisian coasts, some of them have been previously described such as *C. sparusaurati* (Sitjà-Bobadilla, Palenzuela et Alvarez-Pellitero, 1995) from *Sparus aurata*, *C. Globulifera* (Thélohan, 1892) and *C. elongata* (Thélohan, 1895) from *Merluccius merluccius and C. herouardi* (Georgévitch, 1916) from *Sarpa salpa.*

# GILL PATHOGENS OF ATLANTIC SALMON (*SALMO SALAR*): LONGITUDINAL STUDIES IN MARINE FARMS IN IRELAND

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Gill disease in marine farmed Atlantic salmon (*Salmo salar*) represents a significant health and welfare challenge for marine salmon farms in both the Northern and Southern hemispheres (Mitchell & Rodger 2011). The losses due to gill disease include direct mortalities, in some cases poor growth and increased vulnerability to other pathogens. The aetiology of gill disease in marine salmonids is often multifactorial and complex, however, in some cases single pathogens or agents have been identified as causal (Young et al. 2007, Baxter et al. 2011). Gill disease in marine farms in Ireland has been a major cause for concern and the study described in this presentation investigated the involvement of three pathogens (*Neoparamoeba perurans, Tenacibaculum maritimum* and *Candidatus* Piscichlamydia salmonis) which have been previously associated with gill disease, through longitudinal studies on two marine farms. This report is complimentary to a previously published study on the role of hydrozoans in gill disease and aims to elucidate the involvement of these three pathogens with gill disease in marine farm environments.

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O-045

# CONTRIBUTION ON INFLAMMATION/CELL PROLIFERATION MARKERS TO THE STUDY OF RMS PATHOGENESIS

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Red mark syndrome (RMS) is a chronic non-lethal skin disease affecting rainbow trout (*O. mykiss*). It was first noticed in Scotland in 2003, spread rapidly throughout the U.K. and has also been reported in Austria, Germany, Italy, Serbia and U.S.A. Single/multiple skin lesions are macroscopically detectable on the flanks and ventral/dorsal body surfaces, ranging from small pink spots to large bright red areas. Histology shows a lymphocytic/macrophage infiltration in the dermis, enlargement of scale pockets and necrosis of the surrounding connective tissue. Advanced stages are characterised by osteoclastic resorption of scales, and lymphocytes infiltration into the subcutaneous adipose tissue reaching the underling muscle bundles. In the present study, samples of skin and internal organs from naturally infected trouts farmed in northern Italy were evaluated macroscopically and histologically; an immunohistochemical approach was also carried out aiming to study inflammatory cells recruitment and proliferation rate. The evaluation was performed considering the severity of the skin lesion according to Galeotti *et al*., 2011. The following markers were performed on 4 µm-thick sections from formalin/Bouin's fixed paraffin embedded specimens: rabbit anti human CD3 (A-0452, Dako); rabbit anti rainbow trout IgT (Prof. O. Sunyer); rabbit anti salmonid HSP70 (AS05061A, Agrisera); rabbit anti human GM-CSFRα (sc-690, Santa Cruz Biotech.); rabbit anti histamine (H7403, Sigma-Aldrich); rabbit anti-IL-1β (Santa Cruz Biotech.); mouse anti PCNA (2586, Cell Signaling Technology); mouse anti AE1/AE3 Cytokeratin (M3515, Dako); mouse anti E Cadherin (M3612, Dako). Anti CD3 and IL-1β did not cross-react. Anti trout IgT marked a limited number of scattered cells in the dermis, and numerous cells at the base of the intestinal mucosae. HSP70 positive cells were detectable within scale pockets involved in inflammatory changes. Numerous GM-CSFRα positive macrophage like-cells and some positive fibroblasts were scattered in the spongiosum derma, especially surrounding the scales. Anti-Cytokeratin and E Cadherin marked the epithelial cells. PCNA positive cells have been semiquantitatively scored (0=absence of staining; 1=up to 25% or 2=25-50% or 3=50-75% or 4=>75% positive cells) separately in epidermis, dermis and hypodermis. In the two latter mesenchymal areas PCNA positive cells were apart scored in infiltrating lymphocytes, vascular endothelium and stromal fibroblasts. Data were compared by the nonparametric Spearman correlation test among lesions macroscopically graded as initial, intermediate and severe. Any difference in proliferation was found in epidermis and in the 3 dermal components (3 compared groups). In hypodermis any reaction was apparent in endothelium and in fibroblasts but only lymphocytes were PCNA immuno-reactive. Statistic revealed, only for these latter, an increasing percentage of proliferating lymphocytes from initials to severe lesions.

# EFFECT OF WATER TEMPERATURE ON HEALING AND WOUND CLOSURE FOLLOWING ADIPOSE FIN CLIPPING OF ATLANTIC SALMON *SALMO SALAR*

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The aim of this study was to determine what impact routine adipose fin clipping may have on the overall welfare of Atlantic salmon, *Salmo salar* L, and to determine whether this is an appropriate method for batch marking large numbers of fish. This was done by describing the wound closure and initial healing processes following adipose fin clipping conducted at three different water temperatures. Three groups of Atlantic salmon (mean 36g; range 27,7-45,3g; n=66) were held at a constant 4°C, 10°C, and 14°C. Using scissors all fish were 100% adipose fin clipped following a distinct sequence within a 30 min period. Following clipping 6 fish were sampled, using the same sampling sequence, from each group at 11 time points (2, 4, 6, 12, 18, 24, 30, 36, 48, 60, and 72 h) post clipping. Using histology techniques samples were prepared and examined for re-epithelisation. Examination of histology slides indicated that the wounds were closed by an epidermal layer 4 h post-clipping at 10°C and 14°C. In contrast, wound closure took 12 h in fish held at a constant 4°C. An adapted score sheet was used for each epidermal and dermal cell group to determine whether there was a correlation between water temperature and healing rates (time). We found that the there was a positive correlation between water temperature and healing rates for the epidermis, but not for the dermis. The rapid healing rates following adipose fin clipping indicate that this is a low impact method and may be used to batch mark large numbers of fish.

O-047

# LIPOSARCOMA IN CLOWN FISH (*AMPHIPRION OCELLARIS*) PRODUCED IN INDOOR AQUACULTURE

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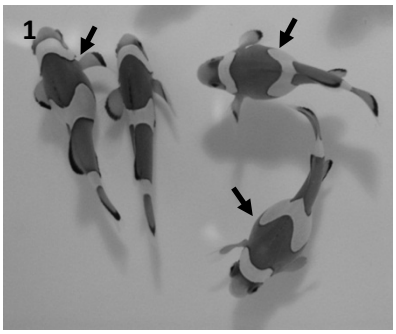


Clown fish (*Amphiprion ocellaris)*, produced and grown in an experimental indoor aquaculture facility, presented soft-tissue tumors consistent with a well-differentiated liposarcoma.
A total of 14 affected fish were examined and the extent of the occurrence was estimated at 1 out of 300 fish. Affected fish had a distended abdomen (Fig 1; arrows). Most fish appeared to behave normally, except for the few with the largest abdomen for which swimming was more laborious.
At dissection, a whitish mass filling the whole body cavity was seen grossly. The mass engulfed all internal organs but easily separated from the muscle tissues. Histological analysis revealed that the whitish mass was composed of round, clear cells with marginated nuclei, a characteristic morphology for lipid-filled cells, conferring them the appearance of mature adipocytes. These cells filled the body cavity, engulfing and breaking down internal organs and musculature. Aggregates of the adipocyte-like cells were present in different tissues and organs, appearing as distinct masses from the main tumor in the abdominal cavity (Fig 2). The morphology of the mass is characteristic of a malignant fatty tumor, liposarcoma. This is the first report of such a tumor in fish.

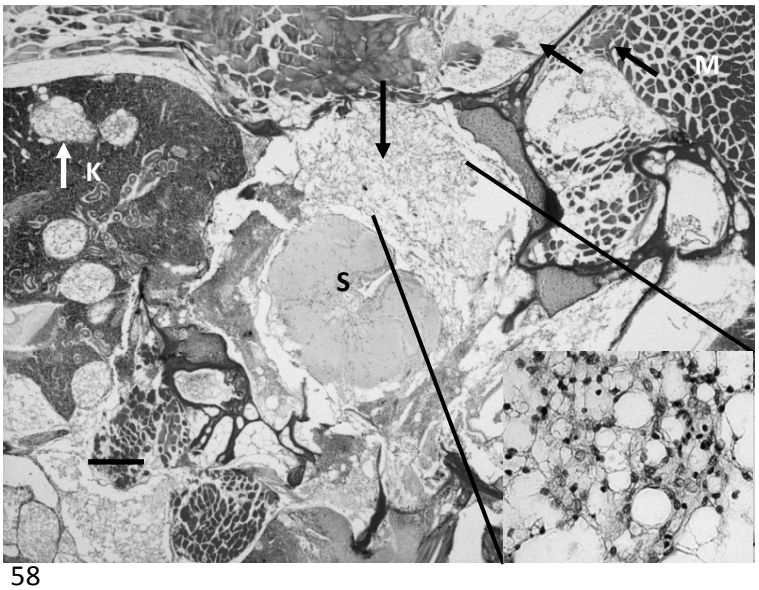


Fig 2: Histological section showing the liposarcoma (arrows) penetrating the kidney (K), surrounding the spinal cord (S) and invading the muscle (M); bar = 500 µm.

# THE COMPARISON OF HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN GOLDEN GREY MULLET *(LIZA AURATUS*) IN SOUTHERN CASPIAN SEA INFECTED BY A NEW EMERGED INFECTIOUS DISEASE

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Following of some several reports about new mortality and observation of clinical signs consist of darkening, abnormal swimming (spiral, corkscrew and belly up) and abdominal distention in Golden grey mullet (*Liza auratus*), about 116 fish samples were captured during July till March 2010 of fisheries cooperatives in Mazandaran and Golestan provinces in Iran. Then 56 samples with mentioned clinical signs were selected with 50-250 gr weight and after anesthesia with Clove powder, fish biometry were done. Then blood samples (0.5-1$^{cc}$) were taken from caudal peduncle of the mentioned fish and transferred into Ependrof tubes containing anticoagulant (one drop or 1$^{cc}$ heparin). Some of the most important hematological parameters were evaluated, including R.B.C., W.B.C., P.C.V. (Packed cell volume), Hb (Hemoglobin) and erythrocyte indices, M.C.V (Mean corpuscular volume), M.C.H (Mean corpuscular hemoglobin), M.C.H.C (Mean corpuscular hemoglobin concentration) and differential of blood cell count. Also some serum indices such as ALT, AST, C3, C4, IgM, Albumin and total protein were measured. Findings in winter captured fish revealed significant decrease ($p<0.05$) in R.B.C, Hb, P.C.V. and (MCHC, g/dl) in infected fish comparison with health fishes. In opposite, (MCV, fl) were more significant increase in infected fish than health fishes but (MCH, pg) was no significant difference between two mentioned groups. Also, total IgM, protein and Albumin have significant decrease ($p<0.05$) in infected fish comparison with health fishes. Although C3 and C4 revealed numerical decrement but have no significant difference between two groups. In opposite, AST and ALT enzymes were more significant increase ($p<0.05$) in infected fish than health fishes. So, according to decrease of (MCHC) and increase of (MCV) in examined fishes, it would be determined that infected fish have been revealed macrocytic hyperchromic anaemia. Also, regarding to obtained results infected fishes have been suffered from severe protein catabolism or dystrophy. In fact, according to clinical signs, haematological and biochemical findings, it could be concluded that a kind of infectious dystrophy chronic disease was occurred between Golden grey mullet in recent years in southern Caspian Sea.

*Key words: Liza auratus, Caspian Sea, haematology, macrocytic hyperchromic* anaemia

O-051

# A COHORT AND CASE-CONTROL STUDY ON RISK FACTORS FOR CARDIOMYOPATHY SYNDROME IN NORWEGIAN SALMON FARMING

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Cardiomyopathy syndrome (CMS) has been known as an economically important disease in Norwegian aquaculture since the 1990'es. Until recently, the aetiology of the disease was unknown, and many different hypotheses towards agent and/or environmental factors have been put forward. A cohort study was therefore designed where data from official registers on monthly production characteristics and case registrations from the Norwegian Veterinary Institute were combined and supplemented with a questionnaire-based case-control survey collecting data on management, in order to identify risk factors for CMS.
In the cohort study, all cohorts with Atlantic salmon that were put to sea and slaughtered out between January 2004 and January 2013 were included. A model was then made where differences between cohorts which got CMS during the production period and those that did not were analysed by a mixed effect multivariate logistic regression. From this model, we found that the probability of CMS increased with increasing time in sea, infection pressure and size of cohort, and that cohorts which had previously been diagnosed with heart- and skeletal muscle inflammation or which were in sea-sites with a history of CMS in previous cohorts had double the odds of getting CMS.
The model was then used to calculate the predicted value for getting CMS for each cohort from which additional data were obtained via the questionnaire-based survey. This value was used as an offset for calculating the probability of CMS in a semi-univariate analysis of each additional risk factor. From this, we found a significant association between CMS and the use of oxygen-logging on sites. The use of oxygen-logging may likely be an indicator that the site has a problem with unstable oxygen-levels on the site, which have been speculated to be a risk-factor for CMS.
Finally, the model was used to calculate probability of CMS in three different hypothetical cohorts: one with excellent conditions, one with poor conditions and one from the median of the dataset. This exercise we believe is a good way of communicating the findings to the farmers, so they can make informed decisions when trying to avoid CMS in their fish cohorts.

# RISK CATEGORISATION FOR AQUATIC ANIMAL HEALTH SURVEILLANCE

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We developed a methodology for categorising (risk ranking) fish farms using farm and disease specific characteristics to develop risk based surveillance for demonstration of disease freedom (EU Directive 2006/88/EC). To inform development of the methodology, the published literature on risk-based surveillance in terrestrial and aquatic animals and of disease characteristics of the 6 fish diseases listed in 2012 under EU Directive 2006/88/EC was reviewed.
The resulting model calculates a quantitative risk score for individual aquaculture sites. The basic principle of the model is to establish risk scores for individual risk themes: A) live fish movement, B) water, C) on-farm processing, D) short-distance mechanical transmission, E) distance-independent mechanical transmission. The scores for these themes are then combined into an overall risk using weights for each theme. The final calculated risk score is a value between zero and one and is intended to indicate the risk of a site relative to the risk of other sites (thereby allowing ranking). The model is suited for assessment of individual fish farms to rank farms to support surveillance to demonstrate disease freedom.
An overview of the project, background for model development and the model itself are presented. Potential model applications and possible solutions for efficient data collection are discussed.

*The work was undertaken in a cooperation Art 36 project "Risk categorization for Aquatic Animal Health surveillance" (CFP/EFSA/AHAW/2011/03) of the European Food Safety Authority (EFSA).*

O-053

# RISK BASED SURVEILLANCE OF AQUACULTURE FACILITIES: FROM THEORY TO PRACTICE

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To guarantee equivalence to the council directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals, Switzerland needs to establish a risk-based surveillance of aquaculture facilities. The Centre for Fish and Wildlife Health (FIWI) therefore initiated a first project in 2009 which aimed at developing a model for risk-based surveillance of fish farms regarding the viral hemorrhagic septicemia (VHS) and the infectious hematopoietic necrosis (IHN). The model included 6 factors to assess the risk of an introduction of VHS and IHN into a fish farm, as well as 7 factors to assess the risk of the spreading of the two diseases from an aquaculture facility. The data used to feed the model were collected by means of a questionnaire that was sent to all aquaculture facilities. Based on the model calculations, the farms were then classified into different risk categories. These risk categories form the basis to determine the control frequencies for an effective disease surveillance in aquaculture facilities.
However, several factors (e.g. factors regarding biosecurity) could not be collected by means of the questionnaire. Moreover, the accuracy of the data provided by the fish farmers is uncertain. Consequently, a follow-up project presented here aims to verify the data collected with the questionnaire, and accordingly the risk categories calculated in the model, by means of aquaculture inspections in 4 selected pilot cantons (Bern, Vaud, Valais and Zurich) covering almost 40 per cent of all Swiss aquaculture facilities. Within the scope of these inspections, missing data (especially regarding biosecurity) have been collected. On the basis of this actualized and newly collected data, the risk classification shall be validated and optimized if necessary. At the same time a control handbook has been developed including the relevant points to be controlled while visiting farms. Performing the visits allows to estimate both, time and financial resources respectively for routine controls of all Swiss aquaculture facilities. This project will provide the authorities in charge with an effective tool that allows an applicable risk-based disease surveillance of the Swiss aquaculture facilities in compliance with the aquaculture directives of the European Union.

# SPATIO-TEMPORAL RISK FACTORS FOR VIRAL HAEMORRHAGIC SEPTICAEMIA IN DANISH AQUACULTURE

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Viral Haemorrhagic Septicaemia (VHS) is an economically very important fish disease in most of the world. When VHS virus was first isolated in Denmark 50 years ago, around 80% of the approximately 800 Danish fish farms were considered to be infected, but vigilant surveillance and stamping-out programmes have led to a drastic reduction in prevalence, and eventually a final eradication of VHS. The disease have now been absent in Denmark since 2009.
Data on outbreaks within the country has been collected throughout the years, and recently all farms have been georeferenced. This has made it possible to use spatio-temporal scan statistical tools to search for clusters of high prevalence. Such tools have previously been applied on terrestrial animal diseases, but this is the first time they are used for aquatic animal diseases.
The analyses revealed a statistically significant cluster in the south-western part of the country, which persisted throughout the study period 1982-2008. Three additional spatio-temporal clusters in different time periods were also identified.
Further statistical analyses were performed on a subset of the farms;
A semi-univariable analysis showed that type of production (marine/freshwater) was not important for VHS, when accounting for whether the farm was situated inside a cluster of high risk.
A further analysis was performed on inland freshwater farms where the effect of year, number of farms in a stream and number of upstream farms on the probability of VHS was investigated. Being situated inside one of the identified clusters or not was also included as a risk factor. The variables; year, inside/outside a cluster and number of upstream farms were all significant risk factors for VHS ($p<0.001$).
The spatio-temporal scan tools provide an easy method for determining high-risk areas, also in aquaculture. Further, some important risk factors were identified. Both are valuable contributions when designing risk-based surveillance as required by the current European aquaculture animal health legislation. The Danish case is the first example of successful eradication of VHS in an endemic area.

O-055

# RISK CATEGORISATION OF FRESHWATER SALMONID FARMS – A CASE STUDY FOR VIRAL HAEMORRHAGIC SEPTICAEMIA

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A model developed to calculate a quantitative risk score for individual aquaculture sites was applied to risk score 74 rainbow trout farms in 2 countries (42 from England, 32 from Italy) for their risk of being infected with viral haemorrhagic septicaemia (VHS). The disease-specific risk score indicates the risk of the site being infected, and is intended to be used for risk ranking of sites to support surveillance for demonstration of zone or member state freedom from a specified disease. The inputs to the model include a range of quantitative and qualitative estimates of risk factors organised into risk themes.
The diversity of infection statuses of farm sites (and corresponding category I-V status according to Directive 2006/88) in the study countries was reflected in the scorings results. It showed that the model clearly is suited to risk rank farms. Requirements for broader application of the method, including options for cost efficient farm data collection, will be discussed.

*The work was undertaken in a cooperation Art 36 project "Risk categorization for Aquatic Animal Health surveillance" (CFP/EFSA/AHAW/2011/03) of the European Food Safety Authority (EFSA).*

# ANTIGENIC PROFILE OF *ENTEROMYXUM LEEI* (MYXOZOA)

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*Enteromyxum leei* is an intestinal myxozoan species affecting a wide range of fish and causing important economic loses in gilthead sea bream farms. The parasite was partially purified from intestinal extracts of infected fish, mainly containing spores and some disporous sporoblasts. Band profiles of *E. leei* were obtained by SDS-PAGE and Western blotting with a polyclonal antibody (Pab) against the parasite. The carbohydrate composition of the parasite's antigens was analyzed in periodic acid/Schiff stained of gels, in periodate and proteinase K treated immunoblots and in Lectin blots. Additionally, the cross-reaction of the parasite with a Pab raised against the polar filament of the myxozoan *Myxobolus pendula* [1] was studied.

Both Pabs detected proteic epitopes on antigenic proteins and glycoproteins of *E. leei* in a broad molecular weight range. Particularly, two glycoproteic bands (15 and 140 kDa), immunoreactive to both Pabs and with glucose and mannose moieties, could correspond to common antigens shared between *E. leei* and *M. pendula*. The 140 kDa band presented also galactose, N-acetyl-galactosamine and N-acetyl-glucosamine, pointing to its possible origin on chitin-built spore valves and to its possible involvement in host-parasite interactions.

Several proteases with apparent molecular weights ranging between 45 and 245 kDa were found in zymographies of *E. leei* extracts, which may have a potential role in the parasite's dispersion and pathogenesis, but still deserve further investigation.

To conclude, the heterologue anti-*M.pendula* Pab has been proved to cross-react with some cnidarian nematocysts in support of the phylogenetic affinity between this phylum and Myxozoa [1]. Interestingly, the 15 kDa glycoproteic antigen matches for its molecular weight with minicollagen, a cnidarian-specific protein of nematocysts with a myxozoan homologue, but still needs to be confirmed.

[1] Ringuette MJ, Koehler A, Desser SS (2011) Shared antigenicity between the polar filaments of myxosporeans and other Cnidaria. *Journal of Parasitology* 97:163-166.

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O-057

# HOST PARASITE INTERACTIONS BETWEEN *KUDOA THYRSITES* (MYXOZOA) AND ATLANTIC SALMON (*SALMO SALAR*) DURING EARLY STAGES OF INFECTION

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*Kudoa thyrsites* is a marine myxozoan with broad geographical and host ranges. The parasite localizes within myocytes and is the cause of post-mortem myoliquefaction. Infections are not known to negatively affect the host but have economic repercussions on fisheries and aquaculture. In certain regions of British Columbia, Canada, infections in farmed Atlantic salmon cause product discards and an overall decrease in the competitiveness of BC's salmon farming industry. Observations of the life cycle of *K. thyrsites* are limited to development within infected myocytes. Infections have been documented within two months after entry to seawater, but no studies have applied intensive sampling during initial exposure. *K. thyrsites* is suspected to migrate through the circulatory system but the route of entry is unknown.

We followed naïve smolts after their introduction to a commercial aquaculture site known to be high risk for *K. thyrsites* infections. During one trial we sampled daily for three days, weekly for five weeks, then approximately monthly for 10 months. Muscle, blood and epithelial tissues were lethally collected for PCR screening, histology, and *in situ* hybridization (ISH). The first indication of infection was in blood collected from fish sampled three days post entry. PCR prevalence increased to over 50% by 71 days. Blood, muscle and gill were predominantly infected between 71 and 100 days, but after 100 days muscle was the most common site of detection. Visual evidence of infected muscle began at 141 days. 64 gill and 25 skin samples were tested for *K. thyrsites* using ISH with a specific DNA probe. A positive signal was observed in 20 to 25 % of gills sampled between 85 and 100 days. For trial two, we repeatedly sampled two groups of tagged fish. Blood and muscle biopsies were collected up to four times from individual fish at 12 week intervals. These fish had high prevalence in blood between 71 and 99 days post entry, but not during three time points from 147 to 237 days. Together these PCR results support the existence of a blood borne stage preceding myocyte infection as a component of the life-cycle of *K. thyrsites*.

# DANCERS IN THE BLOOD: A FUNCTIONAL APPROACH TO UNDERSTANDING THE MOTILITY OF *SPHAEROSPORA MOLNARI* (MYXOZOA) PROLIFERATIVE STAGES IN THE BLOOD OF COMMON CARP

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*Sphaerospora molnari* has been associated with gill and skin sphaerosporosis since the 1980s. Sphaerosporids are widely known for infecting fish and amphibians, this group is also known to have large, motile presporogonic blood stages circulating in the hosts blood. At present, there is limited functional data about early myxozoan parasite development including cell division, nutrition and in regard to blood stages, their motility. Our objective was to investigate proteins involved in cell development, nutrition and motility of *S. molnari* blood stages. We used different microscopy techniques (TEM, SEM and confocal) and protein inhibitors to uncover some of the mysteries surrounding this parasite, its development and unique "dancing" behavior in the blood. In addition, we used total RNA from presporogonic blood stages to detect the genes of interest. We present the preliminary results of these analyses and their implications for our understanding of the biological and physiological requirements of myxozoan proliferative blood stages, and discuss their potential applications for disease management in European aquaculture.

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O-059

# FIRST LOOK AT THE GENOME OF *CERATOMYXA SHASTA*, A MYXOZOAN PARASITE OF SALMONIDS

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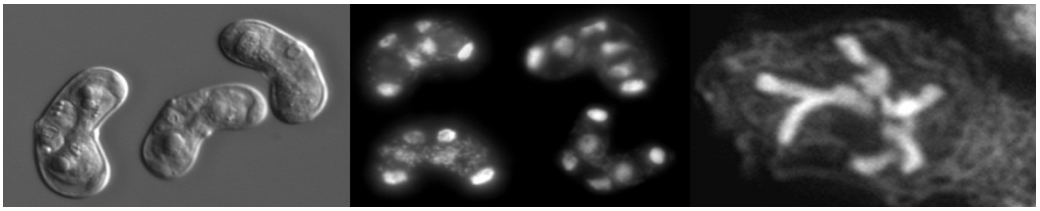
Myxozoans are a common, speciose, yet persistently enigmatic group of parasitic Cnidaria. Here we present the first multi-disciplinary characterization of the genome of a myxozoan, *Ceratomyxa shasta*, an economically and ecologically important intestinal parasite of salmon and trout in North America.

**Methods.** We determined the chromosome number of *C. shasta* through examination of mitotic myxospore sporogenic stages using both standard microscopy and laser scanning confocal techniques. We estimated genome size using flow cytometry, fluorescence microscopy and genome sequencing. We determined the relative copy numbers of ribosomal RNA genes and mitochondrial genes to known single-copy nuclear genes using SYTO9 realtime PCR assays. We sequenced the genome from total DNA extracted from purified myxospores from a single infected rainbow trout, via shotgun sequencing using both a Roche GS-Junior 454 and Illumina HiSeq2000 with 100nt paired-end reads. The genome was assembled using Velvet, SOAP denovo and CLC Genomics Workbench. Genome coverage was estimated using K-mer search strategies, coverage plots and BLAST searches. We identified putative transcripts using Maker, based on the consensus of *ab initio* methods (Augustus) and sequence homology with proteins from another Cnidarian, *Nematostella vectensis*. Putative functions of predicted transcripts were assigned based on BLASTX comparisons with UniProt.

**Preliminary results.** Microscopy suggested that the *C. shasta* genome is organised into 3 pairs of chromosomes. We obtained divergent values of genome size depending on the technique used: ~100Mb (genome sequence data) to 300Mb (flow cytometry and fluorescence microscopy). 454 sequencing produced ~45Mb data in 108k reads, which did not cover the entire genome. Subsequent Illumina data comprised ~400Gb, from which our best draft genome assembly was produced using Velvet and comprised 27,700 scaffolds (total assembly size 102Mb; $N_{50}$=21.3Kb; <1% unknown sequence). Coverage of known single-copy genes was estimated at ~300x. Coverage of the known multi-copy ribosomal rRNA gene array was estimated at ~100,000x; which was only ~330x greater than the single copy gene coverage, much lower than the 1,000x – 100,000x relative abundance expected from previous studies. We will present an update of these results, plus qPCR and genome composition analyses.



# REVEALING CRYPTIC DIVERSITY OF MALACOSPOREAN PARASITES (MYXOZOA: CNIDARIA) USING MOLECULAR PHYLOGENETICS

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Malacosporeans represent a small fraction of myxozoan biodiversity with only three described species belonging to two genera. They cycle between the bryozoans and freshwater fish. In this study, we (i) PCR screen different freshwater/marine fish species from various geographic locations; (ii) perform the rDNA and EF-2 based phylogenetic analyses of all available malacosporean data, and (iii) trace the host species and geographic data on the phylogenetic tree to improve the understanding of the biodiversity, distribution and evolutionary trends within malacosporeans. In all analyses, malacosporeans created a sister lineage to the myxosporeans and showed a partial trend in their clustering according to the host species and biogeography. We discovered the existence of six new *Tetracapsuloides* species, three new *Buddenbrockia* species and one new malacosporean genus. Co-infections of up to three malacosporean spp. were found in one fish specimen of several fish species. Significantly increased species richness in the Malacosporea (5 times) shown in present study points out on the hidden biodiversity within this parasitic group. The finding of a new *Buddenbrockia* species in the marine fish indicates that malacosporean life cycles might exist in the marine environment which would be reasonable due to the fact that the major part of bryozoans are marine species.

O-061

# INTRASPECIFIC VARIABILITY OF FRESHWATER *MYXOBOLUS* (MYXOZOA) SPECIES ON THE BASIS OF SSU rDNA: DOES TISSUE PREFERENCE AFFECT PARASITE SPECIATION?

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*Myxobolus* Bütschli, 1882, involving over 750 species, is the largest genus within Myxozoa. Most species are histozoic parasites infecting freshwater fish. Despite the simplified body organization of the myxospore developing in fish host, the identification of *Myxobolus* spp. is still based on spore morphology mainly; however organ- and tissue location as well as the identity of host species or range of hosts are also essential features for exact species assignment. With the common use of DNA sequence data in species descriptions, the taxonomy of Myxozoa has gathered renewed momentum. In GenBank, approximately 600 *Myxobolus* entries can be found now, over 90% of which are SSU rDNA sequences of about 100 species. Although in the 1990's, authors mainly deposited the DNA sequence of one isolate per species, nowadays, the batch submission of isolates is gaining popularity. Using the GenBank database and our own SSU rDNA data, obtained in the course of studies of freshwater *Myxobolus* species in the last 12 years, it has become possible to analyze intraspecific variability in these parasites. Comparing the sequence data and the additional information about the examined *Myxobolus* species (i.e. host species, tissue location, geographic data etc.), different variability levels were observed. For some species, isolates were 100% identical, whereas for others up to 5% difference was detected among the SSU rDNA of the isolates. The analysis showed that besides geographic location, the parasite's tissue preference was the main feature in correlation with intraspecific variability. The species developing cysts intramuscularly show much higher variability among isolates than those from connective tissue, epithelium or from the tissues of internal organs. Whether tissue preference affects the recombination between genetic clones and thereby has influence on the speciation of *Myxobolus* spp., or if it is only a coincidence, is as yet unclear. But differences in the intrapiscine developmental pathway and in the prevalence of parasite in natural habitats may supply some explanation.

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# MOLECULAR TAXONOMY OF MYXOZOA: CRYPTIC AND NEGLECTED SPECIES

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The phylum Myxozoa is a vast assemblage of microscopic metazoan parasites with simplified morphology and complex life cycles. For many decades, the taxonomy of Myxozoa was based solely on the morphology of myxospores with an emphasis on the number and shape of shell valves and number of polar capsules. With the rapid increase of genetic and genomic knowledge we explore a molecular taxonomy era (mostly based on SSU rDNA). However, it is necessary to combine both morphological and molecular approaches to describe the diversity of myxozoan species.
Thanks to the sensitivity of the PCR method, we are able to detect not only species confirmed microscopically but also myxosporean DNA of overlooked mixed infections and/or morphologically identical but genetically diverse species known as cryptic species. Recently, sequencing of the type species of the genus *Sinuolinea*, *S.dimorpha*, from the type host *Cynoscion regalis* revealed two different SSU rDNA sequences suggesting the presence of cryptic species. Cryptic species are also known in several other myxosporean genera, such as *Chloromyxum*, *Kudoa* and *Cystodiscus*.
We have amplified SSU rDNA of *Unicauda* sp. from *Gnathonemus petersii*. *Unicauda* myxospores present in the infected fish were morphologically identical; however, comparison of sequences from several independently amplified PCR products revealed the presence of three genetically distinct but closely related cryptic species. Analogous results were obtained by sequencingof myxozoan species considered to be *Myxoproteus ambiguus* from the urinary bladder of the type host *Lophius piscatorius.* Molecular data reveals two very different SSU rDNA sequences clustering in two separate clades on the phylogenetic tree. PCR screening of DNA samples of many host urinary bladders from two different geographical regions revealed the presence of both myxosporean SSU rDNA sequences. Detailed morphological examination of all samples, however, suggested the presence of only one myxosporean species – *M. ambiguus* and the morphology of neglected species remains unknown.
Accumulating cases of neglected mixed infections or the presence of cryptic species warrant thorough sequencing of at least three PCR products of newly molecularly characterised myxosporean species.

O-063

# PARASITOLOGICAL, PHYSIOLOGICAL AND MOLECULAR METRICS OF RESISTANCE TO *LEPEOPHTHEIRUS SALMONIS* AMONG PACIFIC SALMON (*ONCORHYNCHUS* SPP.)

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The salmon louse is an important pest in salmon aquaculture throughout the northern hemisphere. Infections on farmed salmon cause significant economic loss and may be of ecological significance to juvenile wild salmonids. The susceptibility to infection varies considerably among species of Pacific salmon however resistance mechanisms remain largely unknown. The purpose of this paper is to review parasitological and physiological measures of susceptibility and to describe novel molecular data that provides insight into putative defence mechanisms. In comparative infection trials, juvenile chum (*Oncorhynchus keta*) and Atlantic salmon (*Salmo salar*) consistently carried more lice when compared with pink salmon (*O. gorbuscha*). Also, higher infection intensities were associated with reduced haematocrit and elevated plasma cortisol in sockeye (*O. nerka*) and chum salmon and whereas inflammatory lesions were more pronounced in the relatively resistant pink and coho (*O. kisutch*) salmon. Transcriptomic evidence from the skin or head kidney of *L. salmonis*-infected salmon indicated differences in susceptibility are associated with inflammation and the acute phase response and with pathways of adaptive immunity. In addition, upregulation of the expression of genes associated with iron retention correlated with resistance to *L. salmonis*. An integrated model of resistance to *L. salmonis* in salmonids and the application of this knowledge in parasite management will be discussed.

# CRAYFISH PLAGUE PATHOGEN *APHANOMYCES ASTACI* - GENETIC DIVERSITY AND ADAPTATION TO HOST SPECIES

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The crayfish plague disease is caused by Oomycete *Aphanomyces astaci*. The native European crayfish species are highly susceptible to this disease and their population sizes are still generally declining, largely due to *A. astaci* epidemics. In this study, variation in the genetics and in the virulence properties of the *A. astaci* strains were investigated. Furthermore, the sporulation of *A. astaci* during the acute infection of the noble crayfish *(Astacus astacus)* and in the chronically infected signal crayfish *(Pacifastacus leniusculus)* was studied. The genetic studies revealed that there was low intraspecific variation in the ribosomal internal transcribed spacer regions of *A. astaci* isolates. In the chitinase genes, high genotype specific diversity was observed among As, Ps, and Pc-genotypes. In infection experiments in laboratory, significant differences were observed in the virulence of different *A. astaci* strains. The tested *A. astaci* strains of PsI-genotype, linked to the signal crayfish introductions, killed 100 % of the infected noble crayfish *(A. astacus)*. The strains of the As-genotype, linked to the first arrival of the disease with unknown original host, were more variable and some individuals survived the experimental infection. The sporulation studies showed that in an infected noble crayfish, the maximum peak in the sporulation occurs 1-3 days *post mortem*. In the chronically infected signal crayfish, sporulation was constant and the highest spore densities were released under stress, moulting and death. The results of this study emphasize that the PsI-genotype of *A. astaci* is highly virulent and it most likely retains its virulence, since its original host species has been imported into and is present in Europe. Furthermore, the carrier crayfish are constantly sources for new spores and therefore pose a continuous risk to the surrounding native noble crayfish populations. Therefore, further spreading of the signal crayfish should be prevented. The carrier status analyses of the noble crayfish should also be considered prior to stockings in order to avoid spreading and mixing of *A. astaci* strains that subclinically infect noble crayfish.

O-065

# BEHAVIOURAL ADAPTATIONS OF ARGULID PARASITES (CRUSTACEA: BRANCHIURA) TO MAJOR CHALLENGES IN THEIR LIFE CYCLE

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Argulids are obligate monoxenous ectoparasites that can theoretically complete the whole cycle on a single fish host. However, in reality they have to find and attach the host many times during their life. Necessity to repeatedly find hosts and mates makes behaviour as important for these ectoparasites as for free living crustaceans. They have to find a host: soon after hatching; when switching to most suitable hosts; when searching for mates; after laying a clutch of eggs on a substrate. Host specificity and low density of host populations makes searching tasks extremely difficult. Two common palearctic species, *Argulus foliaceus* and *A. coregoni*, inhabit many Finnish lakes. *A. foliaceus* live on wide range of fishes, while *A. coregoni* – on salmonids. What behavioural mechanisms could help argulids to find hosts? What factors underlie differences in host specificity? Could argulids modify fish behaviour to make them more available? Argulids are well equipped for swimming, host detection and attachment. We described two host searching tactics based on different sensory modalities: “hovering” (sit-and-wait tactic) in mid-water in the light (vision), and “cruising” in the dark (olfaction and mechanical sense). Use of the two tactics allows parasites search for hosts day and night. We showed that vision is of primary importance, especially in early ontogeny, when parasites preferably attacked the brightest fish. Later, *A. coregoni* developed preference for salmonid fish, while *A. foliaceus* remained nonselective. We found that switching to salmonids was innate and occurred when olfaction became important. Large adults of *A. coregoni*, especially gravid females, were found to be very sensitive to oxygen deficiency. This could explain their preference for salmonids, inhabitants of well oxygenated waters. Smaller *A. foliaceus* easily tolerate low oxygen concentration, which allows them to live on various fishes, including those that could stay in poorly oxygenated waters. Adult argulids leave their hosts rather often (males for mate searching, females for egg laying) and would benefit from high local density of fish. We observed that juvenile rainbow trout attacked by argulids swam slower, stayed closer to each other, and reduced aggression. Such behaviour, facilitating encounter and attachment, resembles host manipulation.

# HOW DO AGONISTIC AND COOPERATIVE INTERACTIONS AMONG FISH AFFECT RISK OF *DIPLOSTOMUM SPATHACEUM* INFECTION?

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Influence of host behaviour on intensity of infection, prevalence, and distribution of parasites within host population attracts increasing attention. Animals avoid relatively large and easily recognizable parasites and microhabitats with high parasite abundance, thus reducing risk of infection. Does this work when parasites are small and virtually undetectable? This is a common situation in fish-trematode interactions, when fish, the intermediate hosts, could hardly recognize tiny cercariae. What other than leaving the risky habitats behavioural mechanisms could mitigate risk of infection? We studied interactions between *Diplostomum spathaceum* cercariae and 0+ rainbow trout, *Oncorhynchus mykiss*, in laboratory experiments on solitary and grouped fish under homogeneous or structured habitats, where fish could behave either agonistically or cooperatively. Given a choice between an area free of parasites and area with high concentration of cercariae, fish faster learned to avoid risky area when in group than as solitary fish. Mean intensity of infection in grouped fish was almost twice lower with higher inter-individual variability. Some individuals in groups remained not infected, while others were heavily infected. Learning and exchange of information were suggested to underlie the observed effects. Gills served as the main entrance of *D. spathaceum* ceracriae into the fish, and ventilation rate of fish host appeared to be a major determinant of infection rate. Therefore, higher ventilation brings more *D. spathaceum* cercariae to the stressed fish. In homogeneous habitats with even distribution of parasites, individual solitary fish got more parasites than fish in groups, probably due to isolation stress. However, an opposite effect, lower mean infection in solitary fish than in grouped fish, was observed in heterogeneous habitats, where fish contested for shelters. Within a group, dominant, presumably less stressed, fish got fewer parasites, than subordinates. Agonistic interactions in groups resulted not only in elevated mean infection rate, but also increased inter-individual variation and led to highly aggregated parasite distribution. Behaviourally induced variation in infection rate could, in turn, cause differential modifications in physiology and behaviour of hosts (host manipulation). Heavily infected fish with strongly modified behaviour are easy prey for piscivorous birds, final hosts of *D. spathaceum*.

O-067

# HOST SPECIFICITY AND LOCAL ADAPTATION OF THE FRESHWATER PEARL MUSSEL PARASITIZING ATLANTIC SALMON AND BROWN TROUT

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There are probably not many parasites regarded as threatened in the IUCN Red List, but the freshwater pearl mussel *Margaritifera margaritifera* is such. This very long-lived (up to 240 years) species used to be very abundant in European salmon and trout rivers, but has later declined so that it is now extinct or endangered with only a small number of vital populations left. The fish hosts of *M. margaritifera* in Europe are Atlantic salmon *Salmo salar* and brown trout *S. trutta*. Traditionally it has been thought that salmon and trout both are equally suitable hosts for the pearl mussel. However, for conservation of the remaining European *Margaritifera* populations, it is of great importance to know whether Atlantic salmon or brown trout (or the local salmon/trout stock) is preferred by a given pearl mussel population. We studied these questions in laboratory and field cage experiments In River Iijoki river system. It appeared that pearl mussels originating from a (former) salmon river infected and developed better in salmon than in trout. On contrary, pearl mussels originating from (former) trout rivers performed better in trout than in salmon. There was no consistent pattern with respect to local adaptation. In one cross infection experiment the local trout was better host than non-local, but results of another experiment indicated opposite. The results indicate clear, previously unknown host specificity in the freshwater pearl mussel. It is worth to mention that in the (former) salmon river where mussels infected better salmon than trout, no salmon has been available for the pearl mussels for 50 years since the building of hydroelectric power plant which stopped salmon migration from/to the Baltic Sea. Thus, our results urge actions to restore the sea-migrating Atlantic salmon of River Iijoki to improve host situation for the threatened freshwater pearl mussel in that river system.

# TRANSCRIPTOMES OF *APHANOMYCES ASTACI* ISOLATES EXPRESS A BROAD ARRAY OF RxLR-EFFECTOR GENES

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Modern high-throughput transcriptome sequencing followed by *de novo* assembly and transcriptome analysis of six crayfish plague (*Aphanomyces astaci*) isolates reveals several effectors and virulence factors involved in their pathogenicity.
The crayfish plague is one of the most harmful animal pathogens; it is threatening all native European crayfish species and limiting their distribution. Mortality in infected native European crayfish populations has normally been close to 100%, but in recent years variation in crayfish plague episode dynamics among different species and populations has been reported. We investigated virulence differences among *A. astaci* isolates by carrying out infection trials in which we monitored symptoms and mortality of several noble crayfish (*Astacus astacus*) populations. In a series of experiments, we observed differences in the aggressiveness of the isolates. The diversity of plague isolates and genotypes was studied by targeted genome sequencing, where the chitinase genes were analyzed from twenty eight isolates. Broad diversity and polymorphism was detected especially among chitinase gene groups CHI1 and CHI2.
Our ultimate goal was to gain a more detailed insight of the crayfish plague transcriptome and to describe the genes responsible for the varying virulence of different genotypes. For this purpose, we conducted RNA sequencing, generated TruSeq libraries and performed 101bp paired-end sequencing using the Illumina HiSeq2000 platform. A total of eight cultured hyphae or zoospores from *A. astaci* isolates were sequenced: 2TB, 6462A, 8866, 8147, SATR1 and SATR2.
We proceeded with *de novo* assembly from six crayfish plague isolates and assembled the transciptomes for them. The generated transcriptomes consisted of around 21,000 different genes expressing up to 50,000 different isoforms and alleles. The known RxLR-motif was screened and identified from the transcriptomes. All the isolates have a unique set of RxLR effectors, up to 110 per isolate, which are involved in the *A. astaci* virulence and modulation responses with their hosts. We will show and summarize these results during the conference and discuss theoretical and practical outcomes of the studies.

O-069

# HOST-PATHOGEN INTERACTION IN BLOOD FLUKE INFECTION IN PACIFIC BLUEFIN TUNA

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Pacific Bluefin Tuna can be infected with two species of blood flukes, *Cardicola orientalis* and *Cardicola opisthorchis*. Both can cause pathology, but *Cardicola orientalis* which is present as an adult mostly in the gills produces more eggs and have greater potential to cause adverse effects. We have followed up the infection after transfer of tuna from the hatchery to grow out conditions. The presence of the parasites and immune gene expression were analysed. We could detect presence of the parasites using molecular methods before the adults and eggs could be detected in heart and gills. There was some evidence for differences in immune gene expression in gills and heart related to infection and time after transfer. Preliminary results suggest that some gene upregulation was correlated with the severity of infection. The implications of these findings will be discussed.

# ENHANCEMENT FOR IMMERSION VACCINATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) WITH LOW-FREQUENCY ULTRASOUND AND POTENTIAL HAZARDS

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Low-frequency ultrasound (LFUS) (between 20 and 100 KHz) is known to increase skin permeability in mammals. Recent research focuses on the application of LFS to improve the uptake of immersion vaccines in fish [1, 2]. However, compared to mammals significant less research has been conducted for fish, leaving unanswered questions about the optimal sonication frequency, intensity, time, and possible side effects. Recent research results suggest that the increased absorption of vaccine particles resulting from LFUS treatment is determined through changes in the skin and that LFS intensities under 400 $mW/cm^2$ would not cause considerable damage to the fish. Hitherto, the potential and efficacy of a LFUS-increased bacterin uptake was not examined for rainbow trout, an important fish species cultured worldwide. Therefore, we exposed rainbow trout (4-7g) to LFUS (37 kHz) with intensities such as those used by previous authors and lower ones. During sonication, fish were treated with an *Aeromonas salmonicida* bacterin (formalin killed, $1{,}6 \times 10^7$ CFU/mL) for 6 min. Our results showed that intensities which modify skin permeability also cause side effects such as erratic swimming and gill bleeding, which raises questions about the ethical practice. Sonication at 105 $mW/cm^2$ did not affect skin permeability, but gill permeability was increased by factor 15. However, erratic movements were still present. Analysis on the persistence of the LFUS-induced increase in gill permeability revealed that 20 min after sonication with 105 $mW/cm^2$ the permeability still increased by factor 3, and after 200 min by factor 2. This means that a certain time after sonication the debilitated protective function of the gill epithelium can make the fish more susceptible to ubiquitous pathogens. Sonication with 57 $mW/cm^2$ enhanced the bacterin uptake via the gills by factor 3 without evident discomfort of the fish during the sonication procedure. Furthermore, a decrease in the albumin to globulin ratio indicated a LFUS-induced inflammatory response that might have an adjuvant-like effect. Funded by: Federal Ministry of Economics and Technology by Decision of the German Bundestag.

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O-071

# CODING MUTATIONS IN UDP-4-GALACTOSE EPIMERASE ARE A CAUSE OF SEROTYPE SWITCHING IN *STREPTOCOCCUS INIAE* FROM FARMED FISH

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*Streptococcus iniae* causes meningitis and generalised septicaemia resulting in rapid mortality in farmed fish in almost all warm and temperate regions. It is also an opportunistic zoonotic pathogen, causing infections in elderly and immunocompromised patients generally through wound-infection during processing of infected fish. Vaccination of farm fish against *S. iniae* is widespread, with most vaccines comprising formalin killed bacterins. However, vaccination is not always successful, with vaccinated fish succumbing to reinfection by novel serotypes. In Australia, use of autogenous monovalent and polyvalent vaccines against *S. iniae* has led to rapid evolution of novel serotypes based on rapid mutation of key genes in the capsular operon. In the present study, we investigated the role of mutation in *cpsG*, encoding a putative UDP-galactose-4-epimerase, a key enzyme in the capsular polysaccharide (CPS) biosynthesis pathway. Three *cpsG* sequence types (ST) were associated with disease outbreaks in vaccinated fish, with each mutation occurring at the same point in the gene and resulting in insertion of 3 repeat amino acids once or twice. Fish vaccinated with bacterin prepared from one ST were not protected against infection by a second ST, but protection was 100% against the same ST. Lack of protection was strongly correlated with lack of cross-reacting antibody in immunised fish, as determined by ELISA. To investigate how changes in *cpsG* may affect capsular polysaccharide production, recombinant epimerases prepared from the three STs were expressed in E. coli, purified and assayed for epimerase activity. The insertion of the repeat amino acid sequences resulted in altered epimerase activity suggesting altered glucose/galactose ratio in the CPS. Further work is required using isogenic mutants to determine how these mutations change the antigenic structure of the CPS in *S. iniae*.

# THE EFFECT OF INJECTION WITH IMMUNOMODULANTS ON THE IMMUNE STATUS AND INTESTINAL MICROFLORA POPULATION OF COMMON CARP (*CYPRINUS CARPIO* L.)

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Whilst the oral application of β-glucans has been shown to improve immunity in cultured fish, the effect of these naturally occurring immunomodulants, when utilised as vaccine adjuvants, has not been extensively investigated. The application by injection also has the advantage in that the exact dose to which the fish immune system is exposed to is known whereas when applied orally, the dose becomes dependent upon the feed rates of individual fish. Our initial studies have revealed different expression patterns for the proinflammatory cytokines, i.e. IL-1β and TNFα, in the intestines of Common carp (*Cyprinus carpio* L.) after injection of MacroGard®, a commercially available β-1,3/1,6-glucan source (approximately 60%), when compared to previously published observations (Falco *et al*. 2012) on the effects after oral application of this immunomodulant. In addition, injection of lipopolysaccharide (LPS) has been shown to be able to manipulate the intestinal microflora of Atlantic salmon (*Salmo salar*) whereas laminarin, a β-1,3-glucan does not (Liu *et al*. 2008).
In the first of our investigations, carp were intraperitoneally injected with either MacroGard® (2mg/kg$^{-1}$), from *E. coli*, LPS (4mg/kg$^{-1}$) or PBS (100µl) and expression levels of several innate immune parameters, e.g. IL1B, TNFa and iNOS, were measured 1 day and 4 days after treatment. In addition, the intestinal microflora content of carp was determined by monitoring the total 16S rDNA gene expression. Initial observations have revealed that there was some apparent change in gene expression of the immune parameters analysed within the intestine however the expression of the total 16S gene was not affected. In a second trial, analysis of the intestinal microflora of carp injected directly into the dorsal aorta with MacroGard® (100µg per fish) as an adjuvant mixed with inactivated *Aeromonas hydrophila* (10^6 CFU per fish), however, showed a decrease in total microflora content after 12 days which returned to similar levels as uninjected controls after 23 days. The differences in the commensal microflora observed in these studies could indicate a close relationship between the bacterial content of the intestine and the activation of specific elements of the fish's immune response.

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O-073

# EFFICACY AND SAFETY OF MULTIVALENT INJECTION VACCINES AGAINST *FLAVOBACTERIUM PSYCHROPHILUM* IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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*Flavobacterium psychrophilum* is the causative agent for rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD) affecting salmonids. Although *F. psychrophilum* is mainly considered a freshwater bacterium causing disease at hatcheries, an increasing number of outbreaks have been documented at sea sites in Norway coupled to reduced salinity. Periods of brackish water due to heavy ice melting and rain is believed to support transmission while unfavorable factors such as high stocking density, reduced oxygen levels and particular material may contribute to outbreaks. Vaccination against RTFS/BCWD, in concert with strict disinfection routines to avoid transmission of *F. psychrophilum* from brood to offspring, are two important prophylactic measures to mitigate the infection pressure and spread of *F. psychrophilum.*
Aiming to develop a challenge model for *F. psychrophilum*, PHARMAQ have collected field strains from outbreaks of BCWD on rainbow trout in Norway. We have further identified an isolate capable of inducing high mortality (>75%) in unvaccinated fish even at a low challenge concentration (100 bacteria/injection). Pre-challenge of a small number of un-vaccinated and vaccinated fish revealed that the isolate may be used in an intramuscular (IM) challenge model for testing of vaccine efficacy at optimized challenge concentrations. A second more comprehensive study testing three different multivalent water-in-oil formulated vaccines against BCWD in challenge tests was then carried out. This study included the vaccines FLAVO IPN (2 Ag components), FLAVO AV4 (4 Ag components) and FLAVO AVM6 (6 Ag components). All vaccines were administered by intraperitoneal injection to rainbow trout (~35 grams) either at 0.05 ml or 0.1 ml doses. 552 degree days post vaccination the fish were randomly separated into four parallel tanks and challenged IM with the highly virulent field strain of *F. psychrophilum*. The challenge resulted in 94.3 % mortality in the negative control while a high level of vaccine induced protection was demonstrated in the vaccinated groups with RPS (relative percent survival) ranging from 63.6-79.5% at study termination. Our results are the first to demonstrate protective immunity induced by a multicomponent vaccine against *F. psychrophilum* in rainbow trout. Study design, vaccine content, antibody levels both in laboratory and field studies, as well as vaccine safety will be presented in more detail during the presentation.

# IMPROVING VACCINE PERFORMANCE THROUGH UNDERSTANDING HOST-PATHOGEN INTERACTION IN YERSINIOSIS

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Vaccine efficacy is usually tested through challenge experiments, which are time consuming and require many controls and as a result high numbers of experimental fish. We propose the use of transcriptional biosignature as an alternative which would improve fish welfare and allow faster testing of vaccines with a direct application for aquaculture industry. Yersiniosis is a bacterial disease affecting Atlantic salmon in Tasmania during hatchery stage and after transfer to the sea. The disease is caused by *Yersinia ruckeri*. While there are commercial vaccines available, outbreaks still occur due to the presence of carriers. Using cDNA microarray we identified the expression of 6 genes in response to infection and 4 genes associated with the protective host response to yersiniosis.
A transcriptional biosignature consisting of predominantly immune-relevant genes (14 up and 3 down-regulated) in the gills of Atlantic salmon after immersion vaccination and before bacterial challenge was identified. This biosignature may be used as a surrogate of protection and therefore as a predictor of vaccine success against yersiniosis. We have tested an effect of a novel inactivation method of Y. ruckeri on vaccine efficacy and showed that the transcriptional signature was a predictor of protection, thus confirming our previous results. This study also aimed to improve vaccine performance through understanding molecular host-pathogen interactions in yersiniosis. We investigated effects of different methods of vaccine application. Survival in an experimental challenge was significantly better after double dip and ip vaccination than control unvaccinated fish. Double dip and ip injection vaccination gave better protection than single dip or bath vaccination. The double dip vaccinated group has significantly better survival than the bath vaccinated group but there is no statistical difference between the single dip and bath. The ip vaccinated fish had a very high RPS of 95.5%. While ip vaccination maybe not practical for salmon industry, it provides a positive control for experimental research. The survival 12 weeks post vaccination further confirmed our previous results.

# FISH IMMUNE RESPONSE TO *MYCOBACTERIUM MARINUM*

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Mycobacterium marinum is a bacterial pathogen that causes a chronic and often lethal fish disease. M. marinum is not fastidious in its host selection and is known to infect more than 150 fish species in freshwater, saltwater and brackish water environments. M. marinum can survive and multiply inside macrophages which aggregate into necrotizing granulomas mainly in spleen and kidney. A recent collaborative study at the University of Maryland has shown that the attenuated M. marinum ΔiipA mutant can trigger a strong immune response without forming granulomatous lesions, and may thus provide the fish with an effective protection from virulent strains of M. marinum.
Two economically important fish species, the European seabass Dicentrarchus labrax and the white grouper Epinephelus aeneus, were used to monitor, evaluate and compare the immune response when vaccinated with heat-killed avirulent ΔiipA mutant or heat-killed virulent M. marinum, and then challenged with a live "high" and "low" dose of the latter. Fish from each treatment were sampled monthly for three months for serological and histopathological analyses. These revealed that D. labrax was significantly more sensitive to the disease than E. aeneus. The vaccinations with high-dose iipA mutant allowed for the highest survival in seabass, with mortalities starting only 3 months after the challenge, whereas mortalities in the non-vaccinated fish started soon after the challenge. No mortalities were recorded in the groupers. In both species, antibody levels increased significantly with time in response to the challenge, which suggests that mycobacteria triggered a strong specific immune response. In addition, one month after challenge, TNF-α expression levels within the challenged groups were the highest, decreasing gradually in the following two months. Such levels were at any time significantly lower in grouper than in seabass.
Although the vaccination did not prevent the development of the disease at our experimental conditions, its outbreak was delayed and its intensity was clearly lowered. The ability of the fish to mount a strong immune response without the formation of granulomas when injected with killed ΔiipA indicates that this attenuated M. marinum mutant may still act as a powerful and safe immunogen, offering a relatively high protection from natural M. marinum infections.

# IDENTIFICATION OF ANTIGENIC PROTEINS OF *FLAVOBACTERIUM PSYCHROPHILUM* USING IMMUNOGLOBULIN OF AYU *PLECOGLOSSUS ALTIVELIS* WHICH RECOVERED FROM BACTERIAL COLD WATER DISEASE

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*Flavobacterium psychrophilum* is the causative agent of bacterial coldwater disease (BCWD) in ayu *Plecoglossus altivelis*, and substantial responsible for economic losses in ayu culture in Japan. In order to develop effective vaccines for the disease, we identified antigenic proteins of *F. psychrophilum* using immunoglobulin of ayu which recovered from BCWD. Ayu weighing 17 g of the average body weight were intraperitoneally injected with *F. psychrophilum* strain GMA0330, which was isolated from diseased ayu. Blood plasma was then collected from the fish at 20 days post-infection. The whole protein extracted from the bacterium was separated by 2-D PAGE and transferred to PVDF membrane. Subsequently, antigenic proteins of the bacterium were detected by western blotting using the plasma. Each protein spot showing antigenicity was subjected to MS/MS analysis using MALDI-QIT-TOF mass spectrometer. Protein identification based on MS/MS data was performed against the genome database of *F. psychrophilum* ATCC49511 and the subcellular localization for each identified protein was predicted with web-based tools (LipoP and PSORTb). In total, 69 antigenic proteins were identified: of these, 46 were putative cytoplasmic proteins, such as elongation factor Tu and heat shock protein 90. Another 23 proteins were identified as putative membrane-bound or secreted proteins which are suitable for vaccine candidates. These include OmpA, Omp 121, M13 family metallopeptidase and M48 family metalloprotease.

# COMPARATIVE GENOME ANALYSES OF *MYCOBACTERIUM* SP. AND *M. MARINUM* ISOLATED FROM FISH AND HUMAN

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*Mycobacterium* sp. and *M. marinum* are causative agents of mycobacteriosis in fish. Earlier, we analyzed the phylogenetic and proteomical similarity between *M. marinum* (two fish isolates and one human isolate) and *Mycobacterium* sp. (012931 strain, isolated from yellowtail in Japan) using multigene phylogenetic analyses and MALDI biotyper. From our results, it seems that *Mycobacterium* sp. is potentially different in terms of genetic make-up and virulence from *M. marinum*. Therefore, we determined the whole genome sequence of *Mycobacterium* sp. 012931 and *M. marinum* fish strains.

Whole genome sequence of the *Mycobacterium* sp. (012931 strain) and two *M. marinum* (MB2 and Europe strains) were determined by 454 GC-FLX and compared with each others and *M. marinum* M (human isolate). The genome analyses including dot plot and insertion/deletion (INDEL) genes were conducted using *In Silico* Molecular Cloning Software (*In Silico* Biology Co.) and Rapid Annotation Subsystem Technology (http://rast.nmpdr.org/).

*Mycobacterium* sp. 012931 contains a single circular chromosome of 5,759,364 bp, which is represented by a single scaffold. Dot plot shows that *Mycobacterium* sp. 012931 shares the highest homology with *M. marinum* strains among *Mycobacterium* species tested in this study. However, there were at least four mutated loci in *Mycobacterium* sp. genome when compared to *M. marinum* genome. The annotated gene number of *Mycobacterium* sp. (012931) and two *M. marinum* (MB2 and Europe) were 5,454, 5,452, and 5,474, respectively. Those annotated genes were classified into 26 subsystems. In virulence, disease and defense subsystem, both INDEL genes of *Mycobacterium* sp. 012931 were associated with PPE (Pro-Pro-Glu) gene cluster in Mycobacteria. Our findings provide a better understanding of pathogenesis of *Mycobacterium* sp. and *M. marinum* that may aid in the development of disease management strategies.

# *RENIBACTERIUM SALMONINARUM*: DISTINCT PATTERNS OF DIAGNOSTIC RESULTS IN MULTIPLE SAMPLES FROM THREE GROUPS OF SALMONIDS AT DIFFERENT STAGES OF INFECTION

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Farming of Atlantic salmon and Arctic char is important in Icelandic aquaculture. Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum*, is endemic worldwide in salmonid fishes, including Iceland. The disease is mainly a problem in culture, in fresh and marine water, although epidemics and clinical signs of BKD are observed in wild fish. Therapeutic measures, including the use of antibiotics or vaccines, have been tried with limited success. Brood stock culling, where fertilized ova from infected females are destroyed, is an useful method in the battle against the disease and therefore sensitive diagnostic tests are important. Little is known about the progression of *R. salmoninarum* infection in Atlantic salmon and Arctic char, especially in the latter one. Arctic char has been considered to be relatively resistant to *R. salmoninarum*.
Results from culture, ELISA and PCR methods, detecting *R. salmoninarum* in multiple organs from three groups, were assembled. In the first group were cultured Atlantic salmon brood fishes with escalating disease when sampled. In the second group were Arctic char fingerlings experimentally infected by cohabitation and by an i.p. injection in the third group. Samples were obtained from the experimentally infected Arctic char 4-6 times in an 8 month period. Different patterns of results were observed for all three groups. With higher ELISA readings, more and more samples tested positive by other methods in group 1. Groups 2 and 3 differed from group 1, as well as from each other. In both groups, there were multiple positive samples by all methods tested at the start of the experiment. ELISA values increased over time, but 8 months after infection all culture samples were negative and the number of samples positive in different PCR tests had declined.

O-079

# USING QUORUM SENSING TO DEVELOP GUT HEALTH SUPPORTING FEED ADDITIVES

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Aquaculture producers are under increasing pressure to reduce the use of antibiotics and chemicals to fight disease and parasitic infestations. We therefore studied the potential of Quorum Sensing Inhibition as an alternative strategy to reduce the impact of bacterial diseases in aquaculture.
Several feed additives, both in aqua- as in agriculture, are composed of constituents that have been described to increase animal production performance by supporting gut health via an immunomodulatory and/or antibacterial effect in the intestinal tract. The development of an antibacterial product often relies on screening components for Minimal Inhibitory Concentration (MIC) values towards certain bacterial strains of interest. Recently, however, Nutriad undertook an effort to analyze the biological activity of components well-below MIC-concentrations, in order to develop a potent new generation of aqua and poultry feed additives, supporting gut health at very low concentrations.
To this end, we analyzed Quorum Sensing (QS) inhibition. QS is a form of bacterial communication that is dependent on population density and that allows bacteria to coordinate biochemical responses often associated with activation of virulent bacterial signaling. We set up a screen to identify highly active bioactive ingredients to compose multi-ingredient products displaying QS inhibiting activity at very low concentrations *in vitro*. In addition, we demonstrated that these products were able to suppress the negative effect of pathogenic QS signaling in simple *in vivo* models.
In a next step, we tested these products in the field: broilers fed the poultry feed additive showed better zootechnical parameters and scored better for veterinary parameters related to health, while the aqua additive was associated with improved survival and productivity of shrimp, exposed to White Spot Virus (WSSV).
Although it is difficult to demonstrate that these improved performance parameters are a direct effect of a reduced QS-signaling in the gut microbiota, we feel that the analysis of biological activity of components at low concentrations, such as QS inhibition, can be a valuable tool to develop efficacious feed additives, and might provide insight in the molecular modes of action of these type of products.

# USE OF IN-VIVO INDUCED ANTIGEN TECHNOLOGY FOR IDENTIFICATION OF *AEROMONAS SALMONICIDA* ANTIGENIC DETERMINANTS SPECIFICALLY EXPRESSED DURING THE INFECTION PROCESS

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*Aeromonas salmonicida* is a major pathogen of fish and the causative agent of furunculosis in salmonids. Like most bacterial pathogens, the expression of its virulence genes is under tight regulation and these are often not expressed outside of the infection process, making their identification more difficult. For this reason it was decided to investigate the virulence factors of *Aeromonas salmonicida* through the use of *In-Vivo* antigen technology. To do so, we exposed *Oncorhynchus mykiss* to sub-lethal doses of *A. salmonicida* before harvesting the serum from the fish. This serum was then adsorbed against an *in-vitro* culture of the bacterium so that only the antibodies uniquely expressed during the infection process would remain. These were then used to screen an expression library of the bacterium in order to identify antigens that are specifically expressed during its pathogenic phase. This study gave us new insights in the virulence of this important fish pathogen as well as new promising leads in the development of vaccines.

O-081

# DEVELOPMENT OF SERO-DIAGNOSIS METHOD OF *STREPTOCOCCUS DYSGALACTIAE* INFECTION USING SURFACE IMMUNOGENIC PROTEIN

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Lancefield group C *Streptococcus dysgalactiae* (GCSD) causes severe necrotic lesions in the caudal peduncle in the genus *Seriola* farmed in Japan. In order to develop a rapid diagnostic method for GCSD infection in farmed fish used at the aquaculture sites, we attempted to identify a surface immunogenic protein that induces an antibody after infection with GCSD by immunoblot analysis by using sera collected from infected fish. A protein obtained from sodium dodecyl sulfate (SDS) extracts of GCSD was identified as *S. dysgalactiae* surface immunogenic protein (Sd-Sip) with a molecular weight of approximately 60 kDa. The *Sd-Sip* gene was cloned in the pCold I vector and a His-tagged fusion protein was expressed in *Escherichia coli* BL21. Expression of the recombinant Sd-Sip (rSd-Sip) was confirmed by immunoblot analysis, i.e., its reactivity to GCSD-infected sera. Latex beads were coated with rSd-Sip and their usefulness for sero-diagnosis of GCSD infection was examined. rSd-Sip-coated latex beads agglutinated on a glass slide by adding GCSD-infected sera collected from farmed amberjack (*Seriola dumerili*) and yellowtail (*Seriola quinqueradiata*), but did no agglutinate by adding non-infected sera or sera immunized by other pathogenic bacteria of fish. These results of the present study suggest that the slide agglutination test using rSd-Sip-coated latex beads is a rapid and effective diagnostic method for GCSD infection in fish at the aquaculture sites.

# CHARACTERIZATION OF *VIBRIO* AND *PHOTOBACTERIUM* SPECIES ISOLATED FROM GREEK AQUACULTURE

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The Vibrionaceae are a family of Proteobacteria containing some of the most important fish pathogens including Vibrio and Photobacterium species. Members of these two genera have been devastating the Mediterranean aquaculture for many years. In this work we present data on the biochemical and molecular characterization of several clinical strains isolated from Greek aquacultures. Most of these clinical strains belong to the following species: *Vibrio anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. fischeri*, *Photobacterium damselae* subsp. *piscicida*, *Photobacterium damselae* subsp. *damselae*. The biochemical characterization was based on the BIOLOG GEN III standardized micromethod which is using 71 carbon source utilization assays and 23 chemical sensitivity assays to provide a phenotypic fingerprint of the bacteria. The strains were grouped using Direct Genome Restriction Enzyme Analysis (DGREA) and the 16S ribosomal RNA of the representatives of each group were sequenced following PCR with universal primers. The sequences were used in phylogenetic analysis. The Greek clinical strains were compared against characterized pathogenic bacteria provided from international collections.
The results of this study are discussed with reference on the severity of each clinical case. The final goal of this work is to develop a biorepository of pathogenic strains belonging to these species to be used as hosts for isolating lytic bacteriophages. A thorough characterization of the bacteria will give us a better understanding on the host specificity of phages and the mechanisms of resistance development, towards phage therapy development.

O-083

# CHARACTERIZATION OF PEPTIDES LOADED INTO MHC CLASS I IN IHNV INFECTION OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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The novirhabdovirus IHNV is the causative agent of infectious haematopoietic necrosis (IHN), a disease whose outbreaks in reared rainbow trout (*Oncorhynchus mykiss*) as well as in other salmonid species can result in very high mortality and economic losses. Attempts to produce vaccines based on attenuated or killed virus as well as recombinant proteins have shown low efficacy, while DNA immunization with plasmids encoding the glycoprotein of IHNV provides good protection. An important defence mechanism against virus infections is cell-mediated immunity. Regarding this, cell-mediated cytotoxicity against IHNV infected cells has been demonstrated by functional assays, and the participation of cytotoxic T lymphocytes (CTL) as effector cells has been confirmed by gene expression profiling and more recently by using antibodies against the specific surface marker CD8. The recognition of virus infected cells and their interaction with effector CTLs is mediated by the presentation of short antigenic peptides from processed viral proteins loaded into major histocompatibility complex (MHC) class I molecules. Those peptides can then be specifically recognized by the T cell receptor (TCR) of CTLs, triggering a clonal expansion of antigen specific CTLs which will further control the virus replication through lysis of infected cells. Thus, the identification of MHC class I loaded peptides as potential TCR epitopes is crucial for the understanding of antiviral immune responses and for vaccine development. In order to predict potential viral peptides that are involved in MHC class I restricted presentation during IHN infection a NetMHCpan computational prediction system was employed. For this, we have uploaded both the sequences of the rainbow trout MHC class I allele UBA*150101, as well as that of the glycoprotein of IHNV, and got several nonapeptides with different binding intensities suggested. Peptides with a high score of binding were chosen to test if in their presence a proper refolding of recombinant denatured subunits of the rainbow trout MHC class I UBA*150101 and the β2-microglobulin (β2m) was induced. Those peptides that were able to induce proper complexing will be further employed for preparation of fluorescent labelled MHC class I tetramers that can be used as a tool for the recognition, isolation and characterization of antigen-specific T cells induced by IHNV infection or vaccination.

# ASSOCIATION OF MHC AND PROLIFERATIVE KIDNEY DISEASE (PKD) IN MULTIPLE NATURAL POPULATIONS OF BROWN TROUT

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Salmonid populations worldwide are threatened by various anthropogenic factors such as construction of dams, pollution and overfishing. During recent years, there is a rising concern that by producing suboptimal temperature conditions; global warming and construction of dams will increase fishes' susceptibility to disease and parasitic infections. Currently, one of the most serious parasitic diseases of salmonid fishes is caused by the myxozoan *Tetracapsuloides bryosalmonae* resulting in proliferative kidney disease (PKD). Importantly, *T. bryosalmonae* is expected to expand its distribution and increase in virulence in increasing temperatures.This parasite infects the kidney of juvenile fish (0+) causing strong inflammatory response, anemia and kidney hypertrophy in water temperature above 16° C. An important question is whether the host populations can adapt to increased pathogen load in the face of elevated temperature regimes fast enough to avoid going extinct? If yes, which host genes are involved? This scientific work aims to address these questions in the context of host-parasite co-evolution by investigating the variation at the major histocompatibility complex (MHC) genome in relation to PKD resistance in multiple natural populations of brown trout. Evaluation of the fitness consequences of allelic variation at immune relevant candidate loci is expected to provide rare insights into the strength of selection.

# GENE EXPRESSION PROFILES IN ATLANTIC SALMON GILLS AFTER VACCINATION AND CHALLENGE WITH *AEROMONAS SALMONICIDA* SUB SPECIES *SALMONICIDA*

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*Aeromonas salmonicida* is a Gram negative bacterial pathogen, causing furunculosis in infected fish. Typical signs of the disease include septicaemia, and ulcerative and haemorrhagic lesions, resulting in mass mortalities and substantial economic losses to the aquaculture industry. Fish gills are a multifunctional organ, one aspect of which is as a mucosal barrier helping to control pathogen entry. Recently, localised intraepithelial lymphoid tissue (ILT) was discovered in the gills, which has been shown to be involved in modulation of the innate, adaptive and mucosal immune responses in fish.

In the present study, gills of Atlantic salmon, vaccinated with a commercial *Aeromonas salmonicida* vaccine, and subsequently experimentally infected with *A. salmonicida* sub sp. *salmonicida* by intra-peritoneal injection, were analysed by qRT-PCR to examine gene expression profiles of some immune-related genes (IgM, IgT and IL-1), with a view to elucidating their functional significance in vaccine-induced immunity following the pathogen challenge.

The relative gene expression (GenEx software) of IgM was significantly higher ($p \leq 0.05$) at 12 days post vaccination (d.p.v.) compared to PBS injected group, but not at 24 d.p.v. The IgT transcript levels changes were almost significant ($p < 0.06$) at both 12 and 24 d.p.v. During challenge, the gene expression of both IgM and IgT were significantly different between groups (i.e. IgM was significantly higher ($p \leq 0.05$) in vaccinated-challenged group compared with the vaccinated unchallenged and the PBS injected un-challenged fish at 4 days post challenge. Conversely, IgT was significantly lower ($p \leq 0.05$) in the vaccinated challenged fish compared to other two groups).

These results highlight the importance of the gill as a mean for assessing immune competence and disease resistance against aquatic pathogens like *A. salmonicicida*. The gill would seem an excellent model for examining both local and systemic immune modulations against septicaemia diseases in Atlantic salmon and has the potential to be used to examine the fish's' response to vaccination. The ability to take gill biopsies from live animals (non-lethal sampling) also makes this a useful model for addressing the 3Rs in animal research.

# *VIBRIO ANGUILLARUM* BACTERIN INTRODUCED THROUGH GILL EPITHELIUM INDUCES INFLAMMATORY RESPONSES IN GILLS OF JAPANESE FLOUNDER *PARALICHTHYS OLIVACEUS*

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*Vibrio anguillarum* bacterin can be introduced by immersion of fish in the water containing the bacterin. However, the route through which the bacterin enter the fish body and the tissue where the local immune responses against the bacterin take place have not been studied in detail. In this study, gene expression levels of inflammatory cytokines (IL-1β, IL-6 and TNFα) and immune-related cell markers (CD4-1, CD4-2, CD8α and IL-8 receptor) were quantified in mucosal tissues and kidney of Japanese flounder *Paralichthys olivaceus* vaccinated by immersion with the bacterin. In addition, the route of entry of the bacterin was investigated by immunohistochemistry. Compared with the control, gene expression levels of IL-1β, IL-6 and TNFα were significantly up-regulated 3- to 15- fold at 3 days post-immersion in the gills of vaccinated fish, whereas these up-regulations were not observed in the intestine, skin and kidney. The expression levels of CD4-1, CD4-2, CD8α and IL-8 receptor genes in the gills showed 3- to 10-fold increases at 3 days post-immersion. *V. anguillarum* bacterin was detected at epithelial cells of the gills at 1, 3 and 6 hours post-immersion, but not at 12 hours post-immersion. These data suggest that immersion vaccination of the bacterin induces local inflammatory responses in the gills. The data also suggest the migration of immune-related cells expressing CD4-1, CD4-2, CD8α and IL-8 receptor into the site. Furthermore, epithelial cells of the gills are probably one of the routes of entry taken by the bacterin, and may play a role in the recognition of antigens in the environment.

O-087

# CHARACTERIZATION OF THE INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS (IHNV) CARRIER STATE IN SOCKEYE SALMON *ONCORHYNCHUS NERKA*

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Infectious hematopoietic necrosis virus (IHNV) is a negative-sense single-stranded RNA virus that is a member of the family *Rhabdoviridae*. This virus is enzootic in British Columbia and is commonly found in sockeye salmon (*Oncorhynchus nerka*) stocks where it is known to cause serious disease. Recently, we demonstrated: the presence of IHNV in brain tissues of sockeye salmon surviving a laboratory exposure, as well as the presence of this virus in asymptomatic juvenile sockeye in the marine environment. Although the virus is detectable in only a small fraction of fish, these results suggest that some individuals within sockeye populations may be lifelong 'carriers' of IHNV. The transcriptional response of these asymptomatic carriers, characterized using the cGRASP 44K salmon oligoarray, were compared to exposed sockeye without detectable virus as well as to the unhandled (naïve) controls. Moreover the physiological consequence of this virus carrier state on the host's ability to respond to subsequent viral infection was investigated using the viral mimic polyriboinosinic polyribocytidylic acid (poly(I:C)). The differential gene expression profiles will be presented and discussed in the context of the role of the IHN virus carrier state in sockeye salmon.

# *YERSINIA RUCKERI* O1 INFECT RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) THROUGH THE GILLS DEMONSTRATED BY A THREE-DIMENSIONAL IMAGING ANALYSIS CALLED OPTICAL PROJECTION TOMOGRAPHY

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The mucosal tissues including gills, skin and intestine are known as the infection route for pathogenic bacteria in fish, however the detailed infection mechanism is not known well. Therefore in order to understand the mechanism of bacterial infection throughout the mucosal tissues, we adopted the optical projection tomography (OPT) as an alternative strategy for immunohistochemistry (IHC), which is a new technique for three-dimensional (3D) imaging of small tissues or fish.
Rainbow trout were infected with *Yersinia ruckeri* O1 biotype 1 ($1 \times 10^9$ cells/ml) for 1 hour at 18°C, and then transferred to clean water. The significant mortality during 21 days was observed. Three fish were sampled at different time points and fixed in 4% paraformaldehyde for OPT, or 10% formalin for immunohistochemistry (IHC). For OPT scanning, the gills were incubated whole with rabbit anti-*Y. ruckeri* polyclonal antibody and Alexa Fluor®594 conjugated goat anti-rabbit IgG, then specimens were scanned by Bioptonic OPT 3001 scanner. The presence of *Y. ruckeri* bacteria in gill tissue was confirmed by IHC using rabbit anti-*Y.ruckeri* antibody.
The OPT results showed that *Y. ruckeri* were observed in connection with the gill filaments 1 minute post infection (mpi) gills and confirmed inside the gill filament by IHC. Furthermore, the *Y. ruckeri* bacteria were isolated from 1mpi blood. It is so fact, which indicates that the single layer of epithelial cells on the gill filament could be the first infective route of entry. After 7 days post infection, the multiplied *Y. ruckeri* were observed in pharyngeal and connecting tissue under the basihyal by OPT, and the septicemia in gill capillary showed by IHC. In contrast, no bacteria were found in control fish gills. These results suggest that the OPT is a strong tool for 3D visualization of infected bacteria using whole tissues which are minutes or days post infection.

O-089

# THE EFFECT OF β-GLUCAN ON NEUTROPHIL EXTRACELLULAR TRAPS IN COMMON CARP

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A novel innate immune defence mechanism against invading pathogens, namely the formation of neutrophil extracellular traps (NETs), has recently been described in fish. These NETs are defined as DNA fibres entwined with antimicrobial peptides, which are able to entrap and kill bacteria. Here we characterised the function of carp pronephros and kidney derived NETs against the opportunistic fish pathogen *Aeromonas hydrophila* in response to the addition of the feed additive β-glucan (MacroGard®). Firstly a time kinetic of NET formation in response to β-glucan-treatment was performed. Therefore, common carp (*Cyprinus carpio*) pronephros and kidney derived cells-each consisting of approximately 45% neutrophils- were isolated, stimulated with 0, 2, 20 or 200 µg/ml β-glucan over 15, 30, 60, 120 and 240 min and subsequently NET-formation was analysed by immunofluorescence microscopy. The results show that NET production occurred very rapid with NETs observed after just 15 min of β-glucan treatment. Furthermore, a significantly higher percentage of kidney-derived cells produced NETs relative to pronephros-derived neutrophils. Secondly the effect of β-glucan on bacterial entrapment and killing by NETs was investigated. The results show that carp NETs are able to entrap *A. hydrophila*, and the addition of β-glucan increased the percentage of entrapped bacteria. However no bacterial killing was observed. Thirdly, host-evasion strategies utilised by *A. hydrophila* to degrade NETs were investigated. For this, carp derived pronephros cells were seeded with or without the addition of β-glucan and *A. hydrophila* was added at a multiplicity of infection of 1 bacterium per cell. The results showed that *A. hydrophila* was able to degrade NETs, which was attributed to the secretion of DNA degrading nucleases by *A. hydrophila*. Interestingly, the addition of β-glucan significantly protected the NETs against bacteria-mediated degradation. The findings of this work shows that carp derived neutrophils are able to produce NETs, which are able to entrap, but not kill *A. hydrophila*. Interestingly the addition of β-glucan significantly stimulated NET production over time and protected the NETs against host evasion strategies utilised by *A. hydrophila* that degrade NETs.

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# RANKING SHELLFISH FARMING AREAS IN ENGLAND AND WALES FOR THE RISK OF DISEASE INTRODUCTION AND SPREAD

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Risk based surveillance (RBS) is undertaken to improve the efficiency of resource allocation. By focusing on high risk animals, farms and regions the likelihood of detecting disease or abnormal mortality is increased. Risk ranking farms or farming areas is fundamental to a RBS system. Cefas has developed a model to risk rank freshwater fish farms, based on the likelihood of disease introduction and spread, in part to fulfil the requirements of EC directive 2006/88 for RBS. To this end farms or farming areas have to be categorised as high, medium or low risk. A project identifying routes of introduction for marine non-native species collated data on depuration plants, the location of ports, marinas, bird colonies and movements of animals for aquaculture. It is important that resources used to develop RBS do not outweigh any efficiency savings. Thus existing datasets were used to develop an RBS for shellfish farms. It was judged most appropriate to risk rank at the farming area, as there are no physical barriers between farms, they are not epidemiologically isolated. Routes for pathogen introduction and spread were classified under 4 themes: i) live animal movements; ii) water, ii) birds and iv) vessels. More than one dataset were used for some themes, for example, under the water theme the number of depuration plants, shellfish holding facilities and proximity to other shellfish farming areas were used. Some data were transformed to minimise excess leverage by outlying values. Each route was scored, on a scale of 0 to 1. Themes, and risk factors within themes, were weighted using expert opinion. Separate scores for introduction and spread were calculated by adding the weighted scores for each theme. Results were plotted on a scattergram (Fig 1) and observed clustering was used to define thresholds between low, medium and high categories. The next step is to obtain weight estimates through a formal expert elicitation process (e.g. Delphi panel). The model can be further developed by ranking farms within farming areas and parameterising the model for specific pathogens. Outputs from hydrodynamic models would provide better estimates of spread by water.

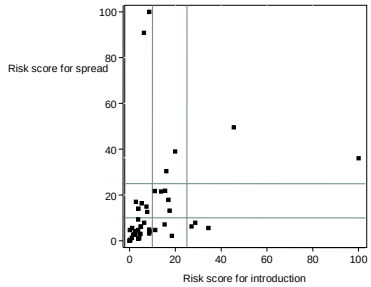


Fig 1. Risk scores for introduction and spread of disease for 45 mollusc farming areas in England & Wales.

O-091

# FIRST COMPLETE NATIONAL STUDY OF THE CAUSES OF MORTALITY IN NORWEGIAN SALMONID FARMS

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Trough compulsory monthly electronic reports form the fish farm management the Norwegian authorities gain very reliable and concurrent data from the salmon farming industry. Facts about the periodic feed consumption, growth, total biomass and mortality is reported at cage level. A reliable and complete overview over the causes of loss of considerable amounts of fish in sea cages has not previously existed at a national level, despite the fact that many of the individual companies keeps records of these facts based on observations noted at a daily basis. A pilot project covering one generation of fish (2009) at 61 different seasites in one geographical area of Norway was studied retrospectively. Trends of the main causes of loss of fish were described. According to this study smolt quality and handling of fish were each responsible for more loss of fish than infectious diseases contracted after the fish were turned to sea.
Subsequent to the results of this study were made known organisations representing the fish farming industry communicated a need for a more in depth and nationwide project in continuation of the regional pilot project.
The Norwegian Food Safety Authority is responsible for the management of the national project, which is financed by the The Norwegian Seafood Research Fund. Relevant data are to be collected in the spring and summer of 2013, covering the generations turned to sea in the autumn 2010 and the entire 2011. Data form both the pilot and the national project will be communicated in the presentation.

# EMERGING GROUP B *STREPTOCOCCUS* IN WILD MARINE FISH IN AUSTRALIA BELONG TO ST261 AND ARE HIGHLY PATHOGENIC IN GROUPER BY INJECTION AND IMMERSION

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Since 2008, 91 wild Queensland groupers, *Epinephelus lanceolatus*, were found dead between Brisbane and Karumba (Gulf of Carpentaria). Most deaths occurred in urbanised areas including Cairns and Port Douglas, with peak mortalities occurring in winter. In many cases, *Streptococcus agalactiae* (GBS) was isolated in pure culture from eyes and internal organs. To determine routes of transmission, infection trials were conducted in farm-reared juvenile grouper. Injection challenge resulted in rapid development of clinical symptoms including exophthalmia. Challenge by immersion with the same isolate resulted in very low mortality, and only when water ammonia levels increased. GBS was isolated in pure culture and detected by PCR in brain, HK and spleen from 100% of trial fish, regardless of challenge dose when challenged by injection. In immersion-challenged fish, GBS was isolated from 25-50% and detected in 25-75% and was dose-dependent. Oral challenge by admixture with feed resulted in development of clinical symptoms in ~ 10% of fish challenged with the highest dose, suggesting a possible natural oral route of infection, but no mortality was recorded. Organs including gills, eye, heart, head kidney, caudal kidney, spleen, liver, muscle, skin, stomach, brain, intestine, pyloric caecae and swim bladder were sampled from all dead or euthanased fish for histology. Bacteria were fluorescently labelled by immunocytochemistry using a polyclonal antibody against type specific carbohydrate of group-B streptococcus. Tissue sections were visualised by scanning confocal microscopy. To determine possible origin of infection, 23 isolates were chosen for complete genome sequencing by Illumina, comprising representatives from fish (grouper, mullet, stingrays), crocodiles and mammals (horse, dog, cat, cow and human) from Queensland and Northern Territory and 23 isolates were chose for genome sequencing by Illumina. From the assembled genomes, strains were typed by MLST and serotypes. All fish isolates from Australia belong to ST261, a type previously found in Nile Tilapia from Israel. The marine strains are not closely related to terrestrial animal or human isolates. Comparison of marine and terrestrial isolates revealed smaller genomes in the marine isolates (1.7 MB compared with 2.1-2.2 MB in terrestrial), with very high numbers of pseudogenes in marine isolates, suggesting long term (and continuing) evolution by genome reduction.

O-093

# PATHOGEN HAZARDS ASSOCIATED WITH THE IMPORT OF PREDATOR BAITS TO THE UK

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The use of angling bait is recognised as a potential pathway for the introduction of pathogens and parasites into aquatic environment but the bait industry is largely unregulated. Fish baits, both marine and freshwater species, are popularly used in angling for large predators. The use of predator baits is potentially an important route of disease introduction as the fish are often used whole and come into direct contact with susceptible species. Businesses supplying predator baits were contacted and a list of bait species compiled. The most popular bait species are lamprey, smelt, mackerel, sprat and herring. Freshwater species such as eel, perch, roach and trout are also used. One supplier of predator baits advertised eels as coming from sustainable sources, as they are mortalities from aquaculture. In addition, rainbow trout intended for human consumption may be diverted for use in freshwater angling.
Disease hazards (for freshwater fish species in the UK) associated with imported bait species used were identified, i.e. pathogens or parasites that are absent from the UK or with limited distribution, OIE listed or a significant threat, and able to withstand freezing (predator baits are sold frozen). The most important hazards identified were viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV) and eels pathogens (eel herpesvirus and rhabdovirus). The persistence of VHSV in the tissues of infected trout has been demonstrated. Whilst the major pathogens of eels have been detected in the UK, their distribution may be restricted. The use of imported fish as bait in freshwater angling is a potentially important route of disease spread since it creates direct routes for the exposure of susceptible wild populations. The use of mortalities from aquaculture is particularly worrying because the animals are highly likely to be infected, and it provides a route for establishment and spread of new pathogens. The introduction of VHSV and IHNV (through diversion of imported trout for use as bait) and eel pathogens need to be investigated further in an import risk analysis to support strengthened biosecurity.

# AQUATIC ANIMAL DISEASE CONTROL IN ORNAMENTAL AQUATIC ANIMAL TRADE AND ORNAMENTAL AQUATIC ANIMAL HOLDING FACILITIES – QUO VADIS?

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Since 2004 ornamental aquatic animals are within the scope of the German Animal Disease Act (TierSG). The German Fish Health Regulation (FischSeuchV) - implementing the Council Directive 2006/88/EC - also includes regulations for the prevention and control of diseases of ornamental aquatic animals. Certain species of ornamental aquatic animals (e. g. koi carp, ornamental shrimp species) are susceptible to the following notifiable diseases, which are listed in the Directive 2006/88/EC:

- koi herpes virus disease (KHVD)
- crustacean white spot disease (WSD).

Other EU regulations and decisions mainly concern the import, intra-community movements as well as placing on the market of ornamental aquatic animals, in order to prevent the introduction or distribution of listed aquatic animal diseases. Experiences in case of KHVD-outbreaks gained after the implementation of the Council Directive 2006/88/EC into national law, show in case for Germany that the existing fish disease control regulations are only to a limited extend suitable to control notifiable ornamental aquatic animal diseases in a sustainable way. Problems occur for example

- in cases of epidemiological investigations after disease outbreaks with respect to traceability – due to the trade structure
- with respect to diagnostic methods to prove KHVD-freedom
- when dealing with KHVD-outbreaks in private garden ponds.

However, it is must be noted that fish diseases, with both susceptible or vector food and ornamental aquatic animal, may be controlled effectively only if minimum disease control measures apply and if these measures are implemented in a harmonized manner across the EU.
Since 2007, a significant decrease in the incidence of koi herpes virus disease can be observed in Germany. This may be attributed to the implementation of minimum control measures according to the Council Directive 2006/88/EC. It should be discussed whether and under what conditions more stringent regulations for ornamental aquatic animal trade or keeping, such as an EU-wide authorisation obligation for ornamental aquatic animal wholesale operations, might provide a further contribution to a sustainable aquatic animal disease control. This should be done within the frame of the Animal Health Law, which is under preparation by now. The Animal Health Law will be a part of the new EU - Animal Health Strategy, which aims to establish a clearer regulatory structure for animal health control across the EU.

O-095

# EFFECTS OF TEMPERATURE ON HEMATOLOGICAL AND HISTOPATHOLOGICAL CHANGES AND SURVIVAL RATE OF JUVENILE *FENNEROPENAEUS VANNEMAI* EXPERIMENTALLY CHALLENGED TO WHITE SPOT VIRUS (WSV)

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Many shrimp farmers were suffering from White Spot Disease (WSD) onset in last decades. Oscillation of environmental factors could lead mortality in susceptible hosts. Our study was aimed to investigate the effect of different temperatures on juvenile *Fenneropenaeus vannemai* experimentally exposed to White Spot Virus (WSV). Five hundred and forty juveniles were distributed among 3 treatments in triplicates, 22, 25 and 30$^{O}$C and experimentally WSV were injected in the shrimps. Our results showed mortality started at 36 hours post inoculation (hpi) in the treatment at 25$^{O}$C ($T_{25}$), meanwhile the mean value of mortality percent at 54 hpi in $T_{25}$ (71.10±17.35) showed the significant difference (P=.045) with $T_{22}$ (3.33±3.33) and $T_{30}$ (Not Observed, NO.). Our results suggest that in site selection, in primary stage of farm designing, water temperature at more than 29$^{O}$C, should be considered as key environmental factor. This finding can lead us that why the White Spot Disease occurred with high mortality in some area when the days of shrimp culture were prolonged until mid-autumn.

Key words:White Spot Syndrome Virus, Temperature, Fenneropenaeus vannemai, Challenge

# SYSTEMIC AMOEBIASIS IN CULTURED SENEGALESE SOLE: DIAGNOSTIC APPROACH AND PRELIMINARY STUDY OF ITS TRANSMISSION

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*Endolimax piscium* is the causative agent of systemic amoebiasis that affects Senegalese sole in some farms of the Atlantic coast of Spain. This amoeba causes a granulomatous inflammatory reaction mainly in muscle but also in different internal organs. When lesions are present, *E. piscium* cells can be identified in histological sections at the periphery of the lesions. In addition, stages of *E. piscium* can be found within the intestinal epithelium, usually disperse and without apparent host response. The minute and inconspicuous morphology of the parasites hampers their detection in routine histological sections, depending on the degree of inflammatory response associated to the development of granulomas. In addition, they can be easily misidentified by degenerated or apoptotic host cells when dispersed in different tissues or when not in a lesional context. In order to facilitate the study of this parasite, specific in Situ Hybridization and Polymerase Chain Reaction diagnostic tests were developed, and the performance of the new techniques was compared with conventional histology. Furthermore, the different techniques were used in combination to further understand the distribution of the parasite. Finally, a preliminary study on the horizontal transmission of this parasite was conducted by cohabitation between infected and healthy fish. As a result, the ISH was the most specific and sensitive technique and it was useful as a reference confirmatory method in intestine samples, not only to confirm difficult cases but also to discard negatives, diagnosed as doubtful by conventional histology. Although PCR-based tests resulted in a fast and reliable screening method, the present data suggests the need of further optimization of the sampling methods, mostly due to discontinuous distribution of the parasites. The route followed by the parasites to breach the intestinal barrier and reach other organs is still unknown, although connective tissue appears to be a preferential site for the localization of the amoebae. Horizontal transmission of the parasite was confirmed after four months of cohabitation, suggesting a slow transmission and a long prepatent period. *E. piscium* DNA was also detected by PCR from water samples taken from the cohabitating tank, thus supporting the hypothesis that transmission occurs through water.

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# REPEATED ZOOSPORE EMERGENCE AS A VIRULENCE FACTOR IN *SAPROLEGNIA PARASITICA*

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The aim of this work is to determine whether the ability to undergo RZE in *Saprolegnia parasitica* strains leads to an increased virulence on salmon parr. We evaluated the *in vitro* development, in absence of nutrients, of 14 strains of *Saprolegnia parasitica.* Two different responses were observed after secondary zoospore encystment: i) germination and ii) the production of a new generation of biflagellated zoospores, termed Repeated Zoospore Emergence (RZE). Then, we selected 6 *Saprolegnia parasitica* strains, where three exhibited RZE and three showed a germinative pattern in order to conduct challenge experiments on Atlantic salmon parr and subsequently determine their virulence. The results show that both the RZE and the germinative patterns are characteristic of the strain but not the species. Strains showing RZE produced significantly higher infection rate and an earlier establishment of the infection.

# RETENTION AND VIABILITY OF MICROSPORIDIAL (*LOMA SALMONAE*) SPORES WITHIN THE BLUE MUSSEL (*MYTILUS EDULIS*)

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*Loma salmonae* is the causative agent of Microsporidial Gill Disease of Salmon (MGDS), which causes chronic inflammatory branchitis and subsequent respiratory distress in affected fish. Chinook salmon (*Oncorhynchus tshawytscha*) are the most susceptible to MGDS and the disease causes high cumulative mortality in caged aquaculture on the west coast of Canada. MGDS cannot be reliably treated or prevented, and since spores are inherently environmentally protected entities, it is difficult to manage and treat MGDS where it is endemic. Extractive species utilized in integrated mutitrophic aquaculture (IMTA), such as blue mussels (*Mytilus edulis*), have shown promise in deactivating diseases such as infectious salmon anemia virus (ISA) and ingesting sea lice copepodids.
Our group has been investigating how the use of extractive species, namely the blue mussel, can influence the viability and movement of *L. salmonae* spores. Studies using our established donor-mussel/recipient-rainbow trout disease model have revealed that *L. salmonae* spores remain infective to naïve rainbow trout following ingestion by blue mussels for up to 1 month. Additionally, it has been determined that mussels do not appear to bioremediate infective spores, as rainbow trout exposed to mussel-filtered water develop MGDS. Spore retention was also studied across a range of ecologically relevant temperatures (10-21˚C). No differences in infectivity or retention were seen across the tested temperature range. We have also determined that feces and pseudofeces expelled from mussels exposed to spores caused infection in naïve rainbow trout, further indicating that mussels do not deactivate *L. salmonae* upon ingestion and excretion. The majority of spores are excreted by mussels 24 hours post-exposure, still viable as determined using an immunofluorescent-antibody test (IFAT) coupled with a propidium iodide (PI) exclusion test. These results verify that if mussels are exposed to *L. salmonae* spores they will be retained for up to 1 month and still be viable; and they will excrete viable spores in their feces and pseudofeces.

# EFFECTS OF GARLIC AGAINST THE MONOGENEANS *GYRODACTYLUS TURNBULLI* AND *DACTYLOGYRUS* SPP. PARASITISING THE GUPPY (*POECILIA RETICULATA* (PETERS))

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Monogenean infections of commercially farmed fishes are responsible for significant economic losses and existing chemical theurapeutants, often stressful to the fish, pose associated risks. Garlic (*Allium sativum*) is a well-known spice which also possesses anti-microbial and anti-parasitical properties (Guo *et al.* 2012). The current work aimed to test the efficacy of garlic-based treatments against infection with monogenean *spp*. in the guppy (*Poecilia reticulata*). Clipped sections of tail fins of guppies heavily infected with *Gyrodactylus turnbulli* were exposed to aqueous garlic extract (7.5 to 30 ppt) and visually observed under a stereomicroscope. Results revealed that exposure to garlic caused detachment of the parasite and cessation of movement, indicating death. Garlic concentration positively correlated with time to detachment and death of parasites, which, at the highest concentration of 30 ppt, occurred at 4.1 and 8.6 minutes respectively. Immersion in aqueous garlic extract (7.5 and 12.5 ppt) was tested in guppies infected with *G. turnbulli*. Prior acute toxicity tests revealed the maximum tolerance levels of guppies to garlic extract to be 12.5 ppt for one hour. Immersion treatment significantly reduced the prevalence and intensity of infection, and, at the higher concentration (12.5 ppt garlic extract for 1 hour), the fish appeared to be cleared of the infection. Oral treatments using dry garlic powder-supplemented diet were tested on guppies infected with *G. turnbulli* and Dactylogyrus *spp*. Fish were fed with food containing 2,4,10 and 20% dry garlic powder for 14 days. Parasite prevalence and intensity was significantly lower in groups fed with garlic supplemented diets at the higher inclusion levels *i.e.* 10 and 20% as compared to the control. Dietary application of garlic did not appear to affect food palatability. Fresh crushed garlic added to a 12 lt aquarium at a level of 1g/lt and applied as an indefinite bath for 14 days was seen to significantly reduce parasite prevalence and mean intensity as compared to the control. These findings show the potential of garlic as a natural alternative to currently used chemical treatments for monogenean infection in the guppy.

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# ASSESSMENT OF THE IMPACT OF A PATHOGEN, *BONAMIA OSTREAE*, ON *OSTREA EDULIS* OYSTER STOCKS WITH DIFFERENT HISTORIES OF EXPOSURE TO THE PARASITE IN IRELAND

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Abstract: The protozoan parasite *Bonamia ostreae* is a pathogen of the European flat oyster *Ostrea edulis* and has decimated stocks throughout Europe over the past four decades. A study of two stocks of *O. edulis* in Ireland with varying periods of exposure to *B. ostreae*, 5 years and 22 years, was undertaken. The two stocks are located approximately 210km apart, on the north and west coast. A number of beds from each stock were screened. The study was carried out over 13 months to investigate seasonality and the role of environmental parameters, population density and size on disease development. Of particular interest was the fact that though stocks of oysters at the two sites had been infected for very differing periods, prevalence of infection in both stocks was very similar. The stock that had been exposed for 22 years had a similar prevalence, intensity and seasonality of infection as the stock infected for 5 years. *B. ostreae* was detected in in both stocks throughout the year with the highest prevalence in spring, possibly related to the increase in water temperature and/or oysters directing their energy towards gametogenesis. Prevalence of infection was greatest in market-sized oysters in both stocks. A decrease in prevalence in larger, older oysters was observed. This may be due to animals with a greater susceptibly to the disease succumbing to the infection by this stage or the larger, and thus older, animals developing some form of tolerance to the parasite. The objective was to determine if varying lengths of exposure would translate into observations of differing susceptibility to *B. ostreae.* The study indicated that over a number of years some resistance to infection will build up naturally but this can be at a very slow pace without some intervention e.g. a selective breeding programme to reduce the input of susceptible oysters to the reproductive effort. In addition *B. ostreae* can also persist long-term in populations where densities are low and population sizes are small.

# *ENDOLIMAX PISCIUM*, CAUSATIVE AGENT OF SYSTEMIC AMOEBIASIS IN CULTURED SENEGALESE SOLE.

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Recently, systemic inflammatory lesions were described in cultured Senegalese sole, *Solea senegalensis*. The condition was characterized by lumps in the muscle, often noticeable at the skin surface, which make the fish unmarketable.
An amitochondriate parasite, possibly an amoeba, was identified in association with the lesions. Amoebic infections involving granulomatous inflammatory lesions and abscesses can affect different animal and human organs, especially the liver and the brain. However, systemic amoebiasis has seldom been reported in fish and the amoebae involved have not been fully characterized. This notwithstanding, a systemic granulomatous infection by a possibly related, amoeba-like organism, was reported in goldfish, *Carassius auratus* (L.), and recently also in tench, *Tinca tinca* (L.) [1-3]. Molecular phylogenetic inference and ultrastructure of the parasite from sole has recently been studied. The results place the organism firmly within the Archamoebae, and relatively close to the genera *Endolimax* and *Iodamoeba*. The parasite from sole has been tentatively described as *E. piscium* [4], although the distance with the closest relative, *Endolimax nana*, is noteworthy (only 62.2 % pairwise identity at the covered SSU rDNA region).
The relationship of this species with the causative agents of systemic amoebiasis from goldfish and from tench described previously is unknown due to lack of genetic data of those organisms. However, some morphological and pathological observations strongly resemble *E. piscium*. Preliminary analysis of a similar parasite isolated from goldfish, obtained from a Spanish pet store dealer, shows them to be closely related species, with 87 % pairwise identity along the SSU rDNA locus. The results point to the existence of a new clade of pathogenic amoebae infecting different freshwater and marine fish and related to facultative parasites from the gastrointestinal system of other vertebrates.

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# *ICHTHYOBODO* SPP. (BODONIDAE) FROM FISH AND CEPHALOPODS: COMPARATIVE ULTRASTRUCTURE, HOST-PARASITE INTERACTION AND MOLECULAR TAXONOMY

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Bodonid flagellates, of the genus *Ichthyobodo*, have long been recognized as significant pathogens of freshwater and marine fish in aquaculture. In contrast, the bodonids and "*Ichthyobodo*-like" flagellates from cephalopods, are poorly known. Key questions remain, for example: (i) what is the true identity of the bodonid from cephalopods, (ii) what is the mechanism of damage to the cephalopod host, and (iii) can infections be passed between fish and cephalopods? These questions are of considerable practical importance when managing the health of captive fish and cephalopods, whether held in exhibit aquaria or for research.

To address these questions, we have recently studied the host-parasite interactions, ultrastructure, and molecular characteristics, of pathogenic bodonids infecting the gills of Giant Pacific Octopuses, *Octopus* (*Enteroctopus*) *dofleini*, that were wild caught, and held at the National Aquarium in Baltimore.

At necropsy, tissue samples were preserved in 10% neutral buffered formalin for histopathology; some gill tissues were subsequently post-fixed in osmium tetroxide for ultrastructural studies, and some were frozen for molecular studies. Histopathology of the octopus tissues showed extensive bilateral gill damage and inflammation, associated with heavy infection with pyriform protozoa attached to the brachial epithilium and bacillary septicemia associated with integumental ulcers and bacterial cellulitis. Scanning electron microscopy showed that the parasites were attached via their cytostome, as is the case in *Ichthyobodo* spp. infections in fish. In the octopus, the attachment of the parasite perforated the mucus covering the brachial epithelium, a type of damage to the surface integrity of the host that does not appear to have been reported previously. Transmission electron microscopy of the octopus tissue showed features consistent with *Ichthyobodo* known from fish, including penetration of the cytostome deep into the cytoplasm of the host cell. Phylogenetic analysis showed that the *Ichthyobodo* species from the octopus was most closely related to one isolated from the dwarf cichlid, *Apistogramma* sp..

The phylogenetic trees showed that many *Ichthyobodo* species can have multiple hosts, and that some *Ichthyobodo* species can infect both freshwater and saltwater hosts.

# ASSESSING THE IMPACT OF PKD/VHS CO-INFECTION ON BROWN TROUT (*SALMO TRUTTA*) IMMUNE RESPONSE MODULATION

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Organisms are continuously exposed to a variety of micro- and macro-parasitic species, hence simultaneous infections often occur in wild, as well as in farm environments. Despite a growing awareness, co-infection studies are still very limited, mainly to a few well established human models. European salmonids are susceptible to both Proliferative Kidney Disease (PKD), an endemic emergent disease caused by *Tetracapsuloides bryosalmonae,* and Viral Haemorrhagic Septicemia (VHS), an OIE notifiable listed disease caused by a *Novirhabdovirus*; but no information is available as to how their immune system reacts in response to a multiple heterogeneous infection.
Naturally PKD-infected brown trout (*Salmo trutta*), after a seasonal outbreak on a UK fish farm, were VHSV-Ia bath challenged in a biosecure facility, resulting in typical macro- and microscopic lesions, although showing a very restricted and delayed mortality. Specifically optimised RT-qPCR surveys assessed the individual pathogen burden, allowing identification of PKD+/VHS+ (co-infected) fish, and detected the host immune response in terms of key marker gene expression in kidney. Histopathology screening provided a further confirmation that a successful co-infection was established, showing typical chronic changes surrounding extrasporogonic *T. bryosalmonae* together with acute hemorrhagic and necrotic patterns due to the viral action. During the course of the co-infection, the modulation of pro-inflammatory and antimicrobial peptides genes was strongly driven by the viral infection, additionally producing a protracted inflammatory status (>2weeks post viral infection - p.i.) that might represent a potential negative/side effect. Earlier activation of the cellular and humoral responses were detected in co-infected fish with a generally stronger up-regulation of Th-1 and antiviral markers. Interestingly, trout resistant to both diseases, obtained only at 2days p.i., showed a rapid and significant induction of Th-1, Th-2 and antiviral response markers. Kidney samples from 7 days p.i. were used for transcriptom profiling, carried out using an Atlantic salmon oligonucleotide microarray (Salar_2, Agilent 4x44K platform), to better assess the differential immune gene expression modulation between single- and co-infected fish.

# IDENTIFYING HEMATOLOGICAL EFFECTS OF CONCURRENT PARASITIC INFECTIONS USING MULTIVARIATE STATISTICS: A CASE STUDY USING RANCHED SOUTHERN BLUEFIN TUNA

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When exposed to a potential stressor, fish physiologically respond in a predictable manner. Yet the combined effects of multiple stressors is often not as clear cut as simply summing together the effects of individual stressors. This is often the case when studying animals in the natural environment, where numerous concurrent stressors may mask the individual effect of each other. Utilizing canonical correlation and split-line linear regression, this presentation will describe the how you can measure the effects of individual parasitic infections on fish within a natural co-infection scenario, as well as the physiological limit where these infections may cause stress.
A case study will be presented using data collected from wild and ranched Southern bluefin tuna. While numerous studies have attempted to isolate the physiological effect of each parasite in SBT, results from these studies are often insignificant or inconclusive. Natural infections often occur concurrently with numerous other parasites, therefore hindering isolation of their individual effects. I will demonstrate how allow for the simultaneous consideration of all parasite infections on a given sample, can identify and account for synergistic and antagonistic associations between individual parasites, while also allowing for determination of an individual parasites effect on one or several response variables, in this case haemtological parameters. In addition, I will describe how using a combination of canonical correlation and split-line linear regression analysis, the threshold limit where infection can be correlated with physiological stress within the host can be calculated.

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# NEW EVIDENCES OF *PARACARTIA GRANI* (COPEPODA, CALANOIDA) INVOLVEMENT IN *MARTEILIA REFRINGENS* (PARAMYXEA) LIFE CYCLE

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Around 10% of the national shellfish production in France is coming from Thau lagoon. Dynamics of the protozoan parasite *Marteilia refringens* was investigated monthly during a year into three suspected host species in this lagoon.
The targeted species were the Mediterranean mussel *Mytilus galloprovincialis*, the grooved carpet shell *Ruditapes decussatus* and the copepod *Paracartia grani*. Samples were first screened by PCR and positive results were then confirmed in mussels by histology and in *P. grani* and clams by *in situ* hybridization (ISH). ISH performed on *R. decussatus* showed *M. refringens* necrotic cells in digestive epithelia suggesting that this species is not involved in *M. refringens* life cycle in Thau lagoon. In opposition, presence of different parasite stages in mussels in histology and ISH positive labelling observed in some copepod sections, indicate that these two species contribute to *M. refringens* cycle at our study site.
The observations of *M. refringens* mature sporangia in spring and autumn in mussel suggest that (i) the parasite has two cycles per year in Thau and (ii) that mussels could release parasites from spring to autumn. PCR detection of *M. refringens* in *P. grani* copepodid stages between June and November supports the hypothesis of the transmission of the parasite from mussels to copepods but also from copepods to mussels. Indeed, a new peak of infection is observed in mussels at the end of summer, when *P. grani* abundance and PCR detection are maxima. ISH analyses performed on *P. grani* copepodites showed unusual parasite plasmodial like cells in digestive tract and gonad from the 3rd copepodid stage. In addition, mussels efficiency retention was measured on all developmental stages of *P. grani* (from eggs to adults). Results suggest that all *P. grani* stages could contribute to the transmission of the parasite to mussels, especially eggs and nauplii which were retained by mussels up to 90 %. The PCR detection of parasite DNA in *P. grani* eggs from *M. refringens* PCR positive females let think that eggs could contribute to the parasite spreading in the water and could allow *M. refringens* overwintering. Our results contribute to better understand relationships between the parasite, bivalves and copepods.

# TEMPERATURE-MEDIATED CERCARIAE PRODUCTION IN A BIVALVE-TREMATODE SYSTEM

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Trematode-mollusc system is sensitive to changing climate, because responses such as parasite development, cercarial emergence and survival, range extension or constriction, host fitness and parasite-induced host mortality can change as function of temperature. Studies examining predicted climate warming effects on mollusc-trematode systems have focused on snail hosts, while bivalve-trematode associations are poorly studied. Two bucephalid trematodes, *Rhipidocotyle campanula* and *R. fennica* are known to infect the freshwater bivalve mussel, *Anodonta anatina*. We investigated long-term temperature-dependent cercariae production in this bivalve-trematode system to assess how this system is likely to respond to predicted global warming. Mussels were from two populations. They were marked and allocated to three temperature treatments – high, intermediate and low. Between May 31 and October 28, 2011, every third week, mussels were individually monitored for cercarial emergence in the laboratory. Our results revealed that temperature-dependent cercaria shedding was species specific. In two populations, the total annual cercariae production was higher in high temperature for *R. fennica* (22000 cercariae per mussel), than for *R. campanula* (1800 cercariae per mussel), but the opposite was found in low temperature (1400 vs 2500 cercariae). *R. campanula* emergence started already in late May, whereas for *R. fennica* not until July. The mean period of cercarial production was about 60 days longer in *R. campanula* than in *R. fennica*, giving probably an advantage for *R. campanula* in northern areas where short summer is limiting occurrence of the parasite. With respect to this study, we can predict that *R. campanula* should be more northernly distributed as it can be better adapted to low temperatures in terms of length of cercarial emergence while *R. fennica* should have an advantage in high temperature habitats in terms of the number of cercariae produced. Host survival decreased with increasing temperature.

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# THE POSITIVE EFFECTS OF TWO PARASITES ON SWIMMING PERFORMANCE IN THE SPOTTED SEATROUT (*CYNOSCION NEBULOSUS*)

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Parasites are often associated with detrimental impacts on their hosts and very few studies have examined their effects on the swimming performance of fish. In this study, we aimed to determine the impacts of two parasite species, *Cardicola laruei* (Aporocotylidae), and *Kudoa inornata* (Myxosporea), on the swimming performance of spotted seatrout, *Cynoscion nebulosus* (Scianenidae). We measured burst (anaerobic) and endurance (aerobic) swimming performance of 18 fish using a swimming flume. Adult *C. laruei* inhabit the lumen of the fish heart and eggs are either carried by the blood to the gills, where they are released into the water, or they become encapsulated in the myocardium, where numerous granulomas may then form. *Kudoa inornata* inhabits the skeletal muscle of *C. nebulosus*. Many of the fish tested were infected with *C. laruei* (72% determined from the presence of granulomas in the myocardium and 89% according to the presence of eggs in the gills). Although we expected that infection by granulomas and eggs would impede fish swimming performance, a significant positive relationship was instead found between granuloma density and endurance swimming performance. At the same time, egg density in gills did not affect swimming performance. All of the fish (100%) were infected with *K. inornata* and an unexpected significant positive relationship was found between myxospore density in the muscle and burst swimming performance of the fish. These results suggest that these parasites aide, rather than impede, host swimming performance.

# EFFECTS OF BODY CAVITY DWELLING PARASITE *PHILOMETRA OVATA* (NEMATODA) ON THE FITNESS AND SECONDARY SEXUAL TRAITS IN THE COMMON MINNOW *PHOXINUS PHOXINUS*

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Parasite infection can have impact on various traits of host, including sexual ornamentation, motor performance, and dominance status. However, such influences have rarely been documented simultaneously. The common minnow is a cyprinid species, in which the males produce bright sexual ornamentation and show dominance hierarchies during the spawning season. In our previous surveys, we have found a minnow population parasitized by the body cavity dwelling nematode *Philometra ovata*. Since *P. ovata* has relatively large size comparing to the host minnow, it is likely that *P. ovata* may significantly reduce the fitness of parasitized minnows. In the present study, our aim was to clarify the effect of *P. ovata* on potential naturally-selected traits (i.e. body condition and swimming performance) and sexually-selected traits (i.e. lateral darkness, abdominal redness, the dominance behavior and courtship behavior) of the male minnows. Our results show that the swimming performance was positively correlated with lateral darkness and abdominal redness, which indicates that highly ornamented males have better swimming performance than less ornamented individuals. Surprisingly, the abundance of *P. ovata* was unassociated with the swimming performance. On the other hand, the male sexual ornaments were significantly correlated with the abundance of *P. ovata* in the body cavity. Thus, males harboring more *P. ovata* in the body cavity had paler abdominal redness and brighter lateral darkness, which thus reduces the contrast of the male coloration. Moreover, infected males showed dominance behaviors in similar level as non-infected ones but were more eager to push females than non-infected males, which indicates that infected males are more active in courtship. To conclude, our study show certain degree of influences of parasite infection on the host traits that are likely under natural and sexual selection.

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# PROTECTION OF ATLANTIC SALMON AGAINST INFECTIOUS SALMON ANEMIA VIRUS (ISAV) INFECTION BY INTRAMUSCULAR INJECTION (I.M.) OF IFNC EXPRESSION PLASMID

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DNA-vaccination experiments have shown strong protection against several virus diseases in fish demonstrating that fish muscle cells take up plasmids and express encoded viral genes that are under the control of an eukaryotic promoter. This inspired us to test if i.m. injection of plasmids expressing type I interferon (IFN) might provide protection of Atlantic salmon against virus infection. Salmon has three type I IFN subtypes, IFNa, IFNc and IFNb, which all induce an antiviral state in cell lines by inducing antiviral genes such as Mx, ISG15 and viperin. In this work we have injected salmon i.m. with plasmids encoding IFNa, IFNb or IFNc under the control of a CMV promoter or with a control plasmid and measured expression of antiviral genes in organs and studied protection against ISAV infection. While all three IFN plasmids induced Mx expression in the muscle at the injection site, only IFNb and IFNc plasmids induced Mx expression in head kidney 1 week after injection. Injection of IFNc plasmid induced antiviral genes (Mx, viperin, ISG15 and ISG58) and receptors for virus RNA (RIG-I, TLR3 and TLR7) in head kidney throughout the 8 week experimental period. Immunoblotting showed increased Mx protein expression in liver with time. Finally, challenge of the fish with ISAV by i.p. injection or cohabitation infection 7 to 9 weeks later showed strong protection of the IFNc plasmid injected, but no protection of the IFNa and IFNb plasmid injected fish. These data suggests that i.m. injection of the IFNc expression plasmid offers a new method of protecting Atlantic salmon against virus infection.

# CYPRINID HERPESVIRUS 3: IMMUNOGENICITY OF DIFFERENT TYPES OF VACCINES BASED ON MOLECULAR METHODS FOR COMMON CARP *(CYPRINUS CARPIO)*

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Cyprinid Herpesvirus 3 (CyHV-3) is a notifiable, highly contagious pathogen that causes massive mortalities of common carp (*Cyprinus carpio*) and the more colourful variety, the koi carp. Intense worldwide trade of these fish made its spread throughout the globe possible, mostly due to lack of veterinary oversight. The virus has been detected in various countries and has affected common carp food production in Europe, Israel, Japan and Indonesia. The virus can be diagnosed with different techniques, but there is no known treatment for CyHV-3. Due to the absence of available chemical treatment for CyHV-3 infection, the development of a suitable protection method for common carp and other susceptible species against this contagious disease is critical for limiting economic losses. Therefore, this study aims to develop and optimize an immunogenic vaccine based on molecular methods to protect common carp from CyHV-3.
A DNA vaccine was prepared by means of a eukaryotic expression vector. It was tested in cell culture to detect mRNA and expressed protein of the CyHV-3 gene. The DNA vaccine will be tested in a shortly upcoming animal trial to assess its protective immunity. This will be done via challenge with native virus 4 weeks post vaccination and continuous sampling for antibody titres.
Similarly, with a bacterial expression vector, we prepared a recombinant protein for use as a subunit vaccine. The originating protein reacted well with an Anit-CyHV-3 antibody in Western Blot analysis, indicating that it could be a good target as a vaccine. Because yields of soluble protein were low, we decided to focus on a truncated form of the protein to increase solubility and therefore practicability for use as a vaccine in a large scale animal trial. After preparation of this truncated CyHV-3 protein, it is going to be tested in an animal trial identical to the DNA vaccine experiment.
The results of our study will be presented and discussed.

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# EFFICACY OF ISA VACCINES POST I.P. CHALLENGE: MORTALITY, CLINICAL SIGNS AND ISAV QUANTIFICATION

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Infectious salmon anaemia virus (ISAV), belonging to the family Orthomyxoviridae, infects and causes disease in sea-farmed Atlantic salmon (*Salmo salar* L.). Outbreaks occur sporadically in most salmon-producing countries worldwide, and may cause high mortality and subsequently large economic losses to the salmon farming industry. Clinically, the infection often appears as a systemic and lethal condition that is characterized by circulatory disturbances including anaemia, ascites, enlargement of the liver and spleen, as well as petechial haemorrhage of visceral organs and the skin. Haemorrhages in the eyes and exophtalmia, may also be seen. The disease may however also develop without the fish showing any external signs of infection.
The Chilean salmon farming industry has been severely affected by the disease, causing the "ISA-crisis" in 2007-2009. In 2008-2010, several outbreaks were also reported in Norway, primarily in the county of Troms. Before 2008, vaccination against ISA was not practiced in Chile and Norway. PHARMAQ increased the focus on developing an ISA vaccine in 2007, and the first ISA vaccine from PHARMAQ was launched in Chile in April 2010. During the vaccine developmental phase, several clinical ISA studies have been performed, and in our presentation we will describe the intraperitoneal (i.p.) challenge model that was used to document vaccine efficacy against ISA, and discuss the correlation between mortality, clinical signs and quantity of ISAV mRNA transcripts in heart tissues of unvaccinated and vaccinated fish post challenge.
Results from our studies demonstrate that the i.p. challenge model provokes similar clinical signs in salmon as manifested during ISAV infections in marine-farmed Atlantic salmon. In addition the challenge model has the ability to discriminate between highly protective and less protective vaccines.

# KINETICS AND SAFETY OF A SALMON ALPHAVIRUS NUCLEIC ACID VACCINE

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Salmonid alphavirus (SAV) is a pathogen that induces Pancreas Disease (PD) in Atlantic salmon (*Salmo salar*) and Sleeping Disease in rainbow trout (*Oncorhynchus mykiss*). PD continues to have a profound effect on fish welfare despite ongoing improvement in management strategies and wide scale fish vaccination. The economic impact of PD is estimated to be in the range of €100 million per year in Norway alone. Novartis Animal Health has recently developed a Nucleic Acid Vaccine (NAV) in the quest for an efficacious solution.
The objectives of the present study were to determine the kinetics, safety and non-integration of the PD NAV into the salmon genome. A total of 369 fish were vaccinated with a 2 or 10 times dose relative to 10 µg per fish, while 197 fish were used as unvaccinated negative controls. Sampling was performed on 12 separate occasions, commencing on day 1 post vaccination with the final samples taken on day 822 post vaccination. To determine the kinetics of the plasmid, samples that comprised gut, spleen, gonad, head kidney and heart as well as epaxial muscle from the site of injection were taken. Additionally, up to day 135, muscle samples from the injection site were taken for microscopic evaluation to determine safety of the plasmid. Fish weights were measured at diverse sample times to determine differences in weight gain. PD NAV was shown to be localized predominantly in the muscle at the site of administration. The number of plasmid copies detected in muscle became negligible over time. This study also provided compelling evidence supporting non-integration of vaccine derived DNA into the fish genome.

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# COMPARISON OF LABORATORY AND FIELD PERFORMANCE OF AN INACTIVATED WHOLE-VIRUS VACCINE AGAINST SALMONID ALPHAVIRUS

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Efficacy of virus-vaccines for fish is commonly tested in various laboratory challenge models. It is not clear how vaccine performance in laboratory models relate to performance under field conditions. Here we evaluate efficacy of an inactivated virus-vaccine based on ALV405, a strain of Salmonid alphavirus (SAV) that was isolated from Norwegian Atlantic salmon (*Salmo salar*). Efficacy of the ALV405-based vaccine was compared to that of a positive control vaccine by intraperitoneal challenge, cohabitation challenge and during natural outbreaks the first and second year of a commercial production cycle of Atlantic salmon. Vaccination with the ALV405-based vaccine provided significant protection against PD under all challenge conditions. Relative differences between test and control vaccines were coherently detected in both challenge models and under field outbreaks, demonstrating that results from laboratory challenge models are relevant for understanding field performance of inactivated SAV vaccines.

# EFFICACY AND SAFETY OF A MULTIVALENT MICRO® DOSE VACCINE AGAINST INFECTIOUS PANCREATIC NECROSIS (IPN) IN ATLANTIC SALMON (*SALMO SALAR L.*)

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IPNV (infectious pancreatic necrosis virus) is a birnavirus causing IPN in salmonids, a disease that often occurs during the first months after transfer to sea. Vaccination is an important prophylactic measure that currently is routinely implemented as part of commercial farming of Atlantic salmon (*Salmo salar* L.). Previous studies on vaccination of Atlantic salmon against IPN have demonstrated that the efficacy of vaccines made by emulsification of inactivated IPNV in water-in-oil formulations is far superior to other vaccine delivery systems/adjuvants. Even though multivalent vaccines are the most widely used in commercial farming, there still are few published reports on the efficacy of these vaccines under laboratory and field conditions.
PHARMAQ has established a cohabitation challenge model for IPNV. By using a highly virulent strain of IPNV, high mortality in un-vaccinated Atlantic salmon has been obtained even when a low number of virus infected shedders were used in the challenge. The cohabitation model has further been used to evaluate the efficacy of ALPHA JECT micro® 6, a multivalent micro dose vaccine against IPN, in a laboratory study. Results showed that ALPHA JECT micro® 6 induce IPNV specific antibodies and a high level of disease protection equal to a similar monovalent IPN vaccine.
Vaccine efficacy and safety of ALPHA JECT micro® 6 has also been evaluated in field trials. Similar to the laboratory study, vaccination with ALPHA JECT micro® 6 resulted in a high level of protection and durable antibody responses lasting throughout the production cycle. Evaluation of side effects from vaccination with the micro® dose formulation (0.05 ml) compared to a 0.1 ml dose revealed that the micro® dose formulation induced lower side effects although the disease protection was equally high between the two vaccines. In summary, our results demonstrate that vaccination of Atlantic salmon with ALPHA JECT micro® 6 reduce mortality caused by IPN under laboratory and field conditions, and that dose reduction improves vaccine safety without reducing the efficacy of the vaccine.

# WHOLE INACTIVATED VIRUS VACCINE PROTOTYPE PROTECTS AGAINST VIRAL ENCEPHALOPATHY AND RETINOPATHY IN EUROPEAN SEA BASS (*D. LABRAX*)

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Viral Encephalopathy and Retinopathy (VER), otherwise known as Viral Nervous Necrosis (VNN), is a severe pathological condition, caused by *Betanodavirus.* The disease is considered as the most serious viral threat affecting marine farmed species in the Mediterranean region, thus representing one of the bottlenecks for further development of aquaculture industry of European sea bass, *Dicentrarchus labrax* (L.). The losses in the field can vary from 11 to 60 % in sea cages and from 11 to 50% in tanks.
The aim of this work is to evaluate the efficacy of a whole inactivated vaccine prototype in a *in vivo* challenge trial.
Two replicates consisting of 30 *D.labrax* specimens weigting 20 grams were immunized (IM injection) with formalin-inactivated supernatant from virus infected cell cultures,and two replicates of 30 fish were mock vaccinated with PBS. Fish were kept in 110 litres fiberglass acquaria with 22‰ salt water at 21° C (gradually raised up to 26° C before challenge).
After 30 days, each fish was intramuscularly injected with infected cell culture supernatant of reference challenge virus.
Classical clinical signs appeared in the mock vaccinated groups, while no signs were observed in the vaccinated fish. Mortality was recorded for 28 days, and at the end of the experiment fish were euthanized and weighed. Relative percentage of survival (RPS) was 76% with 47% mortality in the mock vaccinated. Fish brain tissue was examined for VNN using routine diagnostic cell culture and PCR techniques. Virus was re-isolated in 1 survivor from the vaccinated group and in all the survivors from the mock vaccinated group. Surprisingly, no neutralizing antibody activity was demonstrated in any serum samples collected at the end of the experiment. The prototype vaccine was demonstrated to be effective in reducing mortality, clinical appearance of the disease, and infection prevalence. Moreover fish vaccinated demonstrated a better growth performance compared to mock vaccinated ones.

# HUMORAL RESPONSE TO SYNTHETIC PEPTIDE FROM OUTER MEMBRANE PROTEIN A (*OMP*A) OBTAINED FROM CHILEAN *FLAVOBACTERIUM PSYCHROPHILUM*

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High levels of resistance to florfenicol, oxytetracycline and oxolinic acid have been observed among Chilean *Flavobacterium psychrophilum* isolates and have been associated with the high amounts of antimicrobials used at the Chilean farms to control outbreaks caused by this pathogen. To date no vaccine is commercially available to prevent the appearance of this disease. Among the antigens present in *F. psychrophilum, Omp*A has been used in vaccines against different Gram-negative bacteria, as well as human pathogens. In this study, we synthetized a peptide of 13 residues from *Omp*A sequence and evaluated its ability to develop a humoral response. This peptide was selected based on the hydrophilicity, flexibility and antigenicity to B-cells and was synthesized by the solid-phase method with a multipeptide synthesizer and purified by high-pressure liquid chromatography (HPLC). The peptide was coupled with hemocyanin by the carbodiimide conjugation method with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC). Balb/c mice were immunized with this peptide by intraperitoneal injection and serum was collected. Western-blot assays demonstrated specific reaction and recognition of *Omp*A using whole *F. psychrophilum* protein obtained from different isolates. Actually, challenge studies to assess this peptide-based vaccine are being developed.

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# INTERACTIONS OF HIGHLY AND LOW VIRULENT *FLAVOBACTERIUM COLUMNARE* STRAINS WITH THE GILLS OF CARP (*CYPRINUS CARPIO*)

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This research aimed at obtaining better insights in the interactions of *Flavobacterium columnare* strains of different virulence with the gills. Firstly, a reproducible challenge model generating gill lesions typical for columnaris disease was developed whereby *F. columnare* field isolates were characterized in terms of virulence. Amongst these, a highly (HV) and low virulent (LV) isolate, consistently resulting in 100% and 10% mortality amongst challenged fish respectively, were typified. Secondly, carp (*Cyprinus carpio*) were exposed to this HV or LV isolate and sacrificed at different times post-challenge. Histopathological and ultrastructural examination of fish inoculated with the HV isolate disclosed bacterial invasion and concomitant destruction of the gill tissue gradually spreading from the filament tops toward the base. The gill tissue of the few moribund fish from the group inoculated with the LV isolate displayed the same features as described for the HV isolate, albeit to a lesser degree. The gills of the outnumbering clinically healthy fish following challenge with the LV isolate showed no abnormalities apart from slight oedema. The bacterial number retrieved from the gill tissue as evaluated by plate counts and qPCR was significantly higher for the fish challenged with the HV compared to the LV isolate. TUNEL stained and caspase-3 immunostained gill sections demonstrated a significantly higher apoptotic cell count in the group challenged with the HV isolate compared to the control group. Periodic acid-*Schiff*/alcian blue staining proved that the total gill goblet cell count for the fish inoculated with the HV isolate was significantly higher compared to the control group and the fish challenged with the LV isolate, with a significantly higher increase for the goblet cells containing neutral mucins hence favouring a less acid environment. The bacterial cells of the HV isolate were observed getting encased by acid mucins and herein forming large clusters of bacterial cells packed with neutral mucins resembling biofilm formation. Besides indicating the high colonization capacity and the destructive and apoptotic-promoting features of the HV isolate, the present data point towards the importance of the dynamic host mucin–*F. columnare* interactions warranting their further research.

# NUTRIENT AND BACTERIAL DOSES AFFECT VIRULENCE AND HOST-SPECIFICITY OF THE OPPORTUNISTIC FISH PATHOGEN *FLAVOBACTERIUM COLUMNARE*

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*Flavobacterium columnare* is a gram-negative bacterial pathogen that causes columnaris disease in freshwater aquaculture. Columnaris outbreaks occur at fish farms during summer months and may cause mortality up to 100 %. Virulence of environmental isolates of *F. columnare* has been found to be lower than those isolated during disease outbreaks at fish farms. In order to understand factors selecting for the higher virulence at fish farms, we studied if the bacterial dose, exposure time (transient or continuous), or added nutrients have an effect on the virulence of *F. columnare*.

Three *F. columnare* strains were used in two separate experiments: a non-virulent strain B398 isolated from environment and two virulent strains from disease outbreaks (B185 and B67). In the first experiment zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) fingerlings were individually infected with bath immersion (transient challenge) with 9 different doses of bacterial strains B185, B398, and a mixture of these strains. In the second experiment the bacteria (strains B185, B398 and B67) were added in three doses directly into aquaria (continuous challenge) where zebrafish and rainbow trout were maintained. Longevity of fish was monitored for five days in both experiments, and the infection verified by bacterial culture from gills.

We found bacterial dose to have a positive effect on fish mortality and, rainbow trout to be more sensitive to columnaris infection than zebrafish in laboratory conditions. Increase in nutrients had a significantly positive effect on columnaris infection and fish mortality. The non-virulent strain was able to infect the fish when introduced in continuous exposure, but not in transient challenge. Our results suggest that the continuous exposure to bacteria at fish farms combined with a high nutrient level can promote virulence also in environmental non-virulent bacteria. In addition, the zebrafish can be used as a functional model host to study *F. columnare* virulence and infection dynamics in the laboratory.

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# DETECTION AND QUANTIFICATION OF *FLAVOBACTERIUM PSYCHROPHILUM* SPECIFIC BACTERIOPHAGES IN RAINBOW TROUT UPON DIFFERENT ADMINISTRATION METHODS: IMPLICATIONS FOR DISEASE CONTROL IN AQUACULTURE

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*Flavobacterium psychrophilum* is the pathogen causing the disease rainbow trout fry syndrome (RTFS), which has important implications for aquaculture production and trade worldwide. RTFS can be treated by antibiotic administration, but with the increasing problem of antibiotic resistant bacteria, the use of lytic bacteriophages is a promising alternative approach to disease control in aquaculture. Bacteriophage control of bacterial infections depends on efficient delivery of the phages to the infected organs, and in this study we therefore examined the occurrence and persistence of phages in the internal organs in rainbow trout, following different administration methods. Three phage administration methods using phage FpV-9 were used: phage bath, oral administration of phage-suspension directly into the stomach and feeding with phage-coated feed pellets. Phages were detected in all the four examined organs (intestine, brain, spleen and liver) with all three administration methods, demonstrating that the phages are capable of passing the intestinal wall and entering the bloodstream. The highest phage concentration was found in the intestine where a maximum of $3x10^{10}$ phages $g^{-1}$ was obtained after oral administration of phage-suspension, but also phage addition via phage-coated feed pellets resulted in high phage titers ($5x10^{6}$ phages $g^{-1}$ intestine). The concentration of phages in the spleen was 100 fold lower than in the intestine, suggesting a large phage decay during transport to the inner organs. These results provide the basis for future phage treatment of RTFS.

# POPULATION STRUCTURE OF *FLAVOBACTERIUM PSYCHROPHILUM* IN THE FOUR NORDIC COUNTRIES - DENMARK, FINLAND, NORWAY AND SWEDEN

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The bacterium *Flavobacterium psychrophilum*, recognized as the etiological agent of bacterial cold water disease (BCWD) also known as rainbow trout fry syndrome (RTFS), causes great losses in salmonid farming worldwide. There are few commercial vaccines available against BCWD today and disease control relies mainly on hygienic management and antibacterial treatment. Successful development of vaccines and diagnostic tools requires the identification of virulent clones and knowledge of the population structure of the pathogen. Multi locus sequence typing (MLST), which uses sequence data from a selection of housekeeping genes, has been employed to acquire such information on *F. psychrophilum*. In MLST, variants of each sequenced loci are assigned distinct allele numbers which are combined into a unique allelic profile defining the overall sequence type (ST) of a bacterial isolate. Analysis of 7 housekeeping genes in more than 500 *F. psychrophilum* isolates from the 4 Nordic countries: Denmark, Finland, Norway and Sweden and reference strains revealed 82 different STs. An eBURST analysis with default settings divided the isolates into 12 clonal complexes (CCs) and 31 singleton STs. The data set was dominated by one major CC, comprising almost 65% of the isolates. This CC, with ST10 as the predicted founder, contained almost exclusively isolates from rainbow trout and included ST2, the most predominant ST in the data set and ST79 as subgroup founders. Most ST2 isolates originated from Norway and Denmark while ST79 was mostly isolated in Finland and Denmark. The majority of the Swedish isolates belonged to the major CC. Analysis of MLST data using LIAN, showed substantial linkage disequilibrium, as a consequence of a small number of predominant STs in this population. Indeed, epidemic clones of single STs seem to have risen in frequency within each country causing harmful epizootics. The study showed an expansion of closely related genetic variants of *F. psychrophilum* in a large geographic area i.e the Nordic countries.

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# PHAGE-BACTERIUM INTERACTIONS USED AS A TREATMENT METHOD AGAINST THE FISH PATHOGENIC *FLAVOBACTERIUM COLUMNARE*

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Bacterial viruses (phage) have a large impact on bacterial communities. As a natural weapon against bacterial pathogens, lytic phage can be enriched in their natural habitats and used as antimicrobials. We have taken the first steps towards controlling columnaris disease (causative agent *Flavobacterium columnare)* with the help of phage. We experimentally infected rainbow trout (*Oncorhynchus mykiss*) fingerlings and zebra fish (*Danio rerio*) with virulent *F. columnare* strain and applied phage isolated from fish farming to the experimental aquaria as a treatment. The survival and symptoms of the fish as well as bacterial and phage counts in the tank water were monitored. In both fish species the survival of the phage treated fish was significantly higher compared to the fish not receiving the phage. We also noticed that the tanks treated with phage were either completely free of *F. columnare* or the survived bacteria expressed rough phenotype that we have previously shown to be non-virulent. As columnaris causes external symptoms and the bacterial cells are transmitted in the water, the disease is a convenient candidate for phage therapy. In general, phage are host specific and thus ideal for specific eradication of pathogens, such as *F. columnare*. However, bacterium-phage ratios and applications need further characterization, but our preliminary data suggests that phage can efficiently increase the survival of the fish exposed to *F. columnare*. Alternative biological control methods for bacterial diseases are needed especially because of the increasing problems with antibiotic resistant strains.

# COMPARATIVE GENOMICS OF *TENACIBACULUM* SPECIES

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The genus *Tenacibaculum*, a member of the family *Flavobacteriaceae*, encompasses today 18 species with validly published names, all isolated from marine environments. Some Tenacibaculum species are devastating fish pathogens whereas others, likely harmless, have been isolated from diverse marine animals, macroalgae, tidal flats and sea water. These bacteria are doubtless important for the fish farming industry and for marine ecosystems, likely playing significant roles in organic mater recycling. Despite their ecomomic significance to aquaculture production and their prevalence in different marine environments, little is known about the molecular traits defining their life styles. In order to identify relevant features in relation to their ecological niches, including virulence determinants for the pathogenic species, we carried out complete genome sequencing of all Tenacibaculum type strains and performed extensive comparative genomics. Our results offer significant insight into the evolution of this bacterial genus. These genomic data encompassing a whole genus could serve as references to facilitate further studies, including genotyping tools for epidemiological studies and for a better control of fish pathogens.

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# CHARACTERISATION AND DISTRIBUTION OF SALMONID ALPHAVIRUS SUBTYPE 2 IN NORWAY

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The causative agent of pancreas disease (PD) in Norway has previously been characterized as salmonid alphavirus (SAV) subtype 3. However, in spring 2011, the first case of PD caused by another subtype was detected. This virus is genetically closely related to SAV subtype 2, causing sleeping disease in rainbow trout in freshwater in continental Europe and UK. Due to the association of the new virus with sea reared fish the name marine SAV2 has been suggested.
Following the initial detection, the marine SAV2 has spread rapidly into an area previously free of PD, causing major problems for the fish farming industry in this region. Marine SAV2 found in Norway is very similar to SAV2 isolates found in sea reared salmon in Scotland.
After the initial discovery of marine SAV2 in Norway, PD virus from both historical and ongoing outbreaks of PD has been genetically characterized and the spread of marine SAV2 has been monitored. In this presentation, we will show the distribution of the different subtypes of SAV. In addition, we will present results from phylogenetic studies of the virus, showing that the marine SAV2 virus variant found in Norway has distinct genetic differences compared to isolates of SAV2 from outbreaks of sleeping disease in rainbow trout in fresh water.

# CHARACTERISATION OF INFECTIOUS SALMON ANAEMIA VIRUS ASSOCIATED WITH THE RE-EMERGENCE OF ISA DISEASE IN SCOTLAND IN 2008

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Infectious salmon anaemia virus (ISAV) is an orthomyxovirus and one of the major pathogen of farmed Atlantic salmon (*Salmo salar* L.) with outbreaks reported in all main Atlantic salmon producing regions, including Norway, Scotland, Canada, the United States, the Faroe Islands and Chile. In Scotland, the first infectious salmon anaemia (ISA) disease outbreak occurred in 1998/99 and affected virtually the entire region of Scottish salmon industry. Eventually, the disease was successfully eradiated with high economic costs to the Atlantic salmon industry. A second disease outbreak occurred in 2008/09 and in this instance only affected a relatively small geographic area in the southwest of Shetland Islands. The ISA outbreak isolates were characterized using sequences of complete coding regions of the segment 5 and 6. Significant differences were found between both outbreak isolates, with the ISAV 1998 and ISAV 2009 clustering to different highly polymorphic region (HPR) groups. A challenge experiment with both ISA outbreak isolates administrated to Atlantic salmon by intraperitoneal injection alongside cohabited naïve fish, was performed to investigate differences in cumulative mortalities associated with these viral isolates. Over a period of 71 days, the mortality associated with ISAV 1998 was twice as high as mortalities in fish IP-infected with the ISAV 2009. Cumulative mortalities of fish cohabited with ISAV 1998 IP-injected fish were 3-fold higher than fish cohabited with ISAV 2009 IP-infected fish. The complete genome of the ISAV 2009 isolate was further investigated using next generation sequencing and existence of different virus variant within this isolate will be discussed.

# GILTHEAD SEABREAM AND EUROPEAN SEA BASS CELL-MEDIATED CYTOTOXICITY AND GONADAL IMMUNITY UPON NODAVIRUS INFECTIONS

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Nodavirus (VNNV) is a serious outbreak affecting susceptible fish species such as European sea bass (Dicentrarchus labrax) but uses resistant fish as reservoirs such as gilthead seabream (Sparus aurata) to spread. Though most of studies focus on the interferon pathway and expression of several genes other aspects relevant for the fish immune response and non-immune tissues have received little attention. Thus, both head-kidney leucocytes from seabream and sea bass showed increased innate cell-mediated cytotoxic activity after nodavirus infections. Moreover, sea bass leucocytes resulted unable to lyse nodavirus-infected cells whilst seabream ones were much activated. This pattern was also followed by cytotoxicity-related genes such us perforin, granzymes or natural killer enhancer factor. As this virus seems to use the gonad to be vertically transmitted, we have for the first time evaluated the gonadal immunity. Thus, we found that viral infection decreased the 11KT hormone serum levels in both species whilst the E2 hormone was decreased in sea bass and increased in seabream. At gene level, VNNV infections failed to significantly change the gonads transcription in seabream but greatly up-regulated the transcripts of Mx, IL-1□, IL-6, or antimicrobial peptides in the case of sea bass. Further studies are in progress to understand the reasons behind the different susceptibility of these two species to the VNNV infections and disease.

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# VIRAL SCREENING OF WILD FISH ALONG THE NORWEGIAN COAST

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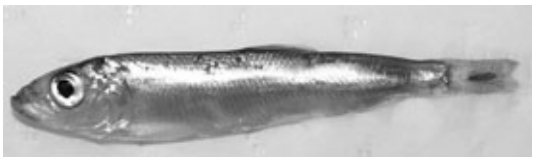
## Sampling and methods

Wild fish are in some cases believed to be the source of infection in viral disease outbreaks in farmed fish. In two ongoing projects samples from 2700 fish of 43 different species have been collected along the Norwegian coast line from Finnmark in the north down to Bergen. All fish will be examined for viral haemorrhagic septicaemia virus (VHSV) by rt-RT-PCR and/or cell culture (BF-2), while a selection will be tested by rt-RT-PCR for infectious salmon anaemia virus (ISAV) and salmon alphavirus (SAV).



## VHSV detection in herring

VHSV was detected in 1 of 451 pooled samples (internal organs from 1-5 fish) inoculated on BF-2 cells. The detection was done in herring and sequencing confirmed genotype Ib identical (99-100%) to VHSV earlier isolated from herring in the south of Norway. The VHSV positive herring were caught in the northern part of Norway (Finnmark), and thereby represents the nothernmost detection of VHSV. Individual organs from herring were therefore further analysed by rt-RT-PCR. 3 herring were found to be positive in all internal organs (brain, kidney, spleen, heart), while 4 were positive in gills only. It is unknown whether this represents a gill infection or if the virus is present only in the mucus layer. Further PCR testing on other fish species are on-going.

## ISAV and SAV

A selection of the same fish material sampled for VHSV-testing will this year be examined for ISAV and SAV. An update on all results will be presented at the EAFP conference.

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# VIRAL LOAD AND HISTOPATHOLOGICAL ANALYSIS OF SEVERAL ORGANS OF SAV-1 CHALLENGED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FED WITH DIETS CONTAINING DIFFERENT LEVELS OF N-3 PUFA

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Salmonid alphaviruses (SAVs) are considered as serious pathogens of both farmed Atlantic salmon (*Salmo salar*, L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum) in Europe, causing pancreas disease (PD) and sleeping disease in these two species. SAVs are composed of a single-stranded positive-sense RNA genome. Vertical transmission of the virus can take place without a vector and therefore are defined as atypical members of the genus *Alphavirus* (family *Togaviridae*).
An increasing demand for fish meal and fish oil for aquaculture has enhanced the focus of research on the substitution of fish based oils and proteins for those of plants, and the effect these have on the fatty acid composition of fish tissues and their immune response. Therefore attention is focusing on role of feed formulations with alternative fatty acid profiles for changing the severity of SAV induced pathology seen during PD outbreaks.
In this study, the effect of different levels of dietary n-3 polyunsaturated fatty acid (PUFA) on viral replication was assayed in 25-g rainbow trout experimentally infected with SAV-1 (isolate F02-148). Fish were fed with either a high (H) (22.4 %) or low (L) (14.4 %) n-3 PUFA diet for four weeks prior to challenging with SAV-1. Samples (heart, pancreas, skeletal muscle, kidney, spleen and gills) were taken at 0 days post-infection (d.p.i.), prior to the infection with SAV-1, and then again on 5 and 15 d.p.i. from uninfected and infected fish.
Histopathological lesions were observed in heart and pancreas of infected fish 5 d.p.i., and significant differences were seen in the pancreas between the two dietary groups by 15 d.p.i. Furthermore, two absolute quantitative PCR protocols based on the SYBR Green I technology were developed to amplify specific fragments within the open reading frame comprising the 3' third of the genome that encodes a 26S sub-genomic mRNA that ultimately produces the structural proteins, i.e. glycoproteins E1 and E2. Statistical analysis was carried out comparing the viral load within the different organs of fish fed with the H and L diets, and the differences obtained (e.g. higher viral load in the heart of fish fed the H diet at 15 d.p.i.) will be discussed further.

*ACKNOWLEDGMENTS: This study was supported by the Scottish Government and EU funded AquaExcel (Ref. number: 0028/02/03/13).*

# VIRAL HEMORRHAGIC SEPTICEMIA VIRUS IN BRITISH COLUMBIA, CANADA: TRANSMISSION FROM WILD TO FARMED FISH

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In the coastal waters of British Columbia, Canada, viral hemorrhagic septicemia virus has been detected for 20 years with many occurrences of mass mortalities among populations of Pacific herring *Clupea pallasii* (Valenciennes) and sardine *Sardinops sagax.* Additionally, the virus has been detected in cultured Atlantic *Salmo salar* and Chinook *Oncorhynchus tshawytscha* salmon. Molecular epidemiology of these isolates revealed all to be of genotype IVa with a close genetic linkage between isolates from pelagic finfish species and farmed salmonids, providing evidence for virus transmission from wild to farmed fish. To experimentally investigate virus transmission between these two species, Atlantic salmon were exposed to VHSV by IP injection or by waterborne immersion and cohabitation with diseased Pacific herring. Disease transmission was quantified by recording mortality, clinical signs, histopathological changes, cellular sites of viral replication, expression of interferon-related genes, and viral tissue titers. Results demonstrate that disease ensues in Atlantic salmon after exposure to VHSV-IVa and that infected salmon can transmit virus to sympatric Pacific herring. These laboratory studies will be discussed in the context of viral transmission, adaptation, and trafficking among populations of farmed salmonids and wild marine reservoirs.

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# ERADICATION OF VIRAL HAEMORRHAGIC SEPTICAEMIA IN DANISH AQUACULTURE

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Viral Haemorrhagic Septicaemia (VHS) virus was first isolated in Denmark in 1963, when more than 80% of the approximately 800 Danish fish farms were considered to be infected. Today, 50 years later, the whole country has applied to obtain status as EU approved VHS free zone. In the intermediate years significant resources have been used to eradicate the disease. The control programs have included strict biosecurity and preventative measures, trade regulations, stamping-out, zoning and intensive inspections and laboratory testing.
During the first decades of control and eradication programs the percentage of infected farms was significantly reduced to less than 20%. The last farms proved difficult to sanitise, and it was only after a large and costly coordinated action from 2009 supported by the European Fisheries Fund including all affected areas that the total eradication of VHS was successfully achieved.
Molecular tracing of the origin of VHSV isolates revealed that despite strict trade regulations and ban on introduction of live salmonids into the country, VHSV seemed to have crossed the boarders into Denmark in at least a couple of cases.
It is the first time that VHS with a targeted approach has been eradicated from an endemically infected country. A crucial factor for the success is the close collaboration between industry, stakeholders, veterinary authorities and scientists. Also a reduction of the total number of active farms and novel farming strategies account for the success, as well as the fact that in Denmark rainbow trout farming would not survive being infected giving a strong incentive for the industry to be involved.
Vaccination was not included in the control in Denmark, but if effective vaccines with marketing authorisation had existed such might have been useful in order to reduce virus load before and during stamping-out. The prevalence of VHSV in fishes living in the waters surrounding Denmark is relatively high and risk management of this threat for reintroducing VHS will be addressed.
Eradication of such a contagious viral disease from a heavily infected country is an inspiration for other areas who might wish to achieve the same goal and make trout production more profitable.

# BACTERIAL INFECTIONS OF KAMCHATKA FISH AND CRABS

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Since 2000 investigations have been carried out on fry salmon from hatcheries, wild pacific salmon (*Oncorhynchus*), flatfish (*Lepidopsetta polyxystra*, *Limanda aspera*), king crabs (*Paralithodes camtschaticus, P. platypus, Lithodes aequispinus*) and Chionoecetes crabs (*C. opilio, C. bairdi*) by bacteriological and histological methods.

Hatcheries had septicemias caused by pseudomonads and mixed infections. At one in particular, coho salmon fingerling regularly displayed gill disease caused by a mixed bacterial flora consisting of *F. psychrophilum* and *P. fluorescens*. Interstitial edema, extensive hyperplasia of the respiratory epithelium and adhesion of gill lamellae in these salmon were found. Accumulation of filamentous gram-negative bacteria was observed between the gill filaments on the surface of the gill lamellae.

Sporadic cases of hemorrhagic septicemia caused by *A. hydrophila* and *A. caviae* were observed in wild mature salmon. Hemorrhage in the muscle and lysis of myocytes were seen in fish with clinical signs of disease. Obligate pathogen *Aeromonas salmonicida* subsp. *salmonicida* was isolated from mature sockeye salmon in four spawning lakes. The prevalence of bacteria *A. salmonicida* was 3,3–36,6%. *A. salmonicida* was confirmed by PCR and three isolates (HE979856, HE979857, HE979858) were placed in the gene bank EMBL. Vibriosis was diagnosed in pink salmon and flatfish from sea waters. *Listonella* (*Vibrio*) *anguillarum* was isolated from 10% of the pink salmon, 12% of the northern rock sole and 32% of the yellowfin sole. Pathological changes in the kidney, gastrointestinal tract and liver were seen.

Experimental infection of salmon fingerling by *A. salmonicida*, *L. anguillarum* and *A. hydrophila* isolated from mature salmon confirmed there virulence.

Investigation of crabs showed that the most common disease is a bacterial shell disease, most prevalent in the fall and winter. The disease is more common in Chionoecetes crabs than in king crabs. In animals with a high degree of damage to the exoskeleton, life threatening severe changes in internal organs was found. The prevalence of damaged animals among Chionoecetes crabs in some years reached 9%, king crabs — 6%. Bacterium *Vibrio, Aeromonas, Pseudomonas* with proteolytic and lipolytic properties were isolated. Diseased crabs displayed large areas of gill necrosis, tegumental gland and lysis of hepatopancreas epithelium.

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# SUSCEPTIBILITY OF TILAPIA (*OREOCHROMIS SPP)* TO *EDWARDSIELLA ICTALURI*

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Bacterial disease outbreaks due to *Edwardsiella ictaluri* continue to have a significant impact on the sustainable production of channel catfish (*Ictalurus punctatus*) and striped catfish (*Pangasianodon hypophthalmus*) farmed in the USA and Vietnam, respectively. Previously tilapia were considered as a non-susceptible fish species but may act as potential carriers of the pathogen in freshwater environments. This study investigated the susceptibility of two species of tilapia (*Oreochromis niloticus* and *O. aureus)* to *E. ictaluri* administered under experimental challenge.

The bacterial challenge strain was recovered from a natural infection of bacillary necrosis of pangasius (BNP) in Vietnamese striped catfish and was identified as *E. ictaluri* using phenotypic, biochemical and molecular assays. Pure cultures of early log-phase growth were administered to tilapia by intraperitoneal injection at $10^7$, $10^6$ or $10^5$ cfu per fish. There were 10 fish per treatment group at 10-15g in weight. A control group was included for each tilapia species and these were treated as per the experimental groups, but did not receive any bacterial challenge. The study lasted for 12 days where fish were inspected daily and mortalities/morbidities recorded together with clinical signs of disease and samples taken for bacterial recovery and histopathology. Mortalities occurred in both tilapia species but were significantly higher in the *O. niloticus* compared with the *O. aureus*. These occurred in a concentration dependant manner with the highest mortality (97%) recorded for the highest bacterial concentration administered. All bacterial isolates recovered from affected fish were identified by biochemical tests and confirmed by PCR as *E. ictaluri.* Grossly, the affected fish presented with necrotic lesions in the kidney and spleen. Multifocal necrosis and granuloma formation was observed in these fish from the pathology sections. No gross or cellular abnormalities were observed in the control fish groups nor were any bacteria recovered from the control fish.

The results from this study would support that tilapia are susceptible to *E. ictaluri* when administered experimentally and that affected fish presented with lesions similar to those described for BNP infections in *P. hypophthalmus*. The impact of *E. ictaluri* infections in farmed tilapia will be addressed.

# MULTIPLE STREPTOCOCCAL SPECIES AS CAUSATIVE AGENTS OF A DISEASE IN FARMED CLIMBING PERCH *(ANABAS TESTUDINEUS)* IN VIETNAM

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A serious infectious disease of unknown aetiology recently broke out climbing perch *(Anabas testudineus)* farms in Vietnam, with moderate to high mortality rates during outbreaks. Natural infection was characterized by darkened body colour, eyes with corneal opacity, listless swimming, ascites, hepatomegaly, and splenomegaly. Pure small and opaque colonies were observed and Gram-positive cocci, catalase negative and oxidase negative, were isolated in brain heart infusion (BHI) agar and blood agar (BA) from internal organs of diseased fish. Therefore, the objectives of this study were aimed at identifying bacterial causative agents isolated from diseased climbing perch, evaluating the pathogenicity of the pathogens, determining the susceptibility of the pathogen isolates to anti-microbial agents, and analysing histopathological changes in infected climbing perch. In this study, conventional and rapid identification systems, and 16S rRNA gene partial sequencing were used to identify the causative agents of the disease. *Streptococcus iniae* made up 69% of the total streptococcal species identified. 26% was *S. agalactiae* and *Gemella spp*. remained less than 5%. Susceptibility of the *S. iniae* and *S. agalactiae* isolates to 15 antibiotics was tested using the disc diffusion method. $LD_{50}$ trial performance of *S. iniae* and *S. agalactiae* showed the virulence of these isolates in climbing perch and fulfilled Koch's postulates. Most infected fish in the experiments showed similar clinical signs to the natural infection. Histopathological lesions of internal organs of cimbing perch infected with streptococcosis showed that hemolysis at the kidney and granuloma-like lesion at the spleen were observed. To our knowledge, this is the first report of *S. iniae* and *S. agalactiae* as pathogens of climbing perch.

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# *AEROMONAS VERONII*, FIRST DESCRIPTION FROM SEA BASS (*DICENTRARCHUS LABRAX* L.) IN GREECE

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During the last three years a new pathological condition has been found to affect farmed sea bass (*Dicentrarchus labrax* L.) in southeastern part of Greece with significant losses. Affected fish exhibit skin lesions resembling furuncles and the most prominent internal finding was the enlargement of spleen and the presence of necrotic areas all over the organ. Microbiological analysis of samples from diseased fish revealed the presence of a non-motile Gram negative bacillus which was subsequently identified biochemically and molecularly as *Aeromonas veronii*. This is the first description of this pathogen from sea bass.

# NOVEL CHLAMYDIA-LIKE EPITHELIOCYSTIS AGENTS IN AUSTRALIAN FARMED YELLOWTAIL KINGFISH *SERIOLA LALANDI*, STRIPED TRUMPETER *LATRIS LINEATA* AND BARRAMUNDI *LATES CALCARIFER*

**M.C. Stride*[1], A. Polkinghorne[2], T.L. Miller[3], J.M. Groff[4], S.E. LaPatra[5], M. Powell[6] and B.F. Nowak[1]**

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Epitheliocystis is now known to affect over 80 difference marine and freshwater finfish species worldwide. In Australia, the condition has been reported from economically important species, such as yellowtail kingfish (*Seriola lalandi*), barramundi (*Lates calcarifer*) and silver perch (*Bidyanus bidyanus*). This condition can cause a severe proliferative host response, including bacterial inclusions in the gills, swollen gills, excessive mucus production and respiratory distress. Affected fish, especially those in commercial culture, will exhibit lethargy, surface gaping and reduced growth. Epitheliocystis can cause up to 100% mortalities of farmed juveniles.

Epitheliocystis has been detected in wild and farmed yellowtail kingfish (YTK) (*Seriola lalandi*), in farmed striped trumpeter (ST) (*Latris lineata*) and in farmed barramundi (BAR) (*Lates calcarifer*). To characterise the epitheliocystis associated bacterium, gills from YTK, ST and BAR were sampled for histopathology, the 16S rDNA region sequenced and comparative phylogenetic analysis performed. Novel 1393 bp, 1396 bp and 1396 bp 16S rRNA sequences could be amplified from YTK, ST and BAR gill DNA (respectively). These bacteria have been named *Candidatus* Parilichlamydia carangidicola, *Candidatus* Similichlamydia latridicola and *Candidatus* Similichlamydia latidicola, respectively, and are only 87-88% similar to the previously published *Candidatus Piscichlamydia salmonis* (AY464422) from Atlantic salmon and Arctic charr. Phylogenetic analysis placed these sequences into two new genera, within a new family of the Order *Chlamydiales*. This is the first molecular characterisation of *Chlamydia*-like bacteria associated with epitheliocystis from any fish species in the southern hemisphere.

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# IDENTIFICATION OF DIFFERENT PATHOGENICITY FACTORS IN *AEROMONAS* SP. AND THEIR CORRELATION TO CYTOTOXICITY AND ADHERENCE TO MUCUS

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*Aeromonas* sp. are Gram-negative bacteria, which are widespread in aquatic environments and commonly found in both fresh and salt water. Motile aeromonads are part of the normal intestinal microflora of healthy fish but are also considered to be opportunistic fish pathogens, which can cause problems under stress or disease and can lead to heavy mortalities in farmed and feral fishes. It is known that the identification of different *Aeromonas* species is difficult with biochemical methods as well as with analyzing 16S rDNA. Many studies show that there are differences in pathogenicity between *Aeromonas* strains. Bacterial virulence is determined by a complex array of virulence factors that allow pathogenic bacteria to cause a disease.
In this study we analyzed the cellular fatty acid profile of 44 *Aeromonas* sp. strains by gas chromatography to check if this method is appropriate to identify different species. Additionally all strains were analyzed for cytotoxicity and their ability to adhere to carp intestinal mucus. By PCR we examined 11 different virulence factors including type III secretion system (TTSS), cytotonic heat-labile (alt), cytotonic heat-stable enterotoxin (ast), cytotoxic heat-labile enterotoxin (act), aerolysin (aero), lipase (lip), serine protease (ser) nuclease (nuc), lateral flagella B (Laf B), haemolysin (HylA) and glycerophosholipid-cholesterol acyl transferase (GCAT).
Our results show that the fatty acid profile of *Aeromonas* strains can be used to a certain degree to distinguish between different species. However, with this method not for all species the results correlated to the biochemical identification. Analysis of the pathogenicity factors showed that there is no correlation between the species and the presence of genes encoding certain pathogenicity factors. Furthermore there is also no correlation between pathogenicity factors and adherence to intestinal mucus or cytotoxicity to EPC- and CCB-cells. These data suggest that for diagnostic use in motile aeromonads an examination of certain pathogenicity factors could be more useful than the exact identification of the species.

# THE EFFECT OF PATHOLOGY ON THE SERUM PROTEOME FOLLOWING INFECTION OF ATLANTIC SALMON, *SALMO SALAR*, WITH SALMONID ALPHAVIRUS 3

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Salmonid alphavirus 3 (SAV3) is the aetological agent of pancreas disease (PD) in marine Atlantic salmon, *Salmo salar*, and rainbow trout, *Oncorhynchus mykiss*, in Norway. This atypical alphavirus is transmitted horizontally causing a significant economic impact on the aquaculture industry. The disease is characterised in terms of histopathology by acute necrosis of the pancreatic acinar cells, cardiomyopathy and skeletal muscle necrosis, fibrosis and degeneration being observed. Whilst significant mortality is observed in the field, one of the biggest contributors of the economic impact PD has is that of muscle damage thus reducing fillet quality. Therefore, there is a large demand for non destructive means to assess the health status of the fish and identify if there may be any disease at a given site. In brief, samples were attained using an established cohabitational PD experimental model using Trojan shedders. In total 12 tanks were used with 9 fish from each being sampled at 0, 2, 3, 4, 5, 6, 8, 10 and 12 weeks post challenge (wpc) with blood and tissues (pancreas, heart, white and red muscle) being sampled and examined using an established semi quantitative scoring system. Proteomics using two dimension electrophoresis, peptide mass fingerprinting and bioinformatic analysis identified 72 protein spots that altered following the experimental infection of which 30 were significantly associated with histopathological lesion scores. A number of protein spots were selected for further investigation by Western blot due to their abundance in serum on the proteomics gels, response to white muscle pathology validated by general linear model analysis and availability of cross reacting antisera. For all proteins subjected to immunodetection by Western blotting the findings of the proteomic analysis were validated by consistent change in detectable bands of the blots. It is anticipated that this could lead to an immunodiagnostic test, not only for PD but also other diseases which cause skeletal muscle lesions in salmon.

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# INTER-LABORATORY TESTING OF FIVE NON-CULTURE ASSAYS FOR DETECTION OF *RENIBACTERIUM SALMONINARUM*

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To evaluate reproducibility and ruggedness of diagnostic assays for the detection of *Renibacterium salmoninarum*, the causative agent of salmonid bacterial kidney disease (BKD), inter-laboratory testing was conducted with five commonly used non-culture assays that had previously undergone bench-top validation in a single laboratory. The assays included a polyclonal enzyme-linked immunosorbent assay (ELISA), direct fluorescent antibody test (DFAT), membrane filtration-FAT (MF-FAT), nested polymerase chain reaction (nPCR), and real-time quantitative PCR (qPCR). Following a training workshop, standardized protocols for each assay were distributed to participants and posted on the U.S. Geological Survey Microbiology website (http://microbiology.usgs.gov/diagnostic_protocols.html #Renibacterium). Inter-laboratory testing was accomplished via blinded analysis of two test panels of kidney tissue and ovarian fluid samples obtained from known infected and uninfected fish. Nine laboratories participated in the testing, and each assay was evaluated independently by at least three laboratories. Results of the first round of ring testing showed relatively high estimated diagnostic sensitivity (≥ 95%) for each of the assays, indicative of a low occurrence of false negative results. Estimates of diagnostic specificity were generally lower (39% to 93%), indicative of a substantial number of false positive results for most assays in the first round. The first round testing results were communicated confidentially to each laboratory, and changes were suggested as needed to reduce misclassification of results. Subsequently, inter-laboratory testing of the second round panel of samples showed fewer false positive results, with estimated diagnostic specificity ≥ 85%, and diagnostic sensitivity estimates remaining high (≥ 93%). This study demonstrated the value of standardized protocols, adequate personnel training and quality control measures for obtaining reproducible results during the use of *R. salmoninarum* diagnostic assays by multiple laboratories.

# SOMETHING SMELLS FISHY

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Dead fish on beaches and river banks are of great public concern as well as generating considerable media interest. The timely investigation of the causes of such kills is often of low priority for government agencies and responses tend to be sporadic and ill-resourced but should form part of any comprehensive disease surveillance.

The Western Australian Fish Kill Response program is a joint initiative between the Department of Fisheries (DoF) and the Department of Water (DoW) and has operated continually since 1998 to gain reliable samples to assist in determining the cause of fish kill events throughout Western Australia.

Each department appoints an incident coordinator and takes a lead role in responding to fish kill events. DoF is usually the lead coordinating agency for marine kills or if the cause is due to disease, introduced aquatic pests or fishing activity. DoW is usually the lead coordinating agency for riverine, estuarine and pollution events. The order of involvement is flexible and is generally dependent on location of each department's field officers at the time of the event.

Because responding to fish deaths creates significant occupational health and safety issues, an intensive 1-day training workshop is conducted every two years for responding field officers, which incorporates sample and data collection, safety and transportation. Since inception, over 600 Officers have been trained.

In 2007 the scheme formed a model on which the Australian "National Investigation and Reporting Protocol for Fish Kills" was developed to provide a consistent national approach, and minimum standards for the management of fish kill incidents.

The reasons for setting up the scheme, the benefits and a summary of the pathology findings over the last decade will be provided.

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# GILL HEALTH MONITORING OF ATLANTIC SALMON IN SCOTLAND

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Amoebic gill disease (AGD), caused by *Neoparamoeba perurans*, emerged in late 2011 as a significant health issue in Scottish aquaculture. Prior to 2011, AGD cases are known to have occurred sporadically in Scotland from at least 2006. It has since become widespread for reasons that are unclear.
The 2012 S0 inputs from eight sites were monitored for the presence of gill pathology as part of a structured sampling programme, which included onsite training. Samples were collected every 1-2 weeks for a period of twelve weeks. These consisted of histology samples and gill tissue for PCR. Gill tissue was submitted for *N. perurans* and *Desmozoon lepeophtherii* PCR, together with gill scores and lice counts in order to assess gill health.
Amoebae were detected at 7 of 8 sites during the monitoring programme, and 4 sites developed clinical AGD. The diagnostic sampling (PCR or histopathology) consistently detected *N. perurans* before significant gill disease became established (gill scores < 0.3). However, it is possible that diagnostic methods could miss early infections, as early lesions in these fish tend to be small and focal. The median time of amoebae detection was 8 weeks post transfer.
*D. lepeophtherii* was detected by PCR at all sites (median time 6 weeks post transfer), but was not always associated with first settlement of *Lepeophtheirus salmonis*, which is thought to be part of their life cycle. There was one site where amoebae were not detected during the sampling period; however gill scores at this site showed a general upward trend. Because gill lesions may be a result of multiple causes, gill scores should always be combined with further diagnostics. Presence of the amoeba was also found on three sites that did not develop clinical AGD, suggesting exposure to the pathogen alone is not enough to cause clinical disease.
Overall the current sampling program is an effective method for monitoring gill health. This allows for adequate treatment of AGD at an early stage of the disease, reducing its impact.

# VACCINATION AGAINST *TETRAHYMENA* INFECTION IN GUPPIES (*POECILIA RETICULATA*) AND ELISA DEVELOPMENT FOR THE DETECTION OF ANTIBODY PRODUCTION

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Immunization against *Tetrahymena* by intraperitoneal (IP) injection was found to be effective (Chettri et al., 2009) although not practical. Oral route is more applicable for delivery in aquaculture. In the present study we tested immunization by anal intubation, where antigen is delivered to the hind gut, the site of antigen uptake. Fish were anally intubated with sonicated *Tetrahymena* in a mixture with Domperidon (DOM), Deoxycholic Acid (DOC) and Free Amino Acids (FAA; valine, leucine, isoleucine, phenylalanine and tryptophane) that enhance uptake from the gut (personal communications, Amos Tandler, National Center for Mariculture, Eilat, Israel). Additional fish were untreated or anally intubated with no antigen (negative controls) or IP immunized (positive control). Booster was applied 3 weeks post initial immunization and after 3 additional weeks the fish were challenged with *Tetrahymena* by IP injection (LD50 dose) or immersion. Mortality was monitored during the next 14 Days. In the IP challenge, mortality of control and anally immunized fish exceeded 50%, compared to 31% in the IP vaccinated group. No clear evidence was found for protection following challenge by immersion in any of the treated groups. To analyze antibody production against *Tetrahymena* by guppies, an ELISA was developed. Anti-guppy immunoglobulin (Ig) was produced in mice by immunization with bead-protein A+G-adsorbed guppy Ig mixed in complete Freund's adjuvant. Flat bottom microtiter plate wells coated with crud *Tetrahymena* extract (Iglesias *et al.* 2003) were incubated with guppy whole body homogenate (as a source for guppy antibodies) followed by incubation with mouse anti-guppy antiserum. HRP-conjugated goat anti-mouse IgG was then applied followed by the addition of substrate (peroxides substrate (3, 3', 5, 5'-tetramethylbenzidine solution plus hydroxide; Bio Rad) and OD measurement at 450 nm. Anti-*Tetrahymena* antibody levels, at a dilution range of 2-to-8 fold, were significantly higher in IP immunized fish compared to fish of all other treatments. The results demonstrate that IP immunization of guppy was partially protective against IP challenge with *Tetrahymena*, but not against challenge by immersion. In addition, immunization by anal intubation was not protective from infection.

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# A MULTIPLEX REAL TIME RT-PCR FOR DIRECT GENOTYPING OF VHSV

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Viral Haemorrhagic Septicaemia (VHS) is a notifiable fish disease, whose causative agent is a rhabdovirus isolated from a wide range of fish species, not only in fresh but also in marine and brackish waters. Phylogenic studies have identified four major genotypes, with a strong geographical relationship. In this study we have designed and validated a new procedure for simultaneous detection and typing of all 4 genotypes of VHSV by real time RT-PCR based on dual labeled probes chemistry, composed by two multiplex systems designed for European and American/Asiatic isolates, respectively, using a combination of three different fluorophores. The reliability of the procedure was evaluated against a panel of more than 80 VHSV isolates covering all known genotypes and subtypes of the virus, resulting in a correct detection and typing of all strains, and without any cross amplification. The efficiency and robustness of the systems were also assessed comparing multiplex systems against each singleplex reaction, yielding efficiency values between 94,2% and 109,7%. Analytical sensitivity was evaluated in a comparative assay with titration in cell culture, observing that both methods provided similar limits of detection. In order to assess repeatability and in-house reproducibility (R & R), an assay on nine different isolates was performed every day for 5 days by several technicians. The level of R& R was hereby demonstrated to be high, since the coefficient of variation between the Ct values was always lower than 4.15%. The procedure was also evaluated directly on fish samples from experimentally infected rainbow trout, and the results reflected that the method is suitable to be used for genotyping VHSV directly in RNA extracted from tissue samples. Finally, the results from the present study demonstrate that the multiplex dual system of RT-qPCR could be a powerful tool for epidemiological analysis of VHSV since an unknown sample could be genotyped in few hours.

# RAPID DETECTION AND IDENTIFICATION OF *MYCOBACTERIUM* PATHOGENS IN FISH USING HIGH RESOLUTION MELTING ANALYSIS (HRMA)

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Mycobacterial infections in fish are commonly referred to as piscine mycobacteriosis, irrespective of the specific identity of the causal organism. They constitute important bacterial infections of fishes, mainly causing a chronic disease with various clinical manifestations. They may result in high mortalities and severe economic losses. Piscine mycobacterioses affect fishes in all environments such as rivers, sea, rearing ponds or even aquaria. Nearly 20 species of *Mycobacterium* have been reported to infect fish. Among them, *M. marinum, M. fortuitum and M. chelonae* are generally considered as the major agents responsible for fish mycobacteriosis. *Mycobacterium* species affecting fish (particularly *M. marinum*) are also responsible for infections in humans, through direct contact of injured skin with infected fishes or contaminated waters. Considering the high impact of this disease and its implications in public health, there is a need for a quick and cheap diagnostic test that would allow both detection and identification of *Mycobacterium* species. In this purpose, we tested the potential of high resolution melting analysis (HRMA) to rapidly identify and discriminate several *Mycobacterium* species involved in fish infections. Targeting both the melting temperature and melting profile of the 16S-23S rRNA spacer region (ITS), the method enabled to discriminate 12 different species simultaneously. Sensitivity tests conducted on *M. marinum* and *M. fortuitum* revealed a limit of detection of 10 genome equivalents. The primers used in this procedure did not lead to any amplification signal with 10 control non-*Mycobaterium* species, thereby demonstrating a high specificity. From these promising results, we expect that this HRMA-based test will be a useful tool for rapid and cheap diagnostic of piscine mycobacteriosis.

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# *MARTEILIA COCHILLIA* SP. NOV., A NEW *MARTEILIA* SPECIES AFFECTING THE EDIBLE *COCKLE CERASTODERMA* EDULE IN EUROPEAN WATERS

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*Marteilia* "type C" was recently discovered, by molecular characterization, in the cockle *Cerastoderma edule* from the Ebro Delta in southern Catalonia (Spain) where it caused high mortalities during the summer of 2008. Further transmission electron microscopic morphological description and additional molecular analyses have been now carried out. Specific ultrastructural diagnosis, based in TEM observations of the parasite, showed the presence of around four secondary cells which seems subdivide into three "proteic masses". Each proteic mass contained two spores, and thus six spores per secondary cell were suspected. The number of secondary cells and number and nature of spores are morphological characters to distinguish different members of the Phylum *Paramyxea*. Phylogenetic analysis placed *Marteilia* "type C" in a well-defined cluster proximal to *M. refringens*. The new data thus corroborate previous results and lead us to formally describe *Marteilia* "type C" as a new species, *Marteilia cochillia* n. sp. affecting bivalves in Europe.

# CYTOKINE EXPRESSION IN RESPONSE TO BATH VACCINATION OF SEA BREAM (*SPARUS AURATA*) JUVENILES AGAINST *PHOTOBACTERIUM DAMSELAE* SUBSP. *PISCICIDA*

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Five genes encoding for five different molecules were analyzed by real time PCR (IL-1β, IL Ir-2, Cox-2, Mx and TNFα) in the liver of sea bream juveniles vaccinated against the marine pathogen *Photobacterium damselae* subsp. *piscicida*. The method of immunization was by short bath and three different bacterins were designed and used to vaccinate fish. Fish were divided into five groups: 1) Group 1, immunized with a formaline-killed vaccine, 2) Group 2, immunized with a heat-inactivated vaccine, 3) Group 3, immunized with a UV-light inactivated vaccine, 4) Group 4, immunized with a commercial vaccine and finally 5) Group 5, fish that received a bath in PBS as a negative control. Gene expression was quantified along four days after fish immunization; results were compared among groups and expressed as an increment upon de negative control value. Results show that the heat-inactivated vaccine stimulates the up-regulation of four of the five analyzed genes, whereas the UV-light inactivated vaccine was the unique vaccine which stimulates the expression of Mx gene in the liver of fish.

# POSTER PRESENTATIONS

# PHYLOGENETIC ANALYSIS AND PATHOGENICITY OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS (IHNV) IN THE NORTH KANTO AREA OF JAPAN

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Infectious hematopoietic necrosis virus (IHNV) is one of the most serious viral pathogens of salmonid fish. Originally enzootic in western North America, IHNV has spread following movement of salmonid fish and eggs to European and Asian countries, and is subdivided into five major genotypes: upper (U), middle (M), and lower (L) genotypes representing North American isolates, a fourth genotype representing European isolates sharing a common source with genotype M, and a fifth genotype representing Asian isolates sharing a common source with genotype U. Furthermore, IHNV isolates in Japan were classified into U and Asian isolates including two lineages, JRt Shizuoka (S) and JRt Nagano (N), and adult-size fish infected with IHNV are now frequently being observed in commercial aquaculture. Since IHNV isolates from cultured salmonid fish in Japan exhibit wide genetic diversity compared with those of other countries, some researchers suggest the diversity of Japanese IHNV continued to increase with changes of its virulence in salmonid farm environments. However, knowledge regarding the temporal shifts in relation to genotype/lineage and pathogenicity of IHNV isolates in Japan is limited. Therefore, in the present study we collected IHNV isolates from diseased salmonid fish at some fish farms in the north Kanto area of Japan from 1981 to 2013, and analyzed the G-protein full-length nucleotide sequences of each isolate. Phylogenetic analysis classified of all IHNV isolates into 3 genotypes/lineages (U, S and N), and isolates from the 1980s to 1990s mainly belonged to genotype U and the S lineage. On the other hand, most isolates after 2000 were classified into the N lineage, and the maximum nucleotide diversity among the N lineage was 4.4%. To evaluate the influence of the evolutionary divergence on virulence of IHNV, experimental challenges for the rainbow trout *Oncorhynchus mykiss* were conducted by bath exposure at $10^3$ $TCID_{50}$/mL using IHNV isolates selected randomly mainly from the N lineage. Results showed that fish mortality rate and symptoms differed among isolates used in this challenge test, and it is suggested that the nucleotide sequences of IHNV isolates in the north Kanto area of Japan continued to change rapidly with its virulence in the salmonid fish farm for at least the last ten years.

P-002

# EXPRESSION OF IPN PROTEINS CLONED IN SALMONID ALPHAVIRUS REPLICON

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Infectious pancreatic necrosis virus (IPNV) was the first virus isolated from fish and it is the etiological agent of a highly contagious disease in salmonid fish. IPN has contributed to significant losses in salmonid culture systems in Norway for decades. Cumulative mortalities may vary depending on virus strain, infectious dose, genetic outfit of the host, farm management and environmental factors such as water temperature. Vaccines have been an important strategy for control and prevention, and improved efficacy of IPN vaccines have been sought for. In this study a heterologous gene expression system based on the replication machinery of salmonid alphavirus 3 (SAV-3) was used for in vitro expression of IPNV proteins. The large reading frame of segment A encodes a polyprotein in which the order of the virus proteins is NH2-pVP2-VP4-VP3-COOH. The lack of presence of VP3 in empty capsids demonstrates that VP2 is capable to make subviral particles (SVPs) alone. The reading frame of the polyprotein (pSAV-segA), pre-VP2 (pSAV-pVP2) and VP2 (pSAV-VP2) were cloned into the SAV-3 replicon. The pVP2 is trimmed to mature VP2 by several cleavages of pVP2 at the carboxy-terminal end. The pVP2 to VP2 conversion is a slow process and probably performed by host cell proteases. The replicon constructs were transfected into CHSE cells and gene expression were detected by immunostaining and confirmed by western blot using specific IPNV antibodies. All proteins were expressed as shown by immunostaining and western blot. The VP2, pVP2 and VP3 proteins were detected from 4 days post transfection (dpt) with optimum expression/detection on 6 dpt.

# CYPRINID HERPESVIRUS 3 INDUCED DISRUPTION OF THE SKIN BARRIER IN COMMON CARP (*CYPRINUS CARPIO* L.) CAN LEAD TO SECONDARY BACTERIAL INFECTIONS

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Healthy fish skin possesses many characteristics which provide protection against invading pathogens and physical stresses from the surrounding environment. The surface of skin epithelium is covered by a mucus layer, formed by large glycoproteins, mucins, and antimicrobial peptides, which prevents the entry of pathogens. Paracellular tightness of skin epithelium is provided by cell to cell contact proteins forming desmosomes, tight and adherent junctions. During viral infections the homeostatic balance of the skin can be disrupted leading to a higher susceptibility to secondary infections. In carp, Cyprinid herpesvirus 3 (CyHV-3) infections are known to induce skin lesions, sloughing off the epithelium and a lack of mucus. Furthermore CyHV-3 induced disease is often associated with secondary bacterial infections. Here we aimed to determine how the skin barrier is challenged under this infection by examining (by RT-qPCR) mRNA expression of genes encoding inflammatory mediators, type I interferons, and antimicrobial peptides, cell junction proteins and mucins as skin defence molecules.
Results showed that in the skin of CyHV-3 infected carp, the infection leads to a reduction in mRNA expression of genes encoding several important components of the mucosal barrier, in particular mucin 5B and beta defensin 1 and 2. Also the CyHV-3 infection was influencing adherent and tight junctions as well as desmosomes by down-regulating the expression of claudins 23, 30, cadherin, and desmocollin 2. In order to confirm these results an *in vitro* skin model was developed based on fin and scale primary cell cultures. This *in vitro* model was able to confirm a virus induced remodelling of epithelial cell junctions.
Finally, the impact of a CyHV-3 infection on the skin barrier was evaluated by monitoring changes in the bacterial flora of the skin during disease conditions. RT-PCR-DGGE showed that CyHV-3 infection caused changes in the bacterial community and the development of secondary flavobacterial infection among some individual fish. This result was confirmed by RT-qPCRs specific for aeromonads, flavobacteria and the total bacterial flora.
In conclusion: we showed that under disease conditions associated with virus infection the mucosal barrier of fish skin was disrupted, resulting in a higher susceptibility to secondary bacterial infection.

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P-004

# EFFECTS OF SIRNA TREATMENT ON CYPRINID HERPESVIRUS 3 INFECTION IN COMMON CARP (CYPRINUS CARPIO L)

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A Cyprinid herpesvirus 3 infection induces disease which causes significant losses in carp aquaculture. Currently no treatment besides preventional vaccinations is available. One of the possible strategies of viral infection management is RNA interference (RNAi), a post-transcriptional gene silencing by, for example small interfering RNA (siRNA), which is showing great potential in human medicine. The main challenge regarding alloherpesviruses is the fact that members of this virus family possess large genomes encoding in the case of CyHV-3 for 156 possible proteins whose functions are not fully explored. Therefore selection of the most suitable single treatment target could be problematic.
The aim of the presented work was to investigate the most suitable treatment strategy and identify the potential of siRNA in treating CyHV-3 infections *in vitro*.
Common carp brain (CCB) fibroblastic cells were used with lipid based transfection. The 21 to 27 bp long siRNA in two concentrations (2μM and 8 μM) were targeting capsid proteins: capsid triplex protein (ORF 72) and major capsid protein (ORF 92) or DNA synthesis enzymes: DNA helicase (ORF 71) and DNA polymerase (ORF 79). Custom modifications phosphothioate oligonucleotide (PTO) and 2'-O-methyl (O-me) modifications were used to increase silencing activity.
A simple readout system based on crystal violet staining of viable cells has been employed to determine the siRNAs treatment potential. The results showed that non-infected cells had OD=0.91 while infected and non-treated infected had OD=0.46. The single target treatment was not effective (OD=0.44 to 0.52), however when oligos were mixed a significant reduction in the number of killed cells was achieved (OD=0.72). Interestingly the poly I:C treatment (resulting in a strong induction of type I interferon responses) used as one of the controls was the most effective (OD=0.82).
In conclusion: we found that a blend of short interfering RNAs specific for viral DNA enzyme synthesis and capsid proteins of the CyHV-3 can be a potent inhibitor of virus replication in fibroblastic cells. Currently, a major obstacle is obtaining better performing single target treatments as well as the delivery of siRNA to specific tissues or organs *in vivo*.

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# MOLECULAR TRACING OF VIRAL PATHOGENS IN AQUACULTURE -A MULTIDISCIPLINARY TRANS-EUROPEAN RESEARCH PROJECT

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Viral diseases threaten sustainable aquaculture production of both fish and molluscs worldwide. In Europe, the most important viral diseases include infection with viruses in the families *Alphaviridae*, *Rhabdoviridae*, *Betanodaviridae*, *Malaco-* and *Alloherpesviridae*. Considerable resources are invested in controlling the spread of viral pathogens in aquaculture. Control strategies have generally been designed based on general knowledge on biosecurity and only to a very limited degree take into account disease-specific transmission patterns. This leads to compromised efficiency of preventive interventions. New EU-legislation requires that surveillance of aquaculture diseases be risk-based, but knowledge on risks and their prevention is poor.
Here we present a new research-project funded under the EMIDA-ERA Net under the EU 7$^{th}$ Framework program, with the purpose of (*i*) increasing knowledge on transmission, prevention and control of viral diseases in aquaculture and (*ii*) developing a generic approach to viral disease control by using information on epidemiological and phylogenetic attributes from several important aquatic animal viruses.
In this project, known nucleic acid sequences will be used as a starting point for clarifying phylogenetic relationships among strains, while new ones will be collated in order to get a better overview of the existing viral populations. Both already-available and newly-acquired sequence and epidemiological data will be organised in the newly established database www.fishpathogens.eu. At the same time, these sequences will be used to build DNA microarrays which will serve to search for possible molecular markers involved in virulence. Statistical models to integrate spatio-temporal epidemiological data and phylogenetic data in analyses of factors of importance to disease spread will then be developed for the different host – pathogen systems incorporated in the project. The models will be used to explore different intervention strategies to reduce future disease incidence.
Despite the broad range of aquatic animal viruses included in this proposal, aquaculture farming share a whole set of epidemiological and management parameters, making them unique from terrestrial animals. By broadening the proposal to several viral pathogens and animal species, generic recommendations and results are anticipated to be produced and to benefit the whole aquaculture industry.

Follow the MOLTRAQ project at: http://moltraq.wordpress.com

P-006

# EVALUATION OF THE POTENTIAL ROLES OF MICRORIBONUCLEIC ACIDS IN THE INTERACTION OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) WITH VIRAL HEMORRHAGIC SEPTICEMIA VIRUS

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Micro ribonucleic acids (miRNAs) are endogenous, 18-22-nucleotide non-coding RNAs that potently mediate post-transcriptional silencing of a broad range of genes. They are emerging as critical regulators of a broad spectrum of biological processes, including immune responses and host-pathogen interactions. Some miRNAs in mammals have been shown to directly inhibit viruses, whereas other cellular miRNAs can be co-opted by viruses to promote viral replication or evade host immune responses. We have previously observed that two miRNAs known from zebrafish, miR-462 and miR-731, were the most highly expressed miRNAs in rainbow trout liver following *Viral hemorrhagic septicemia virus* (VHSV) infection. These miRNAs were also upregulated in the liver and muscle (vaccination site) of fish vaccinated with a DNA vaccine encoding the glycoprotein of VHSV. Recent studies further suggest that the expression of these miRNAs is induced by interferons. In order to investigate the potential role(s) of miRNA-462 and miRNA-731 in host-pathogen interactions, we designed synthetic oligonucleotides called antagomiRs or anti-miRNAs to silence these two miRNAs. These antagomiRs were injected intraperitonealy into rainbow trout fingerlings followed by exposure of the fish to VHSV. Development of disease and levels of infection were analyzed and compared to data from fish treated with control miRNAs. Further analysis of the effect of anti-miRNAs in cell culture will be performed.

# IRIDOVIRUS CARD PROTEIN INHIBITS APOPTOSIS THROUGH INTRINSIC AND EXTRINSIC PATHWAYS

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The grouper iridovirus (GIV) belongs to the genus Ranavirus and family Iridoviridae, whose genome contains an antiapoptotic caspase recruitment domain (CARD) gene. The CARD gene encodes a protein of 91 amino acids with molecular mass of 10, 505 Dalton and shows high similarity to other viral CARD and human ICEBERG. Northern-blotting showed that GIV-CARD expressions began at 4 h post-infection, and the mRNA level significantly inhibit in the presence of cycloheximide but not in the presence of aphidicolin, indicating that this GIV-CARD is an early gene. By immunostaining, it was proven that GIV-CARD-expressing cells effectively inhibited apoptosis. In the intrinsic pathway, UV-irradiated HeLa cells underwent apoptosis. However, Over-expression of recombinant GIV-CARD protein in HeLa cells inhibits apoptosis under UV treatment. In the extrinsic pathway, cells treated with anti-Fas antibody underwent apoptosis and GIV-CARD-expressing cells also inhibit apoptosis under anti-Fas antibody treatment. In caspase assay, the activities of caspase 3、8 and 9 triggered by anti-Fas antibody treatment were reduced by the expression of GIV-CARD gene in HeLa cells. Taken together, these results demonstrate that GIV-CARD inhibits the cell apoptosis through intrinsic and extrinsic pathways.

P-008

# PURIFICATION AND PARTIAL GENOME CHARACTERISTICS OF A HERPESVIRUS INFECTING THE ABALONE, *HALIOTIS DIVERSICOLOR SUPERTEXTA*

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AbHV was purified from infected abalone, *H. diversicolor supertexta* in Taiwan. The genomic DNA from the Taiwanese abalone herpesvirus (AbHV) was obtained and showed 99% (5767/5779) homology in the nucleotide sequence and 99% (1923/1926) in the amino acid sequence with the DNA polymerase gene of the abalone herpesvirus strain Victoria/AUS/2007. Homology of the amino acid sequence with the DNA polymerase of ostreid herpesvirus 1 was 30% (563/1856). The phylogenic analysis showed that the AbHV isolate from Taiwan is a member of the Herpesvirales. AbHV isolate from Taiwan, AbHV-1(Australia isolate/2007) and OsHV-1 were clustered in the same subgroup based on DNA polymerase sequence data. Analysis of large genome fragments of the AbHV virus isolate from Taiwan revealed that the TC04 fragment of the Taiwanese isolate (NCBI accession no. JN083851) had 85.7% (10481/12224) identity to the Victoria/AUS/2007 AbHVscaffold_3172-3200 fragment. The TC02 (NCBI accession no. JF967012) and TC08 fragments (NCBI accession no. HQ890941) of the Taiwanese isolate corresponded to the Victoria/AUS/2007 AbHVscaffold_3197-3033 isolate, and had 66.7% (26,884/40,281) and 61.2% (14,529/23,756) identities, respectively. These results suggested that the Taiwan virus isolate, Taiwan/2004, is distinguishable from Australia isolate/2007.

# HEPCIDIN IS INVOLVED IN THE IMMUNE RESPONSE OF THE GONAD OF GILTHEAD SEABREAM AND EUROPEAN SEA BASS AGAINST NODAVIRUS

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Nodavirus is a vertical transmitted pathogen able to produce the disease in the progeny of infected broodstocks of European sea bass (*Dicentrarchus labrax*). On the other hand, the gilthead seabream (*Sparus aurata*) is an asymptomatic carrier which progeny do not suffer high mortalities but transmit the infection. Within the reproductive organs of vertebrates, regulation of immune response is tightly regulated in order to avoid germ-cell damage. Antimicrobial peptides (AMPs) are increasingly recognized as a critical first line of defense against many pathogens including virus. In mammals, AMPs are expressed in the reproductive tract and might have a role in the innate immune response against pathogens, however, little is known about its presence and role in teleost fish gonad. In this study we have determined the expression of lysozyme and hepcidin genes in the bisexual gonad of gilthead seabream males and in the gonad of European sea bass males and females challenged *in vitr*o with bacteria (*Vibrio anguillarum*), virus (*nodavirus*) or poly I:C. Moreover, after in vivo infecgtion with nodavirus we have also evaluated the bactericidal activity in serum and gonad-homogenates as well as the expression pattern of lysozyme and hepcidin genes in the brain (target tissue for nodavirus), head-kidney and gonad tissues. Our results show that while the gilthead seabream scarcely response to the *stimuli*, the European sea bass up-regulated the expression of hepcidin gene in the gonad both *in vitro* and *in vivo*. Interestingly, the response in terms of hepcidin gene expression is different between the male and female gonad. These data correlated with the bactericidal activity observed in the serum of the European sea bass infected *in vivo* with nodavirus.

*Acknowledgements.*

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P-010

# ABSOLUTE QUANTIFICATION OF *PISCINE ORTHOREOVIRUS (PRV)* CORRELATES WITH MARKERS OF ANTIVIRAL ACTIVITY IN PRV CHALLENGED ATLANTIC SALMON PARR

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Piscine orthoreovirus (PRV) induces Heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (*Salmo salar L*) smolts in challenge studies. Besides causing HSMI outbreaks in more than one hundred salmon farms along the Norwegian coast every year, the virus is also found widely distributed in asymptomatic farmed and wild salmonids. The development of HSMI is reported to be linked to factors which may affect inflammatory control, like feed ingredients and external stressors. HSMI outbreaks commonly occur following smoltification and transfer to sea water pens, whereas PRV distribution has been reported in many Norwegian fresh water cohorts, indicating that the salmon developmental stage may be of relevance for the onset of disease.

In order to quantify the presence of PRV in fish tissues more accurately, we developed an absolute quantification assay. This assay, based on in vitro transcript standard curves and quantitative reverse transcription PCR (qRT-PCR) assays targeting highly conserved regions of PRV gene segments, show good reproducibility and sensitivity for viral copy number calculation.

In the present report, the absolute PRV quantification assay was used to evaluate viral load in tissue samples from a controlled PRV challenge study in parr using low challenge pressure. Virus was found to accumulate for a limited time period after infection, mainly in spleen and head kidney. Increasing viral loads in spleen and head kidney correlated with induced expression of genes linked to antiviral immune response and was followed by a reduction and later disappearance of PRV. These findings indicate that fresh water parr may successfully combat PRV infection.

# PARTIAL MOLECULAR CHARACTERIZATION OF THE ATLANTIC SALMON PAPILLOMATOSIS VIRUS

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Herpesviruses of fish and amphibians have been classified into a novel family, the *Alloherpesviridae*, under a newly established order (*Herpesvirales*) with the herpesviruses of higher vertebrates (*Herpesviridae)* and mollusks (*Malacoherpesviridae*). Presently, the family *Alloherpesviridae* contains four genera with 12 accepted virus species. The genus *Salmonivirus* contains three viruses isolated from different salmonid species, the *Salmonid herpesvirus 1*, *2* and *3.*
Atlantic salmon papillomatosis is a benign skin disease that has been reported since the 1950s in wild and farmed Atlantic salmon (*Salmo salar*) in Scandinavia, Scotland and in the northwestern part of Russia. The disease mainly affects juveniles in fresh water and occasionally migrating adults returning to rivers to spawn. A viral agent, resembling a herpesvirus, has been observed within proliferating epidermal cells by electron microscopy. Attempts to isolate the virus from diseased fish using several fish cell lines yielded negative results.
In this study we provide the very first herpesviral sequences detected in the papillomas from diseased Russian Atlantic salmon. Three partial gene fragments (DNA polymerase, terminase and glycoprotein) were amplified and sequenced from specimens collected in 2011 from different rivers and hatcheries in the Kola Peninsula. The Phylogenetic analyses, based on the partial sequences of the viral polymerase and terminase genes, supported the virus as a novel member of the genus *Salmonivirus* within the family *Alloherpesviridae* as the sister species to SalHV-3. The sequences of the Atlantic salmon papillomatosis virus differ markedly from those of the three known salmoniviruses, therefore the authors propose the Salmonid herpesvirus 4 (SalHV-4) species designation. These data are consistent with previous ultrastructural evidence that the virus is a herpesvirus.

# PISCINE ORTHOREOVIRUS (PRV) REPLICATES IN ATLANTIC SALMON ERYTHROCYTES

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Piscine orthoreovirus (PRV) is a naked virus with a segmented double stranded RNA genome of the *Reoviridae* family. The virus is associated with Heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar L*). Currently, HSMI is diagnosed by typical histopathological changes in the heart and skeletal muscle. The load of PRV in diseased fish examined by RT-qPCR correlates with the severity of HSMI in naturally and experimentally infected salmon.
Studies of the HSMI pathogenesis in salmon challenged with PRV have previously detected the virus in cardiomyocytes by immunohistochemistry, coinciding with the course of the disease. Interestingly, in an experimental challenge we detected distinct patterns of viral presence in a population of blood cells prior to disease onset. This was observed in both the inoculated and cohabitant group.
In the present study, we aimed to characterize the potential target cells of PRV in blood, based on material from two PRV challenge studies conducted at VESO Vikan. Using RT-qPCR we screened for PRV in blood, heart, skeletal muscle, spleen and head kidney. The results showed high PRV levels in blood, especially in the early phases of infection compared to the other tissues analysed. In a follow-up experiment focusing on the initial phases of the infection, PRV-infected blood was used as inoculums. Erythrocytes were isolated using a percoll gradient and screened for PRV by RT-qPCR, demonstrating high virus load in the red blood cell population. Isolated erythrocytes were stained with a polyclonal antibody against a PRV capsid protein and analyzed by flow cytometry.
A population of PRV-positive cells were observed by flow cytometry which correlated with PRV-levels as assessed by PCR. The subcellular localization of the virus was investigated using confocal microscopy, and large cytoplasmic inclusions were observed. These structures resembled viral factories, known to be the main sites for replication of orthoreoviruses. Electron microscopy of erythrocytes isolated during peak infection demonstrated inclusion bodies containing viruses morphologically consistent with reoviruses. Based on these findings we conclude that erythrocytes are main target cells for PRV and virus replication in these cells starts long before pathological changes are observed.

# NEW RNA VIRUS DETECTED IN COMBER (*SERRANUS CABRILLA*)

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Comber (*Serranus cabrilla*) is a species of fish belonging to the family of *Serranidae*, habiting the Mediterranean, the Western Black Sea, Eastern Atlantic Ocean and possibly the Red Sea. This small fish can be found on the shelf and upper slope on rocks, *Posidonia* beds, sand and mud bottoms feeding on fishes, cephalopods and crustaceans.
Eleven samples of SNC were collected from apparently healthy *S. cabrilla* fished during a wild fish monitoring activity and tested by virus isolation in SSN-1 cell. From one sample, a virus grew in SSN-1 cells with an evident cytopathic effect. This sample was inoculated into other cell lines: BF-2 and CHSE cells showed to be susceptible to viral infection, while no viral growth was observed in EPC and GHSB cells.
From the observation at the electron microscope the viruses appeared as pleomorfic enveloped 55-70 nm particles, with an icosahedral capsid of 40-45 nm diameter. Based on these morphological features, the virus could be ascribed to the *Togaviridae* family, a RNA viruses family. Biochemical characterization with 5-iodo-2'-deoxyuridine confirmed the RNA nature of the viral genome. All the efforts made to further characterize the viral genome failed. In particular, the RT-PCR protocol to detect Salmon Alphaviruses (1), to date the only known Togaviruses infecting fish, yielded negative results. Cloning the products of the random primer PCR for viral RNA is in progress.
The infectious agent isolated from the brain of a *Serranus cabrilla* could be a new RNA virus that has never been described in this species.

1 Hodneland K, Endresen C. (2006) Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan ). J Virol Methods. 131(2):184-92.

P-014

# DELETIONS IN THE HIGHLY POLYMORPHIC REGION (HPR) IN THE STALK OF INFECTIOUS SALMON ANAEMIA VIRUS HPR0 HAEMAGGLUTININ PROTEIN ENHANCE VIRAL FUSION AND INFLUENCE THE INTERACTION WITH THE F PROTEIN

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Infectious Salmon Anaemia Virus (ISAV), belongs to the *Orthomyxoviridae* family and is known to cause severe disease in Atlantic salmon. The virus genome is composed of 8 segments of negatively sense, single-stranded RNA. Segments 5 and 6 are coding for two viral surface proteins: the Fusion (F) and Haemagglutinin Esterase (HE), respectively. Previous work has highlighted the existence of an ISAV variant in the Scottish coastal environment, characterised by a segment 6 carrying a longer Highly Polymorphic Region (HPR) located in the near-membrane ectodomain of the protein stalk. This atypical ISAV segment 6 gene sequence, the longest discovered to date is, often referred to as the "full-length HPR" or HPR0. All currently characterised ISAV isolates responsible for disease outbreaks have HPR shorter than HPR0 which strongly indicates variation in this region is important for virulence. While frequently detected in Atlantic salmon gills by qPCR, the ISAV HPR0 type has not been associated with disease. The main objective of this study was to investigate the potential role the segment 6 near-membrane HPR played on the overall ISAV fusion process. More specifically, this work intended to establish whether deletions in this particular region would enhance the ability of the HE protein to induce membrane fusion. For this purpose, the "full length" HE sequence of a Scottish HPR0 (NWM10) was used as a template from which several mutant HE proteins with various deletions within the HPR were engineered. These HE proteins were then co-expressed in CHSE-214 cells in combination with two different F proteins from known pathogenic ISAV. The fusion activity of expressed viral surface proteins was measured by means of two assays based on Erythrocyte Ghost (EGs) haemagglutination, and quantification using either octadecyl rhodamine B (R18) or Fire Fly luciferase (FF).
These results demonstrated for the first time that deletions within the HPR influenced the fusion event and enhanced the capacity of some HE proteins to induce cytoplasmic transfer of a reporter gene. This work represents one of the first functional studies on ISAV fusion in relation to the HPR0 variant. The in *vitro* fusion model presented may also provide us with a first insight into the mechanism of virulence acquisition from an ISAV HPR0 with fusion enhancement acting as a major contributing factor to ISAV pathogenicity

# WHAT ARE THE IMMUNE MECHANISMS UNDERLYING THE RESISTANCE TO SVC VIRUS INFECTION IN ZEBRAFISH RAG-/- MUTANTS?

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To date the major role of vertebrate immune systems has been thought to be played by B and T cells, which use their ability to develop pathogen specific-receptors (through *somatic recombination*), to form the immunological memory and help animals survive pathogenic infections. Here, we use adult *rag1*$^{-/-}$ zebrafish, defective for a necessary gene (*rag1*) in *somatic recombination*, to gain understanding of this species immunological response to the Spring Viraemia of Carp (SVC) virus, lethal to zebrafish. This study shows that mortalities among *rag1*$^{-/-}$ mutants after a first exposure to SVCV are lower than those among wild type (*wt*) fish, suggesting that an enhanced innate response to the virus ensues in *rag1*$^{-/-}$ fish. Now, we are conducting research to study the differences in the response of *rag1*$^{-/-}$ and *wt* fish to a second challenge with SVCV and whether or not the formation of a "trained memory" occurs in zebrafish.

P-016

# INFECTIOUS SALMON ANAEMIA VIRUS (ISAV) IN CHILEAN ATLANTIC SALMON (*SALMO SALAR*) AQUACULTURE: EMERGENCE OF LOW PATHOGENIC ISAV-HPR0 AND RE-EMERGENCE OF VIRULENT ISAV-HPRΔ: HPR3 AND HPR14

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Infectious salmon anaemia (ISA) is a serious viral disease of marine-farmed Atlantic salmon (*Salmo salar*) caused by ISA virus (ISAV). The virus belongs to the genus *Isavirus*, family *Orthomyxoviridae*. This is arguably the most important viral disease of marine-farmed Atlantic salmon because it is associated to social-economic losses, and ISAV remains an emerging fish pathogen because of the asymptomatic infections in marine wild fish and the potential for emergence of new epidemic strains. The first outbreak of ISA in marine-farmed Atlantic salmon in the Southern Hemisphere occurred in Chile in June 2007 and it then presented a classical epizootic curve with a peak in November 2008 followed by a fast drop to December 2010. The outbreak was caused by ISAV of European genotype with a deletion in the highly polymorphic region (HPR) of the hemagglutinin-esterase glycoprotein (ISAV-HPRΔ), identified as HPR7b, but with an 11-amino acid insert in the fusion glycoprotein. We report here the characteristics of the low pathogenic ISAV without any deletion in HPR, designated ISAV-HPR0, that replaced this epidemic strain, and characteristics of the new ISA cases caused by virulent ISAV-HPRΔ that re-emerged in April 2013 in Chile salmon aquaculture.

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# THE CYPRINID HERPESVIRUS-3 (CYHV-3) USES LIPID RAFTS AS A MODE OF ENTRY INTO CARP CELLS

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The Cyprinus herpesvirus-3 (CyHV-3) is a member of the new *Alloherpesviridae* virus family in the *Herpesvirales* order. CyHV-3 has been implicated in a large number of disease outbreaks in common carp (*Cyprinus carpio L.*) which can cause up to 100% mortality. Some members of the *Herpesvirales* order have been shown to utilise lipid rafts as a mode of entry into cells. These lipid rafts have been identified in fish and are described as cell membrane microdomains enriched in cholesterol, sphingomyelin and certain types of protein. Lipid rafts are essential for signalling, trafficking, nutrient uptake, and they have also been implicated in virus entry and exit from a cell. The aim of this study was to investigate if an aquatic herpesvirus also utilises cholesterol-rich lipid rafts as a mode of entry into CCB cells. Firstly, a method was established facilitating the isolation and lipid analysis of cell membrane lipid raft microdomains, and the purified CyHV-3. Interestingly, the results showed that CyHV-3 and CCB cell lipid rafts contained similar lipid profiles, which suggested that during the budding step of the virus's cycle, the lipid envelope may have been acquired from lipid rafts, and therefore indicating that lipid rafts are required at some stage of the replication cycle. Secondly, the role of cholesterol and lipid rafts in virus entry was ascertained. For this, plasma membrane cholesterol was depleted from carp CCB cells with methyl-β-cyclodextrin (MβCD) in order to remove lipid rafts. The addition of MβCD was able to reduce the cholesterol content by 70% for at least 2 hours post incubation. Treated and non-treated cells were infected with CyHV-3 and the infection parameters were evaluated using RT-qPCR and immunocytochemistry. RT-qPCR results showed a significant decrease in the expression of CyHV-3 associated genes 48 and 120 hours post infection. However, cells depleted of cholesterol and then later replenished showed no change in gene expression levels. Similar data was also obtained using immunocytochemistry at 4 and 7 days post infection. The results show that CyHV-3 requires lipid rafts to enter cells and that lipid raft mediated virus entry is therefore conserved amongst herpes viruses.

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P-018

# MOLECULAR CHARACTERISATION OF FINNISH ISOLATES OF INFECTIOUS PANCREATIC NECROSIS VIRUS

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Infectious pancreatic necrosis (IPN) is a highly contagious viral disease of salmonids that has a serious economic impact on aquaculture worldwide. The disease is caused by the infectious pancreatic necrosis virus (IPNV), a non-enveloped double-stranded RNA virus of the family *Birnaviridae*, genus *Aquabirnavirus*. Aquabirnaviruses are divided into two serogroups, A and B, of which serogroup A contains isolates associated with disease in salmonids. Viruses of serogroup A, on the other hand, are categorised into seven genogroups (Blake et al. 2001, Nishizawa et al. 2005).
In Finland, IPNV infections occur regularly in the coastal fish farms, whereas, until recently, IPN in the inland farms has been very uncommon. In 2012, however, IPNV was detected in six inland farms from three different freshwater systems (Kymijoki, Vuoksi, and Kemijoki). The genetic relationships of the 2012 inland IPNV isolates as well as other IPNV strains isolated in the past 13 years were studied in the Veterinary Virology Research Unit of Evira. Based on partial viral capsid protein (VP2) gene sequences, the 2012 inland isolates belonged to genogroup 2 (Ab serotype), whereas the isolates from the coastal fish farms belonged to genogroups 2 and 5 (Sp serotype). Additionally, there were members of genogroup 6 (He serotype) among the isolates studied.

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Nishizawa T., Kinoshita S., Yoshimizu M. (2005). An approach for genogrouping Japanese isolates of aquabirnaviruses in a new genogroup, VII, based on the VP2/NS junction region. J Gen Virol 86, 1973-1978.

# PHYLOGENETIC STUDIES OF THE GLYCOPROTEIN AND POLYMERASE GENE OF PERCH RHABDOVIRUSES

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Molecularly and serologically related rhabdoviruses, called perch rhabdoviruses, have been isolated from perch (*Perca fluviatilis*), pikeperch (*Stizostedion lucioperca*), grayling (*Thymallus thymallus*), pike (*Esox lucius*) and trout *(Salmo trutta sp*) in Europe. The amino-terminal end of the glycoprotein and polymerase gene of isolates dating from 1980 to 2007 were sequenced and phylogenetic studies conducted. Also included in the phylogenetic studies were sequences published in GenBank.
In phylogenetic studies conducted on the amino-terminal end of the glycoprotein three main genogroups were formed. Isolates mostly from France clustered in one genogroup whereas isolates from Ireland in another group. The third genogroup consisted of isolates from different European countries. The perch rhabdoviruses clustered different from the spring viremia of carp virus, tench virus and grass carp virus.
Sequences information for the glycoprotein gene is available for more isolates compared to sequences data for the polymerase gene. This gives a more robust phylogenetic tree.

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P-020

# SURVIVAL OF VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS (VHSV) AND INFECTIOUS HAEMATOPOIETIC NECROSIS VIRUS (IHNV) IN RIVER WATER AND MUD

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In order to model the spread of disease from a point source and develop contingency plans for an outbreak of an exotic notifiable fish disease, data on the survival of the pathogen in the environment are required. There are a limited number of studies published on survival of VHSV and IHNV in natural waters and sediments compared to survival in defined culture medium and such studies are often either single cases or contradictory. To address this, a trial of the survival of two isolates of IHNV (M genotype, isolates from USA and France) and 1 isolate of VHSV (genotype 1a, UK isolate) in mud and raw or filtered river water and maintenance medium at a range of temperatures from 4 to 25°C was undertaken.
As a general rule in all test media virus survival was longer at lower temperatures. Results were similar for both IHNV isolates. IHNV was rapidly inactivated or adsorbed in mud showing a reduction in titre by 4 logs within 1hr and the only significant survival (7 days) was observed at 4°C. IHNV survived in raw river water for relatively long periods; from 21 to 72 days at temperatures between 4 and 15°C; which has clear significance with respect to disease expression.
This survival was enhanced in filtered river water. VHSV showed similar trends but with longer survival in mud at 4°C (49 days). VHSV also showed prolonged survival at temperatures relevant to development of disease in fish. VHSV persisted, although reduced by a 6 log drop, in raw river water for beyond 84 days at 4°C.

# CYPRINID HERPESVIRUS 3: IMMUNOGENICITY OF DIFFERENT TYPES OF VACCINES BASED ON MOLECULAR METHODS FOR COMMON CARP *(CYPRINUS CARPIO)*

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Cyprinid Herpesvirus 3 (CyHV-3) is a notifiable, highly contagious pathogen that causes massive mortalities of common carp (*Cyprinus carpio*) and the more colourful variety, the koi carp. Intense worldwide trade of these fish made its spread throughout the globe possible, mostly due to lack of veterinary oversight. The virus has been detected in various countries and has affected common carp food production in Europe, Israel, Japan and Indonesia. The virus can be diagnosed with different techniques, but there is no known treatment for CyHV-3. Due to the absence of available chemical treatment for CyHV-3 infection, the development of a suitable protection method for common carp and other susceptible species against this contagious disease is critical for limiting economic losses. Therefore, this study aims to develop and optimize an immunogenic vaccine based on molecular methods to protect common carp from CyHV-3.
A DNA vaccine was prepared by means of a eukaryotic expression vector. It was tested in cell culture to detect mRNA and expressed protein of the CyHV-3 gene. The DNA vaccine will be tested in a shortly upcoming animal trial to assess its protective immunity. This will be done via challenge with native virus 4 weeks post vaccination and continuous sampling for antibody titres.
Similarly, with a bacterial expression vector, we prepared a recombinant protein for use as a subunit vaccine. The originating protein reacted well with an Anit-CyHV-3 antibody in Western Blot analysis, indicating that it could be a good target as a vaccine. Because yields of soluble protein were low, we decided to focus on a truncated form of the protein to increase solubility and therefore practicability for use as a vaccine in a large scale animal trial. After preparation of this truncated CyHV-3 protein, it is going to be tested in an animal trial identical to the DNA vaccine experiment.
The results of our study will be presented and discussed.

P-022

# MOLECULAR EVIDENCE OF ANGUILLID HERPESVIRUS-1 (ANGHV-1) IN THE FARMED JAPANESE EEL, *ANGUILLA JAPONICA* TEMMINCK & SCHLEGEL, IN KOREA

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In November 2008 mortality (approximately 0.5% day) of Japanese eel, *Anguilla japonica*, cultured in a recirculating aquaculture system (water temperature 29~30°C) was observed in Yeonggwang-gun in the Republic of Korea. Affected eels had hemorrhagic lesions in operculum and gill, and some specimens showed severe erosion of the pectoral fins. Histopathological findings exhibited hemorrhage around the filamental arteries, fusion of the adjacent secondary lamellae, hyperplasia of the branchial cells and karyorrhexis and hyperchromatosis of nuclear membrane in interlamellar epithelium. Eroded fins and operculum showed severe necrosis with hemorrhage in connective tissue. In molecular analysis, PCR product of the AngHV-1 polymerase gene (expected size 394 bp) was amplified from gills, opercula and pectoral fins of all diseased fish. Sequence analysis revealed that nucleotide and amino acid sequence in the polymerase gene (Accession number: GU205167) were 99% homologous and 100% with the other registered AgHV-1 DNA polymerase genes , respectively in Gen Bank. Hence, the hemorrhagic and necrotic lesions in the gills, opercula and pectoral fins of the affected eels were probably ascribed to AngHV-1 infection.

# THE USE OF DIFFERENT DISINFECTANTS FOR THE DISINFECTION OF FISH PONDS: EFFECTS ON FISH VIRUSES

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Disinfection is one of the most important measures for the prevention of viral infections, especially following outbreaks of viral diseases in fish ponds. Common disinfectants for the disinfection of natural fish ponds are quick lime (calcium oxide) and slaked lime (calcium hydroxide). The liming causes an adjustment of the pH in the alkaline range. It is generally suggested that a pH of 11 or 12 should be achieved for adequate effectivity. However, the effects of these treatments of natural ponds on important viruses have never been sufficiently tested. In an ongoing study, the effects of different concentrations of quick lime and slaked lime were investigated in the laboratory and during disinfection of affected ponds. For this purpose we used a modified sandwich germ carrier method with koi herpesvirus (KHV) and viral heamorrhagic septicaemia virus (VHSV) and tested the disinfection at three different levels of natural and model ponds (water surface, sediment surface and 3 cm deep in the sediment). Furthermore, we tested the effects of six traditionally used disinfectants (formic acid, formalin, quaternary ammonium compound, iodine complex, pH 12 lime wash and peracetic acid) as pure substances on both KHV and VHSV bound to sandwich germ carriers. Initial results show that reduction of KHV infectivity at 3 cm depth takes almost three times longer at pH 11 than pH 12. Furthermore, there are measurable differences between the effects of quick lime and of slaked lime.

P-024

# *EX VIVO* REASSORTMENT BETWEEN IPNV STRAINS

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The infectious pancreatic necrosis virus is the etiological agent of a highly infectious disease affecting several species of wild and cultured fishes. Its virulence has been reported to be modulated in different ways, including reassortment. Natural reassortment was first reported in wild fish (Romero-Brey et al, 2009. J. Fish Dis. 32:585-95) and, since then, our team has been involved in a screening of other populations (wild and cultured), and found specific types of reassortants among wild fish. Therefore, the aim of the present study was to analyze the frequence of *ex vivo* reassortment, what could provide a clue to explain the results from wild populations.
For this purpose, the 2 European (Ab and Sp) and one American (WB) reference strains were used. Three co-infections were assayed on BF-2 monolayers: Sp+Ab, Ab+Wb and Sp+WB. The viruses were initialy mixed 1:1 and inoculated to a final MOI of 0.1, and a second set of experiment was performed using ratios of 2:1 and 5:1. When cytopathic effect was completed, the viral suspension was collected, the crude virus cloned by serial dilutions, and 50 clones subjected to sequencing of a fragment of both genome segments.
In the first set (coinfection ratio 1:1), no reassortants were yielded at any of the 3 dual-coinfections tested. Interestingly, only the wild type Ab/Ab (segments A/B, respectively) was found among the progeny of the co-infection Sp+Ab, and WB/WB from co-infections Ab+WB and Sp+WB. Regarding the second set of experiments, different frequencies of wild types were obtained depending on the type and proportion of coinfecting viruses. Only one type of reassortant was yielded by coinfecting with Ab and Sp (Ab/Sp; 19%), and with Sp and WB (WB/Sp; 4%), in both cases at a ratio 2:1.
These preliminary results suggest that reassortment might be an infrequent phenomenon in IPNV, coinciding with our pervious observations *in vivo*. Furthermore, some special conditions may be necessary to allow reassortment, and hosts may play an important role in this process. Nevertheless, new experiments are being conducted including new strains to be tested *ex vivo*, and *in vivo* assays will also be performed.

# FUNCTIONAL CHARACTERISATION OF THE NON STRUCTURAL PROTEIN 2 (NSP2) OF THE SALMON ALPHAVIRUS (SAV)

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Pancreas Disease (PD) is one of the most significant viral diseases of salmonids aquaculture in Scotland and worldwide, responsible for considerable economic loss. In Atlantic salmon, it was first described in Scotland in 1976 and is characterised histopathologically by pancreatic and cardiomyocytic necrosis and skeletal myopathy. Outbreaks are associated with variable mortalities ranging from 0.1-63% in the period of 1989 to 1994 in Ireland, however survivors often fail to reach optimal weight and have to be graded out as runts. Salmonid alphavirus (SAV), identified a the viral agent of PD and belonging to the family *Togaviridae*, has a single stranded positive sense RNA genome of approximately 12 Kb. The 3' third comprises the structural proteins, including the capsid, E3, E2, 6K and E1 and is polyadenylated whilst the 5' two thirds code for the non-structural proteins nsP1-nsP4. There are three untranslated regions, one at the 5' end, one between nsP4 and the capsid and another at the 3'end followed by a poly A tail.

In alphaviruses infecting other vertebrates nsP2 has been described as a multifunctional protein with proteinase activity in the C-terminal domain, termination cessation of minus strand synthesis, correct folding of the nonstructural proteins and interferon inhibitor.

Very little is known about the function of the SAV nsP2. Two subtypes 1 SAV isolates, a cell culture adapted F93-125 and a low passage filed isolate 4640 have very similar genome sequence but very different ability to replicate and induce interferon (IFN) in vitro. To elucidate the detailed function of SAV nsP2, two expression plasmids encoding for nsP2 isolated from SAV1 F93-125 or 4640 were constructed and used to characterise the cellular localisation and nsP2 ability to interfere with the IFN response. Stable cell lines clones over-expressing nsP2 F93-125 or nsP2 4640 under the control of doxycyclin (Tet-off system) were isolated by stable transfection of CHSE-TOF and nsP2. The permissivity of these clones to a range of fish viruses was investigated and compared with the parental CHSE-TOF cell line.

P-026

# *IN VITRO* VIRAL COEXISTENCE DECREASES STRIPED JACK NERVOUS NECROSIS VIRUS (SJNNV) BUT FAVOURS RED-SPOTTED GROUPER NERVOUS NECROSIS VIRUS (RGNNV) REPLICATION

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Viral Nervous Necrosis Virus (VNNV) is the aetiological agent of the Viral Nervous Necrosis (VNN), a wide spread disease affecting different marine and freshwater fish species. The Striped Jack Nervous Necrosis Virus (SJNNV) and the Red-spotted Grouper Nervous Necrosis Virus (RGNNV) are the only genotypes of this viral group recorded in the Iberian Peninsula to date, and a high percentage of wild specimens carrying simultaneously both genotypes has been recently reported. The coexistence of two viruses may affect the course of both viral infections. In this study, several assays, including viral genome quantification by two absolute real-time PCR protocols and viral titration, have been performed to characterise the effect of the RGNNV-SJNNV coexistence (coinfection and superinfection) on the replication of each genotype on E-11 cells. The results obtained showed the partial inhibition of the SJNNV replication in presence of RGNNV, whereas the RGNNV replication got favoured in either coinfection or superinfection with SJNNV. In addition, no competence for cellular receptors between these two genotypes has been observed.

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# SUSCEPTIBILITY OF JUVENILE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*) TO DIFFERENT VIRAL NERVOUS NECROSIS VIRUS (VNNV) ISOLATES

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Viral Nervous Necrosis (VNN) is a neurological disease caused by the Viral Nervous Necrosis Virus (VNNV), a member of the *Betanodavirus* genus, *Nodaviridae* family, with a genome composed of two single-stranded positive-sense RNA molecules (RNA1 and RNA2). This virus causes high mortalities in cultured European sea bass (*Dicentrarchus labrax*), especially during larval and juvenile stages. In this study, the susceptibility of 5-g juvenile European sea bass was evaluated by intramuscular injection ($10^5$ $TCID_{50}$/g) using isolates belonging to the RGNNV and SJNNV genotypes as well as a reassortant isolate (RGNNV RNA1/SJNNV RNA2) obtained from Senegalese sole (*Solea senegalensis*). In these experimental infections, the cumulative mortality was determined. Furthermore, quantification of viral genome, by absolute real time PCR, and infective viral particles, by virus titration, was performed from brains of dead and survivor fish (30 days post-inoculation). In addition, anti-VNNV antibodies in sera from survivor animals were determined by indirect ELISA. Typical symptoms of VNN and mortality were only recorded in fish inoculated with the RGNNV (47% cumulative mortality) and the reassortant (33%) isolates. However, high levels of viral genome and infective viral particles were recorded in brain of survivor fish inoculated with the SJNNV isolate, although did not cause mortality or clinical signs. Specific antibody response, measured by indirect ELISA, was only observed in the VNNV-inoculated groups, with titres of 1/1024, 1/4096 and 1/8192 for RGNNV, SJNNV and reassortant inoculated animals, respectively.

*ACKNOWLEDGMENTS:*

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P-028

# UTILISATION OF MONOCLONAL ANTIBODIES AND IMAGE ANALYSIS TO INVESTIGATE DIFFERENTIAL CYPRINID HERPESVIRUS-3 STRUCTURAL PROTEIN EXPRESSION KINETICS *IN VITRO*

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Cyprinid herpesvirus-3 (CyHV-3), also referred to as koi herpesvirus (KHV), causes a fatal disease in common and koi carp. The virus is composed of at least 40 structural proteins of which few have been characterised and little is known regarding their immunogenicity in carp. The current investigation was undertaken to determine the expression kinetics of two of these proteins, a CyHV-3 glycoprotein and a capsid protein, using purified monoclonal antibodies (MAbs) specific for these at standardised concentrations (20 μg $mL^{-1}$). Following a time course of infection of CyHV-3 (isolate H361; MOI 0.01 - 0.02), in koi fin (KF-1) and common carp brain (CCB) cells, differential expression of the glycoprotein and capsid protein was seen using an indirect fluorescence antibody test (IFAT) examined by confocal microscopy. In order to quantify differences in expression a macro was developed, which enabled virus-associated fluorescence and cell nucleus-associated fluorescence to be separated from stacks of scans ($n$=25), captured through fixed cultures of cells, and quantified independently by image analysis. Early expression (≥ 4 hpi) was noted for both viral proteins and, as expected, there was significantly greater expression ($p \leq 0.05$) of both at later stages of infection (3-7 dpi) than during earlier stages (1-24 hpi). However, >1 dpi the abundance of capsid protein increased dramatically (> 8-fold in CCB cells and 10-fold in KF-1 cells by 5 dpi), whereas glycoprotein expression remained relatively low until later stages of infection (≤ 2-fold by 5 dpi). Despite much greater recognition of the capsid protein by MAb 20F10 in infected cells, notably higher absorbance values ($OD_{450\ nm}$) were observed for MAb 10A9 (specific for the glycoprotein) when screened against purified whole CyHV-3 virus by ELISA. A dominant band of ~100 kDa, recognised by MAb 20F10, was also recognised by CyHV-3-infected carp anti-sera in Western blot, indicating that the capsid protein was produced in abundance during infection *in vitro* and was immunogenic in fish. Investigations are now on-going to evaluate this capsid antigen's potential use in serology and application in conjunction with an inactivated CyHV-3 vaccine, for differentiating infected from vaccinated animals (DIVA), similar to DIVA approaches successfully applied for notifiable diseases such as Avian influenza virus (AIV).

# DEVELOPMENT AND VALIDATION OF A SERONEUTRALISATION TEST ALLOWING THE DETECTION OF ANTIBODIES SPECIFIC TO KOI HERPESVIRUS (KHV)

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Koï Herpesvirus (KHV) is the etiological agent of a contagious disease regulated in Europe (Directive 2006/88/EC) affecting common carp and varieties such as koï and ghost carp. This virus appeared almost simultaneously in Europe, the United States, Israel and Japan in the late 90s, with a geographical extension highly favored by the international trade. The most marked clinical signs are large skin ulcers, excess mucus production and hemorrhages in the fins. Symptomatic infections usually occur between 18 and 28°C, with mortality rates reaching 100%. Outside this temperature range, the virus can persist in a latent state in infected hosts which remain asymptomatic, contributing to the virus spreading. The KHV detection in these healthy carriers is difficult using the direct diagnostic methods recommended by the World Organization for Animal Health (OIE, 2012).
The objectives of this work were to develop and validate an indirect and non-lethal seroneutralisation (SN) test allowing the detection of KHV specific antibodies from sera of carps.
After phases of development, optimization and standardization, assessment of the analytical and diagnostic performance of this method was done using sera from healthy or experimentally infected carps. The KHV strain 07/108b used was efficiently neutralized by sera form carps infected with European, American and Taiwanese KHV isolates but no neutralization was observed using sera specific to other herpesviruses (Chanel Catfish, Herpesvirus Anguillae, Cyprinid Herpesvirus type 1). 100% of repeatability was obtained and diagnostic sensitivity and specificity calculated were respectively of 1 and 0.983, with a resulting relative accuracy of 0.988. Applied to KHV experimentally infected koï carps, we were able to detect 100, 95 and 65% of positive individuals respectively at 40 days, 3 months and 21 months post-contamination. Similar analyses were done on naturally infected carps, showing that the proportion of positive individuals increased with the time elapsed since the onset of the clinical signs. This SN test could be used in a close future to improve the epidemiological surveillance and control of KHV disease in Europe.

**Reference**

OIE. Manual of diagnostic tests for aquatic animals. Edition 2012.
Council Directive 2006/88/EC, 24 October 2006.

P-030

# RGNNV TYPE BETANODAVIRUS ISOLATED FROM TURBOT JUVENILE WITH NO SIGNS OF VER

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During routine sampling, as part of a systematic surveillance program, a betanodavirus strain was isolated from farmed juvenile turbot with unspecific symptoms and very low mortality. Both NNV RNAs were sequenced and phylogenetic analysis indicated that the strain showed 99% identity with the RGNNV genotype. Presence of an IPNV-type virus was also revealed, but only after nested-PCR, suggesting a low viral load; in addition, different Vibrio sp were isolated from some of the individuals. Since the presence of NNV virus in wild populations in this area has been demonstrated from a previous surveillance program (http://www.magrama.gob.es/app/jacumar/planes_nacionales/Documentos/100_IF_GESAC_EPIDEMIOLOGIA_Anexo_I_GALICIA.pdf) we decided to analyze the potential risk of this virus for cultured fish.
Two experimental infection tests (via immersion and intraperitoneal route) were conducted in order to determine the susceptibility of turbot juveniles (2 and 5g) to the NNV isolate at 15 and 18 ºC (range of turbot rearing temperatures in our area). The results obtained indicated that although the viral isolate was able to replicate in the turbot tissues, it did not induce clinical disease in this fish species. These findings suggest that the existence of a reservoir of NNV-RGNNV type in wild fish in the area represents a low risk for the turbot farming industry.

# CHARACTERISATION OF A SEROGROUP C AQUABIRNAVIRUS FOUND IN HIGH PREVALENCE IN IMPORTED *GARRA RUFA* DESTINED FOR THE FOOT SPA TRADE

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The period from 2010 to early 2012 saw a rapid rise in the importation of *Garra rufa* into the UK for use in the fast growing ichthyo-therapy industry. During this period disease screening was undertaken on *G. rufa* experiencing mortalities and on subsequent targeted import checks. A number of significant bacterial species were found including known pathogens of fish and potential pathogens of humans (Verner-Jefreys *et al*., 2012). Also, from 5 of the 11 assignments sampled, virus isolates showing identity to serogroup C aquabirnaviruses were isolated. There is limited information on the biology of the serogroup C aquabirnaviruses. The original isolate (BSNV) in this group was identified from blotched snakehead cells in culture and there have been subsequent isolations from sampled fish of the species giant snakehead, *Channa micropeltes* and marble goby, *Oxyeleotris marmorata* from several south-east Asian countries. Given the association with diseased fish and the pathogenic nature of other members of the aquabirnavirus genus (e.g. IPNV) the recent isolates from *G. rufa* were characterised to assess the potential risk posed.
In cell culture the isolates showed a preference for growth in BF and FHM cell lines with no growth in EPC, KF, CCB, CHSE, RTG, SHK or TO cell lines. Full CPE was reached faster at 25°C (average 3 days in BF and 15 days in FHM) compared to 20°C with little or no CPE at 15°C and 10°C. Incubation at 60°C reduced titre by 1 log in the first hour; up to 3 logs after 6hrs and fully inactivated the virus (limit of detection being 17.6 $TCID_{50}$/ml) after 24hr. Sequence analysis of a partial fragment of the RNA dependant RNA polymerase gene (VP1) identified 2 separate isolates showing 80% identity to each other; 82 and 86% identity to BSNV and 66% and 73% identity to IPNV (A2/Sp).
The results of *in vivo* challenge by intraperitoneal injection and cohabitation in carp (*Cyprinus carpio*) and tench (*Tinca tinca*) at 15 and 24°C, currently ongoing, will be discussed with relation to potential risk to native species.

Verner-Jeffreys DW, Baker-Austin C, Pond MJ, Rimmer GSE, Kerr R, Stone D, et al. Zoonotic disease pathogens in fish used for pedicure. 2012. Emerg. Infect. Dis. 18:1006-1008 http://dx.doi.org/10.3201/eid1806.111782

# FISHPATHOGENS.EU/NODA: A FREE AND HANDY ONLINE PLATFORM FOR BETANODAVIRUS TARGETED RESEARCH AND DATA SHARING

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Betanodaviruses are responsible for a severe neuropathological disease known as Viral Nervous Necrosis (VNN) that can affect a broad range of fish species worldwide with high economical and ecological impacts. Betanodavirus genome consists of two single stranded positive sense RNA molecules, which are susceptible to reassortment events. The phylogenetic analysis of both genetic segments allowed the identification of at least four betanodavirus genotypes, namely RGNNV, SJNNV, BFNNV, TPNNV, as well as the two reassortants RGNNV/SJNNV and SJNNV/RGNNV. The wide geographical spread of betanodaviruses and their intense circulation *via* fish trade, highlight the need of a platform for sharing epidemiological and molecular data.
Fishpathogens.eu is a website developed and maintained by the European Union Reference Laboratory for Fish Diseases (EURL-FISH). It has been previously launched for Viral Haemorrhagic Septicaemia (VHS) and Infectious Haematopoietic Necrosis (IHN), and has proven to be a valuable tool for experts targeted research. With the aim of collecting molecular and epidemiological information on betanodavirus, relevant for its control and research study, we extended FishPathogens.eu to VNN. The present database will be a useful tool to better understand betanodavirus diffusive dynamics and ecological features, as well as VNN epidemiology.

# TEMPERATURE SENSITIVITY OF THE RGNNV AND THE SJNNV BETANODAVIRUSES AND THEIR NATURAL REASSORTANTS

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Betanodaviruses are small naked viruses responsible for Viral Nervous Necrosis (VNN), an infectious disease affecting a broad range of fish species worldwide. Their genome consists of two single-stranded +RNA molecules: the RNA1 segment contains two ORFs and encodes the viral polymerase and the B2 protein, while the RNA2 molecule encodes the coat protein. The segmented nature of the betanodavirus genome facilitates the occurrence of reassortment events. Indeed, in Southern Europe the presence of the RGNNV and the SJNNV genotypes and the two deriving reassortants, namely RGNNV/SJNNV and RGNNV/SJNNV, has been reported. Among different phenotypic properties, temperature adaptation appears to be very important for betanodaviruses ecology, and reflects their genogrouping.
To explore the replication capacities of betanodaviruses with different genomes in response to temperature and to understand the role of genetic reassortment on viral phenotype, RGNNV, SJNNV, RGNNV/SJNNV and RGNNV/SJNNV native isolates were fully sequenced and cultivated *in vitro* at four different temperatures (15°, 20°, 25°, 30° C) to develop growth curves. Experimental records were validated through extensive statistical analysis, and linear mixed models were used to investigate the influence of the genotype, temperature, exposure time and their possible interactions on viral replication.
Laboratory results, fully substantiated by the statistical analysis of linear mixed models, demonstrated that viral titers increased with temperature, but significant differences existed between genotypes. Interestingly, the high temperature (30°) heightened phenotypical differences between betanodaviruses with diverse genomes, and viruses containing the polymerase gene of the RGNNV genotype showed the best replication fitness. In particular, at 30° C the RGNNV and RGNNV/SJNNV strains presented significantly higher titers than the SJNNV and SJNNV/RGNNV virus ($p<0.01$). Notably, there was no significant difference ($p>0.10$) between growth kinetics of RGNNV-RGNNV/SJNNV and SJNNV-SJNNV/RGNNV pairs at each temperature tested, suggesting that viruses clustering within the same genotype according to the RNA1 segment, possess similar kinetics in response to temperature.
The data gained might have practical implications, as they could help infer the viral phenotype according to the genetic data; as well, they may contribute to a better understanding of the betanodavirus ecology and of the impact of water temperature variations on viral kinetics and disease control.

P-034

# SENSITIVITY OF TOPMOUTH GUDGEON (*PSEUDORASBORA PARVA)* TO THE EXPERIMENTAL INFECTION OF CYHV-3

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Cyprinid herpesvirus 3 (CyHV-3; koi herpesvirus - KHV) is the causative agent of very serious and contagious fish disease which has aflicted world aquaculture since the end of twentieth century. Natural outbreaks of the KHV disease affected only koi carp and stocks of edible common carp so far. The aim of this study was to investigate if other cyprinid species, namely topmouth gudgeon (*Pseudorasbora parva*), could be infected by koi herpesvirus. The challenge test was done by cohabitation of topmouth gudgeons with koi carps threated with intraperitoneal injection of medium harvested from infected CCB cell line. Naïve koi carps were placed to the same tanks as a positive control. During 30 days post infection no gudgeon died while 70% of naïve koi died. At the 21st d.p.i., when 50% of koi carps had been died, six specimens of gudgeon were transferred from infected tanks to the tank with naïve koi carps. Fish were then observed for 27 days. No any koi cohabited with gudgeons from infected tanks neither died nor showed any symptoms of the disease. Presence of the virus in tissues of died as well as surviving fish was examined by nested PCR.

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# ISOLATION IN CELL CULTURE OF *BETA-NODAVIRUS* FROM SEA BREAM (*SPARUS AURATA* L.) LARVI IN GREECE AND CYPRUS

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Isolation in cell culture of *beta-nodavirus* from sea bream (*Sparus aurata* L.) larvi suffering from viral encephalopathy and retinopathy (VER) is described. SSN-1 cell line was inoculated with homogenized whole larvi. Extensive cytopathic effects (CPE) developed approximately 5 days post inoculation and were observed after several passages in the same cell line. Results from the molecular and phylogenetic analysis are presented. This is the first report on isolation of *beta-nodavirus* in cell culture from sea bream in Greece and Cyprus.

P-036

# FIRST ISOLATION OF KHV IN ITALY FROM IMPORTED KOI (*CYPRINUS CARPIO KOI*)

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The present work describes the first Italian KHV outbreak where the infectious agent was isolated and genetically characterized. At the beginning of April 2012, a batch of 109 koi (*Cyprinus carpio koi*) was imported from Israel by a wholesaler facility located in North-Eastern Italy. Fish originated from different farms: 95 medium size koi came from two KHV-free farms, whereas 14 large size koi were provided by a farm in which vaccination was routinely applied. Fish, shipped separately, were maintained together in the same tank. Three weeks after the introduction of fish, an increased mortality was recorded. Typical clinical and pathological signs of koi herpesvirus disease (KHVD) were observed: enophthalmia, skin erosion, gill hemorrhages and necrosis, pale and enlarged kidney. The histological analysis of symptomatic koi allowed the detection of pale intranuclear inclusions with margination of chromatin in gill epithelium and intestinal lamina propria in the early phase of infection, followed by extensive necrotic degeneration. Viral isolation yielded positive results only for gill samples from symptomatic fresh fish immediately processed on cell culture (CCB) and KHV isolation was confirmed by immunofluorescence. Conventional and real time PCR substantiated the presence of Cyprinid Herpesvirus type 3 (CyHV-3) belonging to U/I lineage. Mortality in the koi tank reached 79.8 % at the moment of the confirmatory identification and the few surviving fish, mainly large size koi, were culled by the Official Veterinary Inspector. After the first detection of clinical signs, the Official Inspector carried out an accurate epidemiological investigation, collecting information on animal movements to establish the origin and prevent viral spreading. Epidemiological data and laboratory results suggested that the virus likely originated from Israel. The role of vaccinated carp as possible asymptomatic carrier of wild type CyHV-3 is discussed in this paper.

# CLINICAL CARDIOMYOPATHY SYNDROME (CMS) IN ATLANTIC SALMON, *SALMO SALAR*, IN IRELAND

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Cardiomyopathy syndrome (CMS) was confirmed in a population of farmed Atlantic salmon in Ireland in 2012. Clinical presentation was of lethargic fish with congestion and skin oedema, ascites and visceral organ petechiae. Histopathology was confined to the cardiac ventricle and the liver and the piscine myocarditis virus (PMCV) was detected via RT-PCR in affected fish. Sequencing of the ORF3 gene from positive material indicated that the virus showed significant similarities with previously published Norwegian isolates. Further characterisation of the virus is ongoing and will be presented.

P-038

# *IN VIVO* TRANSCRIPTOMIC STUDY OF 39 VIRAL GENES AND 17 HOST GENES IN PACIFIC OYSTER, *CRASSOSTREA GIGAS* INFECTED BY OSTREID HERPESVIRUS 1: COMPARING TWO OYSTER FAMILIES PRESENTING DIFFERENT LEVELS OF SUSCEPTIBILITY

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Since 1991, high rates of mortality among Pacific oysters spat have been associated with the detection of a herpesvirus called ostreid herpesvirus 1, (OsHV-1) and reported in different countries, including France. However, no study has been performed to understand and follow viral gene expression during the viral infection whereas gene expression in other herpesviruses was widely studied.
An in vivo transcriptomic study was performed during an OsHV-1 infection in *Crassostrea gigas* spat in order to better undrstand interactions between the host and its pathogen. In this context, 39 viral genes and 17 host genes were selected and analysed by real-time PCR using two oyster families presenting different levels of susceptibilty to the virus.
First virus RNAm transcripts were detected at 8 h post injection (hpi) in the most susceptible family whereas in the less susceptible one first transcripts were observed at 12 hpi. After 12 hpi 39 viral genes were detected in the most susceptible family. However, in the less susceptible family, RNA transcripts of all the 39 virus genes were detected only at 26 hpi. In addition, analysis of the relative expression of the host gene encoding the Myeloid differentiation factor 88 showed an up-expression in the most susceptible family at 26h pi (R=120 in the most susceptible family versus R=21 in the less susceptible one).
This study focused on the detecion of RNAm transcripts in *Crassostrea gigas* spat challenged with OsHV-1. First results confirm the replication of the virus during infection and therefore information on the viral life cycle of OsHV-1 and the defense mechanisms against the virus can be drawn.

# STUDY OF THE BETANODAVIRUS INFECTION IN SOLE USING REASSORTANT (SJNNV/RGNNV) AND PARENTAL STRAINS

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Viral encephalopathy and retinopathy is one of the most threatening diseases affecting fish all over the world. The aetiological agent of this pathology belongs to the *Nodaviridae* family, genus *Betanodavirus.* These viruses have been traditionally classified into four genotypes, being SJNNV (Striped jack nervous necrosis virus) and RGNNV (Red spotted nervous necrosis virus) the most spread ones. Reassortants between both genotypes have also been isolated in the last years from different species, including Senegalese sole. In order to analyse the role of the genetic reassortment on the susceptibility of sole to nodavirus infection, we carried out an experimental infection with three different strains: a reassortant RGNNV/SJNNV isolate (SpSs-IAusc160.03) and two strains from the parental genotypes SJNNV (SJNag 93) and RGNNV (ERV378/102-5/04). Senegalese sole of 1 and 5 g were infected by immersion and intramuscular injection, respectively. Experiments were terminated after day 31 (immersion) and 41 (i.m. injection), when all fish were dead. Virus from dead specimens was recovered on cell culture, and determination of the viral load in the fish tissues was carried out by the traditional $TCID_{50}$ method, and quantitative real-time RT-PCR. The level of viral replication in different tissues was also monitored in fish challenged by immersion and sacrificed daily from days 1 to 10 p.i.
Although the mortality recorded was very high in the three groups, 100% mortality was achieved earlier in the fish infected with the reassortant strain, this group also showed the highest viral titres. However, the highest number of genome copies per gram of tissue was recorded in the RGNNV-infected fish. Regarding viral genome detection in tissues during the first days of infection, both SJNNV capsid-strains were detected in brain by real RT-PCR from the first day, even though only fish inoculated with the reassortant strain showed a gradual increment of the RNA copy number, up to day 10. The RNA of the RGNNV strain was not detected until day 4 p.i., but once the virus reached the brain, the copy number increased rapidly, yielding the highest virus load.
These results seem to indicate that the reassortant strain is the best suited for infecting sole, even though the RGNNV and SJNNV wild types were also able to replicate and to cause mortality in this fish species.

P-040

# HIGH OCCURRENCE OF BETANODAVIRUS IN MEDITERRANEAN *LABRIDAE* FISH

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Viral encephalopathy and retinopathy (VER) is one of the most devastating diseases for marine aquaculture and represents a threat for wild fish, as well. A severe outbreak of this disease in wild fish has recently been reported in Southern Italy (1). Following this episode, the monitoring activities performed routinely by our laboratory have been implemented in that region. From May 2012 to February 2013 a total of 58 wild healthy fish belonging to the *Labridae* family were collected from the Ionian and Southern Adriatic sea to be tested for betanodavirus presence. Fish were caught during scuba diving excursions (n=27) and with experimental fishnets (n=31). Fish species collected were: East Atlantic peacock wrasse (*Symphodus tinca*) (n= 53), axillary wrasse (*Symphodus mediterraneus*) (n=3), five-spotted wrasse (*Symphodus roissali*) (n=1) and brown wrasse (*Labrus merula*) (n=1). A total of 91 samples, which included 58 brain and 33 eyes were analyzed by real time RT-PCR (rRT-PCR), virus isolation, histology and immunohistochemistry. The rRT-PCR yielded positive results in 25 fish (n=24 *S. tinca*, n=1 *L. merula* and n=1 *S. roissali*): 24/58 samples of CNS and 10/33 eyes were found positive. Two viruses from 2 specimens of brain and one eye of two *S. tinca* were isolated and characterized as RGNNV genotype. Phylogenetic analysis highlighted a close genetic relationship between these isolates and the viruses detected in the same area, in particular those obtained from dead fish collected during the outbreak of 2011. Positive brain and eye samples showed the absence of histological lesions referable to VER and only sporadic immunopositive cells.
Wrasses are generally found near rocks mainly in eel-grass beds in all the Mediterranean Sea. They feed small invertebrates, crustacean, mollusks, and organic debris. They are frequently predated by carnivorous species such as groupers and sea bass. Since no clinical signs were ever observed in analyzed samples, the data obtained suggest that fish belonging to the *Labridae* family could act as a reservoir for betanodavirus. Further studies will be needed to confirm the high observed prevalence of the infection in *Labridae* fish (43%) and role in VER epidemiology.

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# GLYCOPROTEIN AND NON-VIRION PROTEIN GENES OF VIRAL HEMORRHAGIC SEPTICEMIA VIRUS ARE NOT MAJOR DETERMINANTS OF HOST-SPECIFIC VIRULENCE IN TROUT

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Viral hemorrhagic septicemia virus (VHSV) is a rhabdovirus belonging to the *Novirhabdovirus* genus. The European VHSV of genotype Ia is very virulent for trout and salmon species, but it is not virulent for yellow perch. In contrast, the VHSV genotype IVb that invaded the Great Lakes in United States is highly virulent for yellow perch, but not for trout. To determine the host-specific virulence of VHSV in trout, we made an infectious clone of VHSV-Ia and created chimeras using existing infectious VHSV-IVb clone. Six chimeric VHSVs were generated in which the glycoprotein (G), non-virion-protein (NV), or both G and NV genes of VHSV-Ia genotype were replaced with the analogous genes from the VHSV-IVb, and vice versa. Viable viruses were recovered in all cases and used to challenge groups of rainbow trout. The parental recombinants rVHSV-Ia and rVHSV-IVb were virulent and avirulent, respectively, as expected. All chimeric VHSV-Ia viruses with substitutions of the G, NV and G +NV of VHSV-IVb were highly virulent (100%), without loss of function. In reciprocal exchanges, chimeric VHSV-IVb viruses with the G, NV and G +NV substitutions of VHSV-Ia were avirulent in trout, with no gain of function. These results suggest that the G and/or NV genes of VHSV are not, by themselves or in combination, sufficient to determine host-specific virulence in trout.

P-042

# COMPARATIVE GENOMICS OF HERPES VIRUSES, A PARADIGM FOR HOST PARASITE CO-EVOLUTION

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Classically, the Herpesvirales order is divided into 3 famillies, the *Malacoherpesviridae*, which infects molluscs, the *Alloherpesviridae* found in fishes and amphibians and the *Herpesviridae*, which infects mammals, birds and reptiles (the latest being subdivided into 3 subfamillies α-, β-, and γ-*herpesvirinae*). Herpes viruses (HVs) are classically considered as an ancient class of viruses having originated over 400 million year ago. HV evolution roughly reflects host divergence. However, lateral host switches have also been encountered in HV evolutionary history. This is supported by highly variable genome size, GC % and incongruent host parasite phylogenies. One may consider HV as a paradigm of viral evolution, reflecting either (i) a rapid spread of HV as a consequence of an extended host switch followed and supported by adequate host adaptation mechanisms or (ii) an extremely ancient radiation of a distant common ancestor, leading one to consider HV as a model of “ancient viruses”, according to Forterre’s (Forterre, 2009) or Claverie's definitions (Claverie, 2006). In addition, HVs may vary their level of virulence or develop mechanisms to escape from the host immune systems: for instance, HVs have been reported to be responsible for dramatic economical losses in aquaculture where molluscs, including oysters, are currently infected by virulent variants of HVs.
Understanding the mechanisms underlying evolution of viral virulence as well as the acquisition of novel host specificities of HVs is thus today a major goal both from a fundamental and an applied economic perspective. If comparative genomics offers new opportunities to infer phylogenetic relationships between more or less distant species, novel tools are yet needed to reconstruct long evolutionary history. In this context, we will present a novel approach to study the long term evolution of HVs, based on comparative genome organization. This approach enabled us to draw conclusions on the evolutionary history of this ancient viral group and to develop new tools for homogenous HV genome annotation.

# FIRST ISOLATION OF PERCH RHABDOVIRUS IN SWITZERLAND

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Perch (*Perca fluviatilis*) is a fish species of increasing interest for the Swiss fish farming industry. New farms using recirculation systems have been set up in recent years to produce this species. In one of these farms elevated mortalities occurred in imported fish two to three weeks after stocking. This phenomenon was found repeatedly. Fish showed an aberrant swimming behavior (spiraling swimming) but no other macroscopic signs of a disease. Analyses of fish from the last event revealed a mild infection with the protozoan *Ichthyobodo necator* on the gills. However, the infection load could not explain the mortalities.
Bacteriological examination showed no bacterial growth. Additionally, pooled samples from inner organs and CNS were incubated on cell cultures to investigate for viral infections. Virus growth was detected in BF-2 cells but not on EPC cells. In immunofluorescence, antibodies against viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV) and infectious pancreas necrosis virus (IPNV) did not react, however, a positive result was obtained with an antibody directed against perch rhabdovirus. Subsequently, additional samples were taken from the same stock, one from fish showing aberrant swimming and one from fish displaying normal behavior. A further sample from older fish from another tank in the same recirculation system was included. These latter fish did not experience any problems. Pools of inner organs and pools from CNS were inoculated separately. Virus growth was found in BF-2 cells inoculated with CNS-material from fish showing clinical signs only. The origin of the virus remains unclear so far. Imported perch had been tested for viruses including perch rhabdovirus before transport and confirmed to be virus free. However, samples had only been taken from inner organs and not from CNS. Another possibility might be a covert infection in the farm with a virus load in resident fish too low to be detected by cell culture methods.
At present further steps are undertaken to characterize the virus. The respective results will be presented.

P-044

# APPROACHES TO PROBIOTICS FOR AQUACULTURE - PREVENTION OF VIRAL DISEASES USING ANTIVIRAL BACTERIA

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Generally, normal intestinal bacteria play an important role in inhibition of the growth of pathogenic bacteria in the intestine, and stimulating the immune response of the host animals. Mortalities of larvae and juveniles of cultured fish due to viral diseases remain a major problem of aquaculture. Although vaccines are useful to control viral diseases, because of a premature immune system, they are not effective during early stages of larval growth. Therefore alternative strategies to prevent the viral diseases are required for sustainable aquaculture and seed production. In a series of our studies of microbial ecosystem, we reported that many bacteria producing antiviral substance could be isolated from aquatic environments. Fish intestinal bacteria such as *Aeromonas* spp. and *Vibrio* spp. producing anti-viral substances were isolated from intestinal contents of masu salmon, Japanese flounder and barfin flounder. *Aeromonas* strains produced anti-infectious hematopoietic necrosis virus (IHNV) substances and *Vibrio* strains showed anti-IHNV, *Oncorhynchus masou* virus (OMV), hirame rhabdovirus (HIRRV), barfin flounder nervous necrosis virus (BF-NNV) activities. When *A. hydrophila* strains M-26 and M-38 were mixed with food pellets and fed to rainbow trout and masu salmon, both bacteria became dominant in the intestinal microflora and anti-IHNV activity was observed in homogenates of intestinal contents. These rainbow trout and masu salmon showed more resistance to the artificial IHNV challenge test. Barfin flounder fed *Vibrio* spp. strains 2IF6a and BI-9715 with *Altemia* sp. showed anti-IHNV, OMV, HIRRV and BF-NNV activities in the intestinal contents. Larvae fed the *Vibrio* spp. showed a higher survival rate than the fish cultured using the virus free seawater and non-treated seawater. In case of Japanese flounder larvae fed with *V. alginolyticus* strain V-5 or V-23 manipulated rotifer showed anti-OMV activities in the intestinal contents and rearing water in the tank. These results show that, by manipulating diets with anti-viral substance-producing bacteria, resistance of fish larvae to viral disease can be improved thereby helping to ensure regular food production through aquaculture.

# NOVEL CARDIAC NEUROPATHY IN ATLANTIC SALMON (*SALMO SALAR* L.) AFFECTED WITH VIRAL CARDIAC DISEASES

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The heart is considered the powerhouse of the cardiovascular system where neural control is equally important for the power and functionality of the heart.
The cardiac pacemaker is the neural tissue responsible for initiation and control of heart beat. The action–potential starts in the autonomous pacemaker cells at sino–atrial (SA) junction and propagates impulses to other parts of the heart. In teleost (Atlantic salmon), pacemaker tissue has been identified at the junction of *sinus venosus* and atrium and is composed of ganglion cells, specialized or spindle shaped cardiomyocytes and a network of nerve fibers. Heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and pancreas disease (PD) are important viral diseases of marine farmed Atlantic salmon which mainly affect the heart. The cardiac diseases also affect the conduction system where pathological changes have been observed in several mammalian diseases.
The present study examined the histopathological effects of above mentioned viral diseases in the pacemaker tissue of Atlantic salmon. The ganglionitis and neuritis (termed as cardiac neuropathy) were identified in the pacemaker of investigated diseases (CMS, HSMI and PD) in addition to vacuolization in the ganglion cells. Few degenerating ganglion cells were also identified in HSMI– and PD–affected hearts. Melanin deposition was also observed in and around the ganglion cells. The necrosed cardiocytes were also observed in close vicinity of pacemaker tissue in CMS–infected hearts. Additionally immunohistochemistry identified the neurogenesis (as identified by PCNA) in the ganglion and nerve cells of HSMI– and CMS–affected hearts. It is likely that Atlantic salmon heart with cardiac neuropathy exhibit cardiac arrhythmia and may be the cause of mortality. Due to the fact that despite severe degenerative heart lesions, CMS-affected fish remain alive and die mostly at harvesting stage, suggesting similar mechanisms of cardiac arrhythmias in teleost (Atlantic salmon) as identified in other mammals.
The cardiac neuropathy in Atlantic salmon (teleost) is a novel finding but requires further assessment for its functional implications.

P-046

# ISOLATION AND IDENTIFICATION OF A NOVEL ISOLATE OF RANAVIRUS IN CHINESE GIANT SALAMANDER, *ANDRIAS DAVIDIANUS*

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The Chinese giant salamander (*Andrias davidianus*) is one of the national protected and cultured species in China. Recently, a severe epizootic has been occurring in cultured Chinese giant salamander with a wide range of size in China, and caused huge economic losses. The typical clinical signs of diseased animals exhibited skin and subcutaneous hemorrhage, ulceration of the hind limbs, and multiple hemorrhagic spots in the visceral organs. Light microscopy demonstrated tissue necrosis and cytoplasmic inclusions, suggestive of a viral infection in kidney, spleen and liver. A virus was isolated from the diseased animals and was determined pathogenecity after experimental infection, resulted in reproduction of definite pathological signs in health animals. The virus electron microscopy revealed virions with the size 140-180 nm in diameter in the renal tubular epithelial cells in kidney lesions of the natural diseased animals and in the virus-infected EPC cells. The whole major capsid protein (MCP) of the isolated ranavirus was cloned and sequenced (GenBank: JN615141) resulted in a similarity of 98-99% in the selected sequences from ranaviruses in GenBank, which suggested that the isolated virus was a novel ranavirus strain in giant salamander. The phylogenetic tree analysis demonstrated that the isolated virus was genetically the same as CGSV (Chinese giant salamander virus: CGSV, HQ684746) reported by Dong et al. (2011).

# AN EPIZOOTIC CAUSED BY CYPRINID HERPESVIRUS 2 INFECTION IN CULTURED GIBEL CARP, *CARASSIUS AURATUS GIBELIO* IN CHINA

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An epizootic with severe mortality emerged in cultured gibel carp, *Carassius auratus gibelio*, in Jiangsu province of China in 2009. The diseased fish showed severe hemorrhages on the opercula, anterior abdominal and at the fin bases. Severe hemorrhages were also presented in visceral organs including liver, spleen, intestine and kidney. A herpesvirus was isolated from moribund gibel carp and identified as Cyprinid herpesvirus 2 (CyHV-2) by means of experimental infection, histopathological and electron microscopic examination, cell culture, PCR assay and sequence alignment analysis. Experimental infection proved the high pathogenicity of the isolated virus to apparently healthy gibel carp.
The histopathological changes observed were necrotic lesions in the spleen and kidney, and the necrotic cells often contained nuclei with marginated chromatin and pale intranuclear inclusions. Ultrastructural examination of the kidney tissue and the purified virus revealed typical herpesvirus-like particles measuring 170~200 nm in diameter with an envelope and a nucleocapsid measuring 110~120nm in diameter. The virus could cause significant CPE in Koi-Fin at the early passages, but it could not be detected beyond 5 passages. The specific primer pair was designed based on the helicase gene sequence of CyHV-2 and used to amplify viral DNA by polymerase chain reaction (PCR). The PCR amplification product with a size of 357 bp was obtained and then sequenced, which shared 100% nucleotide sequence identity with the published sequence for CyHV-2(EU349287).This study provided solid data for the emerging cyprinid herpesvirus 2 infection in gibel carp in China and set up a fundamental basis for the further studies on the diagnosis, prevention and treatment of the disease.

P-048

# IMMUNE AND METABOLIC RESPONSES TO CHRONIC VIRAL DISEASE IN ATLANTIC SALMON (*SALMO SALAR*)

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The Salmonid AlphaVirus (SAV), the etiological agent of pancreas disease, is recognized as a serious pathogen of farmed Atlantic salmon (*Salmo salar).* This disease results in loss of weight followed by poor growth of surviving fish, as such it is viewed as a chronic disease. SAV and other chronic disease causing viruses affect the heart and skeletal muscle tissues and at present the mechanisms by which pathology occurs is currently unknown. This project is focused on both the temporal expression of a number of key antiviral / immune genes and key molecular markers for protein metabolism at different time points post infection. For this work an experimental infection was performed and skeletal white muscle, heart and liver samples were collected at 0, 4, 8 & 12 weeks post infection. Gene expression by RT-qPCR analysis showed maximum viral load in the muscle tissue 4 weeks post infection, this coincided with maximal expression of immune genes. Relating to positive signals for protein synthesis and deposition several genes in the Insulin Growth Factor (IGF) pathway were also shown to be modulated during this time course. Our results show a highly significant up-regulation in antiviral immune genes at 4 weeks post infection which decreases by weeks 8 and 12. Taken together, these observations increase our understanding of salmon poor growth during chronic viral infection, and will serve as a basis to develop strategies to manage this chronic viral wasting disease.

# THE INVESTIGATION ON HISTOPATHOLOGICAL CHANGES OF FOUR ORNAMENTAL FISH SPECIES DUE TO EXPOSE OF CAUSATIVE VIRUS OF VIRAL NERVOUS NECROSIS (VNN) DISEASE

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The epizootic diseases could be considered as most important threats in hatchery and cultured Ornamental fish farms that may face with mortality occurrence and economic lost. Viral Nervous Necrosis (VNN) as emerging disease was approved in Golden grey mullet of the Caspian Sea in recent years. According to importance of Ornamental fish industry and national development programs, new research project was applied to determine and identify sensitive of most important ornamental fish in front of VNN virus. Study on histopathology changes of some important ornamental fish such as Guppy, Zebra, Oscar and Gold fish to acute virus of VNN was main objective in this research that carried out in Virology Lab of Inland Water Aquaculture Institute in Aug.2010. So after adaptation period challenge affaires were done on them with supernatant of affected mullet fish with Bath challenge and Intravitreous injection in Guppy and Zebra fish and I.P method in Oscar and Gold fish. Then all exposed fish were investigated and mortality cases and clinical signs were recorded daily. Suspected samples were taken from affected fish with abnormal swimming behavior, lethargy, exophthalmia and skin darkening and then delivered to laboratory for histopathology and Indirect Fluorescent Antibody Test (IFAT). In comparison of control group, some histopathological lesions such as edema, inflammation, hyperemia, necrosis and pyknotic in nuclear of cells and clear vacuolation were observed in brain tissue and less than in retina. Also, IFAT findings as a confirmation test approved completely mentioned histopathology results. Therefore it could be concluded that mentioned ornamental fish species could be sensitive to acute virus of VNN. So prevention and control executive protocol would be essential in order to preservation and conservation of ornamental fish industry in the country.

Key words: Ornamental fish, Viral Nervous Necrosis, Histopathology, Fluorescent Antibody Test, Guppy, Zebra, Oscar, Gold fish

P-050

# HISTOPATHOLOGICAL OBSERVATION OF LYMPHOCYSTIS DISEASE IN FRESHWATER ANGELFISH, *PTEROPHYLLUM SCALARE* (LICHTENSTEIN, 1823) IN NORTH OF IRAN

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Lymphocystis disease has a worldwide importance and distribution. So far, it has infected over 125 species of fish belonging to 42 families including wild, cultured and ornamental fish, which is characterized by the white nodules mainly occurring on the fish fins and skin, decreasing the commercial values of the fish. In IR.Iran, lymphocystis disease was reported in a female flower horn fish as first report with clinical signs of white-colure masses on the head and gills in Uremia province in April, 2008. In current study, in annual investigation on some ornamental fish farms in Mazandaran province during 2010-2011, an unknown disorder as lymphocystis like-virus was observed in angelfish, *Pterophyllum scalare*. Some nodules with white till yellowish color were observed on upper and lower lips of affected fish with soft density. No sudden mortality was reported and infected fish have serious problems in feeding and some emaciated fish were reported. Then fixed specimens were taken and then structural features of the nodules tissues were studied using histopathological methods. The results indicated that numerous fibroblasts turned round and hypertrophied in the nodules tissue beneath the epidermis of fish body surface, the cytoplasm became incompact and vacuolated. The hypertrophied cells with the size of 10～18 μm showed no capsule outside the cell membrane, and only a few basophilic substances were found in the cytoplasm of some cells; The hypertrophied cells with the size of 18～20 μm had capsules outside the cell membrane and basophilic inclusion bodies in the cytoplasm; The cells more than 20 μm possessed the typical structural features of lymphocystis cells of fish. In this study, although attempt for virus isolation was unsuccessful other comprehensive diagnosis tools such as bacteriology, parasitology and mycology assays were carried out and no bacteria, protozoa or fungal pathogens were obtained. So confirmation of mention disease needs more investigation in the future.

Key words: Lymphocystis disease, Pterophyllum scalare, histopathology, Iran

# HISTOPATHOLOGY STUDY OF THE CULTURED RAINBOW TROUT'S (*ONCORHYNCHUS MYKISS*) FRY MORTALITY SYNDROME IN SOME COLDWATER HATCHERIES AND REARING FISH FARMS IN I.R. IRAN

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In recent decade, rate of fry and juvenile mortality increased dramatically in some provinces in Iran.During 15 months, from Nov.2009 till Feb.2010, 104 tissue specimens consist of liver, kidney, spleen, pancreas, intestine, and gill from 59 diseased fries as well as 45 affected finger ling and suspected adult fish from 7 provinces were collected for histopathological studies. All samples were collected from moribund fry and adult fish and then were processed in schedule and standard protocol of OIE. Finally, The 5 µm sections were stained using H & E staining method, and studied by light microscope. The clinical signs were skin darkening, exophthalmia, ascites, erratic swimming and whirling, lethargy, gathering nears the outlet of the ponds and presence of the fecal casts in the anal area of the fries. Microscopic findings revealed histopathologic changes as follows; congestion, inflammation of the basal membrane of secondary lamellae, hyperplasia, fusion of secondary lamellae and clubbing in some cases in the gill. Congestion of blood vessels, degeneration of kidney cells, necrosis of hematopoitic tissue and tubules, increasing of melanin pigments and inflammatory cells infiltration were observed in kidney. In liver congestion of blood vessels, increasing of fat in hepatocytes, congestion and dilation of sinusoids with increased monocytes, increasing in melanomacrophage number, vacuolation of hepatocytes and necrosis were seen. Bile duct neoplasia (cholangioma) also was present in some cases. Spleen showed congestion, hemosidrosis, melanomacrophage centers increasing and necrosis in some cases. The changes of pancreas tissue were congestion, degeneration and necrosis of asinar cells and islets of Langerhans. Congestion of submucusal layer, fusion of mucosa layer, necrosis and detaching of the mucoid columnar epithelium were observed in the intestine tissue. Regarding to findings of clinical signs and histopathological assay, it could be concluded that the causative agent of the fry mortality is likely to be a viral agent with the signs similar to IHN disease. However for confirmation of the isolated agents, it should be examined by standard confirmatory tests such as IHC, FAT and ELISA. Also further investigation using PCR or RT-PCR could be considered for definitive diagnosis and confirmation of the causative agents.

Key words: Rainbow trout, Fry mortality Syndrome, Histopathology, Iran

P-052

# CAN VIRAL NERVOUS NECROSIS (VNN) DISEASE BE CONSIDERED AS A NEW INVASION OR NEW ZOONOTIC DISEASE? ASSESSING THE ZOONOTIC POTENTIAL OF AQUATIC ANIMAL DISEASES

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A zoonotic disease could be an infectious disease that can be transmitted from animals to humans. A number of infectious diseases, including viruses, bacteria, and parasites, can be transmitted from animals to people through a variety of infection routes, including animal bites, vectors (i.e., insects), and animal-to-human contact (i.e., inhalation of respiratory droplets or skin-to-skin contact). In general the disease usually exists in animals but can infect humans either directly or via a vector. Within aquatics the perception is that there are few zoonotic diseases considered globally as important for those that are recognized the number of cases per year is small compared to other zoonotic diseases such as campylobacteriosis or salmonellosis. Whilst this might be correct, there is a possibility that this is an underestimate, due to poor awareness and surveillance. However, for those that are diagnosed the consequences can be severe including death. Many diseases found in aquatic animals can be classified as Emerging Diseases, defined by (WHO) as, “An emerging disease is one that has appeared in a population for the first time, or that may have existed previously but is rapidly increasing in incidence or geographic range”. One attribute of emerging diseases is that information on the zoonotic potential is limited, yet where a potential exists it is essential to ensure that information is disseminated to other professionals and the public effectively and quickly. This can be done by a qualitative risk assessment. Questions which need to be answered in carrying out the assessment are:
•What is the aetiology?
• What is the distribution?
• What is the prevalence?
• What is the incidence?
• What is the epidemiology?
• What clinical disease is caused?
• Are diagnostic tests available?
• Is there any zoonotic potential?
• What are the potential sources of human exposure?
• Would zoonotic disease be detected?
Regarding to increscent of spread rate and worldwide appearance of Viral Nervous Necrosis disease in many suspected aquatic hosts it could be considered as emerging disease and new Zoonotic disease. So with using a risk assessment algorithm after Spearman the zoonotic potential of (VNN) disease will be assessed and some of the difficulties highlighted.
Key words: Viral Nervous Necrosis Virus Disease, VNN, Zoonotic disease

# MEASUREMENT LEVELS OF CERTAIN ESSENTIAL AMINO ACIDS IN THE BLOOD PLASMA OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Optimum utilisation of the amino acids of the diet for the production of fish flesh is among the key issues of research in fish nutrition. It is necessary to maintain a proper nitrogen balance in the diet in order to reach the desired weight gains and to prevent the health problems that result from a lack or poor balance of essential amino acids (EAA) or non-essential amino acids (NEAA). With this in mind, seeking to improve our activities related to rainbow trout health diagnostics, we focused the first part of the research programme on three branched amino acids (BCAAs): leucine (Leu), isoleucine (Iso) and valine (Va). The purpose was to identify their physiological range and their variability with growth, and to use correlation analysis for assessing their relationships and the interactions between some unbranched amino acids. Besides their anabolic effect on tissues and their indispensability in a heavy catabolic state, these amino acids also have, among other effects, a significant impact on the metabolism of saccharides and lipids. Owing to their favourable characteristics, supportive of the production of fish flesh, they should be determined with utmost attention, especially in cases of deeper skin lesions of unclear aetiology or where the muscle fibres damaged by the infectious agent need to be repaired. The initial objective of the research was to measure the levels of the above amino acids in the peripheral blood plasma of 132 rainbow trout (f. kamloops) weighing 455 ± 120 g. A direct non-parametric method was used for determining the physiological boundaries. Leu, Iso and Va levels decreased with time and with the growth of the fish, but after 86 days they increased slightly and showed a higher variability. The significant (close to very close [p=0.0000]) correlations were found between Leu and Iso, between Iso and Va and between Leu and Va.

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# SERUM CERULOPLASMIN AND HAPTOGLOBIN IN ATLANTIC SALMON, SALMO SALAR, DURING SALMONID ALPHAVIRUS 3 INFECTION

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The serum proteins, ceruloplasmin (Cp) and haptoglobin (Hp) have been shown in a number of species of vertebrates and invertebrates to be important components of the innate immune system and are described as acute phase proteins (APPs). Furthermore, Cp and Hp are up regulated during a number of infections and diseases of teleostei. The aim of this work was to determine serum concentrations of both of these proteins during pancreas disease of salmon resulting from infection by salmonid alphavirus (SAV) and to examine the influence of diet on their APP responses to infection. Samples were obtained using an established cohabitation PD experimental model using Trojan shedders infected with SAV3. In total 12 tanks were used, with three replicate tanks for each of the four diets examined (A, B, C and D). Blood was taken and serum removed from nine fish from each tank at the following time points post challenge; 0, 2, 3, 4, 5, 6, 8, 10 and 12. Serum Cp concentrations were determined via p-phenylenediamine oxidase (PPD) activity and Hp concentration by colorimetric assay based on the residual peroxidase activity of a haptoglobin-haemoglobin complex at low pH. Concentrations of these proteins were analysed using statistical analysis system (SAS) software by procedure GLM using time as a factor and a contrast test for diet effect significance. From this analysis it was determined that both Cp and Hp concentrations were significantly increased four weeks post introduction of Trojan fish. By week 10, Cp values had fallen back to basal concentrations while Hp remained elevated until the final sampling time point (W12). In addition, it was found that one of 4 diets had a significant effect on Cp and Hp concentrations, with individuals fed that diet showing a significantly earlier up regulation of serum concentration.

# EFFECTS OF FUNCTIONAL DIETS IN CULTURED SEA BASS (*DICENTRARCHUS LABRAX*): CHANGES IN INTESTINAL MICROBIOTA AND PROTECTION AGAINST VNN DISEASE

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The wide and frequent use in aquaculture sector of chemotherapy to control bacterial infections has resulted in the development and spread of antibiotic resistance. Moreover, vaccines for controlling most parasitic and viral diseases are not available or are poorly developed. Under this scenario, novel strategies are needed for a sustainable development of aquatic cultures. The inclusion of immunostimulants in fish diets (functional diets) is one of the main strategies to achieve this goal. The objectives of this study have been to evaluate the effects of functional diets in terms of changes in the gut microbiota of fish and protection against Viral Necrosis Nervous (VNN) caused by betanodavirus. For this purpose, we performed a controlled laboratory trial in which cultured sea bass were fed control diet (controls) and two functional diets containing essential oils (B and C) during a four week-period. Subsequently, we analysed and compared the autochthonous intestinal microbiota of fish applying pyrosequencing of PCR-amplified 16S rDNA gene and challenged fish with betanodavirus using an intramuscular (im) injection challenge model.
Intestinal community consisted of two dominant bacterial genus, *Dysgonomonas* and *Ralstonia*, but effects of diet on this dominance were observed. The genus *Dysgonomonas* (Bacteroidetes) significantly decreased in samples from fish fed functional diets, recovering high proportion levels at the end of the study period. However, *Ralstonia* (Beta-proteobacteria) proportion significantly raised in samples from fish fed diet C, maintaining high levels along the study period. Evaluation of the efficacy of the diets against VNN disease was based on differences in mortality in fish after challenge. Diet C yielded an apparent benefit for sea bass in terms of diseases protection under the assayed conditions since mortality in this group was lower than in controls.
In conclusion, administration of functional diets causes changes in the composition of gut microbiota of fish. Functional diet C could be a good candidate to be administered to sea bass before stressing periods. The developed protocol could be used to study the composition and diversity of bacterial communities in the fish intestine and its impact on infection, immune system and general fitness of cultured fish.

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# EFFECT OF MEDICINAL HERB (*ORIGANUM ONITES* L.) ON THE GROWTH PERFORMANCE AND ANTIOXIDANT STATUS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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The microbial diseases cause economic losses in aquaculture. The use of commercial antibiotics for disease treatment produces undesirable side effects. In some countries regulations on the use of antibiotics are strict and only few antibiotics are licensed for use in aquaculture. In recent years especially after the ban on the use of antibiotics in animal feed in the European Union since January 2006. Many medicinal herbs have evolved potent defense against pathogenic bacteria, there is a growing interest in these herbs as sources for natural antibacterial agents (Chakraborty and Hancz, 2011). For alternative biocontrol measures essential oils have emerged as a potential alternative to antibiotics in animal feed. E.O. is good potential alternative to growth promoters (Hammer, 1999). Essential oils is produced *oregano* plant and bacteriostatic effects found in oregano are due to its high content of phenolic compounds, particularly carvacrol (Oflaz vd., 2004; Abdel-Tawwab vd., 2010).
The aim of this study was to determine the impact of feeding rainbow trout diets with medicinal herb on the growth performance, antioxidant activity and cytological and histological indicators of the liver. *Origanum* oil was added to the ingredients of tested diets to represent control, 0.0, 0.125, 1.5, 2.5, 3 ml/kg diet. Fish (average 15-20 g) were distributed to various treatments at a rate of 70 fish per 400 L aquarium and fed one of the experimental diets for 12 week estriplicate.

# DIET-INDUCED SUMMER GUT SYNDROME IN FARMED ATLANTIC SALMON IN TASMANIA IS CORRELATED WITH ADVERSE ALTERATIONS IN GUT MICROBIOTA

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The aquaculture industry has made substantial progress in reducing the fishmeal content of feeds for carnivorous species, driven by demand for improved sustainability and reduced cost. Soybean protein concentrate (SPC) is an attractive replacement for fishmeal, but intestinal disorders have been reported in Atlantic salmon (*Salmo salar*) fed these diets at high seawater temperatures, with preliminary evidence suggesting SPC induces these disorders by altering the intestinal microbiota. We compared the intestinal microbiota of marine-farmed *S. salar* fed experimental diets with varying levels of SPC in mid- and late-summer. Terminal restriction fragment length polymorphism (T-RFLP) and 16S rRNA clone library analysis revealed the microbiota adherent to the intestinal tract of salmon is complex at the population level, but simple and highly variable at the individual level. Temporal changes were observed with the bacterial diversity increasing in the intestinal tract in late summer. A *Verrucomicrobia* was the most frequently observed ribotype in early summer, whilst an *Aliivibrio* was the most frequently observed ribotype in late summer. Feeding SPC to salmon increased the bacterial diversity of the intestinal tract and resulted in the presence of bacteria not normally associated with marine fish (*Escherichia* and *Propionibacterium*). These diet-induced changes to the intestinal-microbiome could be ameliorated by inclusion of a prebiotic (mannan-oligosaccharide or MOS) to the diet. None of the experimental diets induced inflammation of the intestine as assessed by histopathology and expression of inflammatory cytokines. Our results support the “dysbiosis” hypothesis that SPC adversely affects the intestinal microbiota of Atlantic salmon.

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# DEVELOPMENT AND APPLICATION OF REVERSE TRANSCRIPTION QPCR TO QUANTITATIVELY ANALYSE FISH ASSOCIATED MICROFLORA POPULATIONS.

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Although there are a wide range of methologies available for the analysis of fish associated microflora populations, their sensitivity and limitations can impact greatly on the data gained. The use of qualitative molecular methodologies is highly popular within nutritional studies of fish however these techniques only give data as to changes in microbial diversity and not alterations in the frequency of a particular species or genus. Whilst quantification of bacteria can be achieved when performing culture based analysis, this is reliant upon the growth of isolates which is not always possible. Reverse-transcription real-time PCR (RT-qPCR) offers the ability to quantitatively analyse the presence of bacteria that may not grow in culture conditions. Using common carp (*Cyprinus carpio* L.) as a model species, bacterial groups and genera representative of part of a "normal", i.e. disease free, intestinal microflora population have been selected for analysis by RT-qPCR. Based upon the 16S rDNA gene, assays have been designed for quantitative analysis of *Aeromonas*, *Pseudomonas*, *Lactobacillus*, *Vibrio*, *Enterobacteriaceae*, and total bacteria populations. The assays were screened against multiple bacteria isolates taken from carp and tinfoil barb (*Barbus schwanenfeldii*) to ensure their specificity.
This method is currently under trial to analyse changes in the authochthonous population of the intestine of carp fed with either a diet lacking β-glucan or a diet containing 0.1% MacroGard®, a commercially available β-1,3/1,6-glucan source (approximately 60% β-glucan) over a 7 week period.

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# INFLUENCE OF B-GLUCAN ON THE INTESTINAL MICROFLORA OF TINFOIL BARB

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β-glucan is used in fish nutrition as an immunomodulant. Because the value of feeding β-glucan could not be proofed till now, we examined the effect of β-glucan on the bacterial community in the gut of tinfoil barb (*Barbus schwanenfeldii*). One group of barb received feed supplemented with 1% MacroGard®, a commercially available β-1,3/1,6-glucan, one group received feed supplemented with 0,1% MacroGard® and one group received feed without β-glucan. All fish were fed for 14 days at 1% of their body weight per day. Separated sections from first and second segment of the gut were analysed.
We used classical microbiological techniques as well as Denaturing Gradient Gel Electrophoresis.
No differences in the diversity of bacterial species in the gut could be detected. The main intestinal bacterial species were motile aeromonads and pseudomonads. Lactic acid bacteria, *Bacillus* sp., enterobacteriaceae (mainly *Citrobacter* sp.) and *Vibrio* sp. could be detected to a lesser extent. The proportion of motile aeromonads and pseudomonads increased after feeding with feed supplemented with 1% MacroGard®. In barb fed without β-glucan supplementation, the proportion was 40% and in barb fed with feed containing 0.1% MacroGard the proportion was 31% of the total amount of bacteria in the gut. In barb fed with feed containing 1% MacroGard® the amount of motile aeromonads and pseudomonads increased to 62%. The proportion of lactic acid bacteria and Bacillus sp. did not change due to the feeding. In barb fed without β-glucan and fed with feed containing 0.1% MacroGard® an amount of 14% respectively 8% enterobacteriaceae could be detected. Also the amount of Vibrio sp. increased to 5% after feeding with feed containing 1% MacroGard® compared to an amount of 8% in barb fed without β-glucan and 10% in barb fed with feed containing 0.1% MacroGard®.
Our results suggest that feeding tinfoil barb with food supplemented with 1% β-glucan leads to a reduction of specific facultative pathogenic bacteria like Vibrio sp. and Citrobacter sp. in the intestinal microflora and can therefore be health promoting for tinfoil barb.

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# INTESTINAL MICROCOMMUNITIES IN FARMED RAINBOW TROUT *ONCORHYNCHUS MYKISS*

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The diversity and function of the intestinal microflora has been intensively studied in humans and homeothermic animals, however little attention has been paid to this area in fish. Some studies have been carried out that show the microbial community of the fish intestine to be highly dependent on bacterial colonization during early development, environmental conditions and dietary changes.
Fish are subjected to various forms of stress during the farming cycle including temperature fluctuations, handling stress and dietary manipulations, which result in the occurrence of profound physiological changes in the host.
The gastrointestinal tract has been suggested as a major portal of entry for many infectious diseases and yet the role of the gut microflora in maintaining fish health is poorly understood.
In this study, the intestinal microflora of rainbow trout subjected to varied dietary modifications was assessed using a combination of conventional and molecular microbiological techniques. The data generated has contributed to a genetic microbial library where bacterial species present in the fish intestine were identified and then compared with the microbial gut samples from fish under varied dietary regimes. This data will provide the foundation from which to study not only the microbial communities themselves but also their stability during dietary changes within the fish. Insights into the composition of bacterial species present in fish subjected to dietary modifications will help to both broaden our understanding of the normal intestinal microflora of farmed fish species, and how this community responds to dietary alterations.

# IMPACT OF COMMERCIAL DIETARY SUPPLEMENTS ON THE GROWH OF THE RAINBOW TROUT *ONCORHYNCHUS MYKISS* AND ITS SUSCEPTIBILITY TO INFECTION WITH *AEROMONAS SALMONICIDA*, THE CAUSATIVE AGENT OF FURUNCULOSIS

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In recent years, the fish farming industry has witnessed an increase in the adoption of feed additives as an environmental and consumer friendly alternative to the use of antibiotic drugs to fight against bacterial infections such as furunculosis, caused by the bacterium *Aeromonas salmonicida*. However, under the umbrella term of "feed additives" are comprised a wide array of products and molecules whose mechanisms of actions are often complex and not fully elucidated and scientific literature still only provides limited insight into their effectiveness. To alleviate this problem, the present study was designed to test the potential of two commercial feed additives, a phytogenic enriched formula (Digestarom® P.E.P MGE, BIOMIN, Austria) and a mixture of three components (organic acids, phytochemical and Per4rizer®) with an expected synergistic action (Biotronic® TOP3), to determine their ability to improve the growth performance of the rainbow trout *Oncorhynchus mykiss* and its resistance against *A. salmonicida*. To do so, fish were fed restrictively for 175 days with either commercial feed or one of the supplemented feed and their weight was recorded once monthly. Then, they were challenged with a clinical isolate of *A. salmonicida* through one of three different routes of infection: intra-peritoneal injection with $7x10^3$ CFU of bacteria resuspended in 100µl of 0.9M PBS; two hours balneation in a solution containing $10^5$ CFU per ml bacteria. Infected fish were then returned to their aquarium so that the remaining ten fish could be infected indirectly by horizontal transmission from cohabitation with these infected fish. Mortalities were recorded for 35 days after which the experiment was terminated. This allowed us to concluded that, while the supplemented feed had little effect on the growth of the fish, the mortalities were indeed significantly lower in the tanks fed the supplemented food (37% in the tanks fed the commercial feed, 18% in the tanks fed Digestarom® P.E.P MGE; 20% in the tanks fed Biotronic® TOP3) suggesting that these feed additives indeed had a protective effect against *A. salmonicida*.

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# THE EFFECTS OF DIETARY SOY PROTEIN CONCENTRATE (SPC) LEVELS ON GROWTH, COMPOSITION AND IMMUNE FUNCTION OF ATLANTIC SALMON (*SALMO SALAR* L.) PARR VACCINATED WITH A COMMERCIAL *AEROMONAS SALMONICIDA* VACCINE

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Soy protein concentrate (SPC) as an alternative to fish meal (FM) is considered a premium protein source of great potential because of its competitive price and high nutritional properties. It can substitute high levels of FM in Atlantic salmon post-smolt diets without causing negative effects on growth and intestinal integrity. In the current trial four experimental diets, containing 35, 50, 65 or 80 % of the dietary protein from SPC, were fed to Atlantic salmon (*Salmosalar* L.) parr. Growth and innate immunity were assessed after 97 days of feeding, then the fish were vaccinated with a commercial *Aeromonassalmonicida* vaccine to determine any dietary effects on fish immune response 7 and 34 days post-vaccination. Evaluation of the health status of the fish was performed by measuring basic haematology (haematocrit, total white blood cells and differential leucocyte counts) and several other immune responses (i.e. plasma lysozyme, anti-protease and alternative complement activity, plasma protein, total and *Aeromonassalmonicida* specific immunoglobulin M levels, and oxygen radical production by head kidney macrophages). Carcass and bone proximate composition, phosphorus and mineral analysis were assessed at the end of the trial.

It was shown that the immune response of experimental fish did not appear to be size dependent since the linear decline of fish growth and total mineral content, with increasing dietary SPC inclusion levels, was not followed by a concomitant decrease in their immune response. Decreased ash levels were attributed to the linear decrease of Ca2+, Mg2+ and Mn2+ with increasing SPC inclusion in the diets. Moreover, feeding Atlantic salmon parr on diets containing up to 65 % dietary protein from SPC enhanced salient components of innate immunity such as plasma alternative complement and lysozyme activity while also plasma total IgM levels; with the diet containing 50 % protein from SPC giving the best performance both in terms of growth, carcass and bone composition of the fish and immune response. Diets containing 80 % of dietary protein from SPC were still able to promote some non-specific immune responses, but to a lesser degree than diets containing 50 and 65 % protein from SPC, however plasma total IgM levels in these fish were found at lower levels than in fish fed the control diet. This study reveals the need for increased supplementation of certain minerals in diets with increased protein levels from SPC. Furthermore the immunostimulatory effects of medium to high dietary SPC inclusion on Atlantic salmon parr requires further investigation, and the best way to investigate this would be through performing disease challenges.

# THE INFLUENCE OF SUBSTITUTION OF DIETARY FISH OIL WITH PLANT OILS ON INTESTINAL HEALTH IN ATLANTIC SALMON

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Salmonids are indigenous carnivorous, but fish meal and fish oil are increasingly replaced by ingredients from terrestrial sources in the feeds for farmed salmonids due to expanding production and lack of marine resources. Fish oils are rich in marine omega-3 fatty acids considered to be beneficial for human health in general and for the prevention of intestinal inflammation and colon cancer in particular. On the other hand, omega-6 fatty acids that are present in many vegetable oils may promote intestinal carcinogenesis in rodents and humans, while their effect in Atlantic salmon includes lower transcription levels of certain stress and antioxidant-related genes.
The aim of the present study was to investigate the influence on intestinal health in Atlantic salmon of diets with different vegetable oils with varying omega-3/omega-6-ratio as partial substitutes of fish oil in the feed. A feed trial lasting for 28 weeks included three different feeds where 80% of the fish oil was replaced by plant oil blends including either olive oil, rapeseed oil or soybean oil as the main lipid source. These plant oils have intermediate or low omega-3/omega-6-ratios compared to fish oil having a high omega-3/omega-6-ratio. The protein and carbohydrate fractions were identical in all the feeds, and feed containing fish oil as the only lipid source served as negative control.
Nine fish in each of the four group were sampled, and the results from morphometric analyses including measurements of the height and width of the folds in the mid intestine and distal intestine as well as quantitative PCR of mRNA expression of selected immune-related genes (CD3, MHCII, COX2, TGF-β, TNF-α, IL1-β, IgM, IgT and NOD2) in the pyloric caeca, mid intestine and distal intestine will be presented in the poster.

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# INFLUENCE OF KYNURENIC ACIDE (KYNA) ON THE MACROPHAGE AND LYMPHOCYTE ACTIVITY IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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The role of the kynurenine pathway in the immune function is very important issue in comparative immunology. There is considerable amount of evidence showing the interactions between the kynurenine pathway, immunocompetent cells, cytokines and the nervous system. Activation of the immune system is associated with increased concentrations of physiologically active kynurenine pathway metabolites including L-kynurenine (L-KYN), quinolinic acid (QUIN), and kynurenic acid in both the blood and the central nervous system. Kynurenic acid (KYNA) is an endogenous metabolite of tryptophan. In this experimental study we examined the influence of different doses of KYNA on the spleen macrophage and pronephros lymphocyte activity in rainbow trout. Fish was fed 7, 14 and 28 days with commercial pellets containing 0, 2.5, 25 and 250 mg of KYNA (Sigma) per kg of feed. The pronephros and spleen from 10 fish of each group were separated for isolation of macrophages and lymphocytes. Phagocytic ability and potential killing activity of spleen macrophages were examined by spectrophotometric assay. The lymphocyte activity was examined by proliferative response on the mitogens concanavaline A (ConA) or lipopolisaccharide (LPS) by MTT assay. Results of our study indicate that KYNA modulate the macrophage activity depending on the doses and time of application. The similar pattern was observed in lymphocyte activity, where the modulatory effect of KYNA on the proliferative response of lymphocytes was observed.
The preliminary results showed that KYNA supplementation in the diet of rainbow trout provide a further feedback mechanism in modulating the immune responses in fish.

# INFLUENCE OF DIETARY ADMINISTRATION OF THE β-HYDROXY-β-METHYLBUTYRATE (HMB) ON THE INNATE IMMUNITY AND PROTECTION AGAINST MOTILE *AEROMONAS* SEPTICAEMIA IN FINGERLING OF CARP (*CYPRINUS CARPIO*)

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β-hydroxy-β-methylbutyrate (HMB) is a metabolite of the amino acid leucine. In recent years a number of experimental studies showed that HMB significantly increased the cell-mediated and humoral-mediated immunity in mammalian and fish. Motile *Aeromonas* Septicaemia (MAS) is among the most common bacteria in freshwater habitats throughout the world, causing diverse pathologic conditions that include acute, chronic, and latent infections. MAS often concur with viral infections such as Spring Viraemia of Carp. The aim of the study was to examine the influence of feeding with leucine metabolite HMB on the innate immunity and on resistance against MAS in fingerling of carp (*Cyprinus carpio*) grown in a intensive system of culture. The juvenile carp were reared in circular tanks, 200 L each, with water temperature maintained at about 22°C. The fish of approximately 100g were fed with commercial carp feed using automatic band feeders. The carp were fed with 100 mg HMB per kg body weight per day for 4 weeks. The control group was fed pellets without HMB. The disease challenge test using *Aeromonas hydrophila* were conducted after 4 weeks of feeding. Briefly, 100 fish from each control end experimental group were given a single intraperitoneal injection of 48h growth of *A. hydrophila* (0,2 ml). Mortalities were tabulated and the presence of pathogens was confirmed by isolation from the kidney. The results showed that HMB stimulated the macrophage and lymphocyte activity. Also HMB increased the lysozyme activity and total immunoglobulin (Ig) levels in serum.
The challenge test showed that dietary supplementation of HMB decreases the mortality after experimental infection. Feeding with HMB resulted in a lower cumulative mortality (25%), compared to the control group (75% cumulative mortality).

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# GLUCOSE UPTAKE IN THE TRUNK WHITE MUSCLES FROM SEVERAL FISH- GLUCOSE UTILIZATION OF FISH I

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Most fish have limited ability to utilize carbohydrates when compared to mammals. In fish, skeletal muscle is the major site for glucose uptake, because it represents more than 50% of the body weight. It is reported that the number of insulin receptors in skeletal muscle of fish is significantly lower than in mammals. There are, however, only a few detailed reports on the insulin resistance of the skeletal muscle from various fish species. In this study, the rate of glucose (2-deoxy-D glucose : 2DG) uptake of the trunk white muscle isolated from several kinds of fish was evaluated in the presence or absence of insulin. Fish were used for this experiment after preliminary breeding for about two weeks.
Omnivorous goldfish (*Carassius auratus*), herbivorous grass carp (*Ctenopharyngodon idellus*), carnivorous three-lips (*Opsariichthys uncirostris*), rainbow trout (*Oncorhynchus mykiss*) and shortnose sturgeon (*Acipenser brevirostrum*) were used in this experiment.
The rate of 2DG uptake of the skeletal muscle from mouse (used as a control) was 8μmol/mg tissue in the presence of 2 mIU/mL human insulin. Insulin stimulated the highest values of 2DG uptake in fish muscle were as follows (relative value of mouse): goldfish ; 1/4, grass carp ; 1/1, three-lips ; 1/8, rainbow trout ; 1/40, sturgeon ; 1/2.
These results suggest that in the insulin resistance of skeletal muscle, (1) most fish have considerable higher than that of the mouse, (2) carnivorous fish have significant higher values compared to that of herbivorous fish, (3) the value of the skeletal muscle from sturgeon was of the same order as that described for mouse, although sturgeons are opportunistic omnivores.

# THE TRANSLOCATION MECHANISM OF GLUT4 IN GOLDFISH AND RAINBOW TROUT TRUNK MUSCLE- GLUCOSE UTILIZATION OF FISH II

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Most teleost fish are characterized by a limited efficiency to use carbohydrates. However, the exact mechanism of the poor utilization of carbohydrate by fish is still unclear. The purpose of this study, we pay attention to the insulin resistance in the muscle cells of fish, and investigated transfer mechanism of glucose transporter type 4 (GLUT4) in the muscle cells.
Goldfish and rainbow trout were used in this experiment. Fish were administrated orally pioglitazone (10 mg/kgBW/day; PI3 kinase is activated) and metformin (25~250 mg/kg BW/day; AMP kinase is activated). After three weeks, the rate of 2-deoxy-D-glucose (2DG) uptake in the trunk white muscle and changes in blood glucose and insulin concentrations in the fish administered glucose intraperitoneally (glucose tolerance test : GTT) were investigated. Moreover, it is in fixed quantity by a western blot method about on the activity of Akt, PI3 kinase, AMP kinase in muscle cells and the level of GLUT4 on the cell surface. The following results were observed. 2DG uptake rate of control goldfish and rainbow trout was 1/4 and 1/40 of that of the mouse, respectively. That is, fish have very higher insulin resistance compared to mammals. Pioglitazone and Metformin increased the rate of 2DG uptake and promoted the recovery of blood glucose levels in both fish. Moreover, we also found in fish that (1) these drugs induce insulin-independent translocation of GLUT4 to the cell surface which is maintained in insulin resistance, and (2) muscle Akt and PI3 kinase pathways activated by pioglitazone and AMP kinase pathway activated by metformin, respectively.

# THE EFFECT OF FUNGAL-DERIVED β-GLUCAN ON IMMUNE FUNCTION, IMMUNE GENE EXPRESSION AND DISEASE RESISTANCE OF *PANGASIANODON HYPOPHTHALMUS* TO *EDWARDSIELLA ICTALURI*

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*Pangasianodon hypophthalmus* (striped catfish) is an important fish species cultured in Southeast Asia, especially in Vietnam. Rapid expansion and intensification in the culture of this species has resulted in increased disease problems, especially with *Edwardsiella ictaluri*. Immunostimulants are food additives used by the aquaculture industry to enhance the immune response of fish; β-glucans are now commonly used for this purpose. The aim of this study was to determine the effects of dietary fungal derived β-glucan on immune function, immune gene expression and ultimately, resistance to *E. ictaluri* in *P. hypophthalmus*.

Two trials were performed. In the first trial *P. hypophthalmus* were fed for 28 days with 0.0%, 0.05%, 0.1% or 0.2% fungal-derived or 0.1% commercial yeast-derived β-glucan. The fish were then challenged with *E. ictaluri* by immersion for 1 h (8 x$10^4$ CFU/mL); the percentage cumulative mortalities (PCM) recorded at 14 dpi. Immune function was measured prior to, and 14 days post-infection (dpi). A number of statistically significant differences were found in the blood parameters (haematocrit and white blood cell count) and immune responses (complement, head kidney phagocytic function and respiratory burst, total plasma protein, total IgM) as a result of immunostimulation and/or the *E. ictaluri* infection.

In the second trial fish were fed 0% or 0.1% fungal derived β-glucan or 0.1% commercial yeast derived β-glucan for 14 days before challenging with *E. ictaluri* by immersion (1 x$10^5$ CFU/mL) for 30 min. At 1 dpi, the expression of immune genes in liver, kidney and spleen were analysed by qPCR. Significant differences were found in the level of expression of precerebellin-like protein, transferrin and C-reactive protein in liver, 2a MHC class II in liver and spleen, complement factor B and interleukin-1β in kidney between infected and uninfected fish. Transferrin was the only gene affected by diet, being reduced by β-glucan feeding. Significant differences were also found in the PCM between fish fed the basal control diet (30% ±12%) and the two immunostimulated groups [0.1% fungal-derived β-glucan (17% ±8%) and commercial yeast-derived β-glucan (16% ±5%)] at 14 dpi.

# DESCRIPTION OF *HEPATOSPORA* SP. INFECTING PEA CRAB (*PINNOTHERES PISSUM*) – A COMMENSAL OF MARINE MUSSELS (*MYTILUS* SPP.)

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The pea crab (*Pinnotheres pisum*) is considered a commensal organism living amongst the soft tissues of marine mussels (*Mytilus* spp.) and other bivalve molluscs. Here we describe a microsporidian pathogen within the hepatopancreatic tubules of *P. pisum* found within mussels collected in the United Kingdom. The pathogen occurred within an interfacial membrane inside the cytoplasm of hepatopancreatic epithelial cells and elicited tubular degeneration during advanced infection. Histologically, the disease caused by the microsporidian was indistinguishable from that observed in Chinese mitten crab (*Eriocheir sinensis)* infected by *Hepatospora eriocheir* (Stentiford et al., 2011). However, ultrastructurally, the microsporidian was significantly different in that it was diplokaryotic throughout all observed stages of its life cycle. Partial sequencing of the SSU rRNA gene confirmed the close relationship (99% similarity) between the pea crab parasite and *H. eriocheir*, and to another *Hepatospora* sp. described infecting the European edible crab (*Cancer pagurus*). This description provides a further example of a gut-infecting microsporidian pathogen residing within the family Hepatosporidae and suggests that the family forms a growing clade with members of the family Enterocytozoonidae. The latter also contains parasites infecting the gut of marine crustaceans but also parasites of aquatic vertebrates (fish) and humans. The placement within the genus *Hepatospora* (despite ultrastructural distinctions to the type species *H. eriocheir*) takes in to account the potential for plasticity in closely related microsporidian taxa and supports the possibility for closely related organisms with the *Hepatospora-Enterospora* clade to exist in both a wide range of ecological niches and in a divergent range of host taxa. We predict that the clade may grow significantly given improving tools for the taxonomic placement of novel Microsporidia from aquatic habitats.

P-071

# BIVALVE MOLLUSCS PATHOLOGY: RESULTS OF THE ITALIAN MONITORING PLAN FROM 2007 TO 2012

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Italy is the third European producer of molluscs. Like in all animal production, bacteria, parasites and viral pathogens are a potential cause of economical losses. A monitoring programme to check the presence of pathogens which are included in the OIE list or in Annex IV of the Directive 2006/88/EC and to ensure an early detection of abnormal mortality events, is in force for many years in Italy.
The aim of this paper is to highlight the results of the 2007 to 2012 monitoring plan when a total of 68605 diagnostic tests were performed.
In order to check the presence of *Bonamia spp.* or *Marteilia refringens* in flat oysters, 2259 individuals were tested; *Bonamia spp.* was detected in 42 of them (P 1.8%, 95%CI 1.3-2.5) while *Marteilia refringens* in 33 (P 1.5%, 95%CI 1.0-2.0). *Bonamia exitiosa* was detected in 2007 and 2010 and *Bonamia Ostreae* in 2007 and 2008, but neither of them has ever caused mortality events. *Marteilia* was detected in 2007 and 2008 and only in one case associated to low mortality (less than 5%).
The presence of *Marteilia refringens* in mussels was checked in 42184 farmed individuals and was confirmed in 263 (P 0.6%, 95%CI 0.5-0.7). This parasite was detected in the Adriatic Sea (prevalence less than 1%) and in the Tyrrhenian Sea (prevalence 10-20%) each year during the 2007-2012 monitoring programme, but never caused any death event.
*Perkinsus olseni* was constantly detected in the considered period in farmed manila clams of north eastern regions of the country (24162 individuals, 11663 of them positive, P 48.3%, 95%CI 47.6-48.9) but severe mortality was reported only in the southern part of the Venice Lagoon in 2011 (about 100%).
Ostreid Herpes Virus was detected in 2010, without mortality, in off-shore long-lines along the Adriatic coast, in pacific cupped oyster imported from France.
In 2012, an episode with 50% rate of mortality was detected in a lagoon in Sardegna, in juveniles.
The impact and frequency of infectious diseases in production areas are underestimated: this is due to the lack of epidemiological data and the infrequent determination of abnormal mortality episodes' causes. For this reason it is important to maintain the monitoring activities, despite the low frequency of mortality associated to the presence of pathogens reported in Italian mollusc production.

# FIRST DETECTION OF *MINCHINIA* SP. (HAPLOSPORIDIA) INFECTING COCKLES *CERASTODERMA EDULE* FROM GALICIA (NW SPAIN)

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Haplosporidians are obligate protozoan parasites of invertebrates: Some species cause mass mortalities in molluscs. This presentation reports on a haplosporidian infecting cockles *Cerastoderma edule*. A sample including 140 individuals was collected from a cockle bed located in Cambados (ría de Arousa, NW Spain) within a cockle health surveillance programme. A sample of haemolymph was taken from the adductor muscle of each cockle; then a piece of tissues containing visceral mass, foot, gills and mantle, was excised and processed for histopathological analysis. Additionally, DNA was extracted from paraffin blocks and haemolymph samples to characterise the haplosporidian parasite; PCR assays were performed with primers described by Renault et al. (2000) to amplify the SSU rDNA of haplosporidians.
Plasmodia, uninucleated and binucleated cells of a haplosporidian-like protist were observed in histological sections of 6.4% the cockles; moderate to heavy infection, involving abundant plasmodia, was found in 5% of the cockles.
The parasites were observed free throughout the connective tissue, mainly in the digestive gland, gills and gonad area. Small plasmodia and uninucleated and binucleated cells were seldom observed within host haemocytes. The infection was associated with heavy haemocytic infiltration of the connective tissue and necrosis in some areas, especially in the digestive gland. Molecular analyses showed that the most similar sequence in GenBank was *Minchinia mercenariae* with an identity value of 98%; followed by *Minchinia tapetis* and *Minchinia chitonis* with 91% and 88% of identity value, respectively; lower values corresponded to the other haplosporidian genera. These results support the allocation in genus *Minchinia*. This is the first record of *Minchinia* genus infecting Galician cockles. Further characterisation is needed for species identification, although the absence of spores makes it difficult. Longshaw and Malham (2012) suggested that *M. tapetis* and *M. mercenariae* could be the cause of the large foci of heavy haemocyte infiltration observed in cockles *C. edule* from The Netherlands, United Kingdom and Spain. The infection described in this study was not associated with large haemocytic foci but with heavy haemocytic infiltration of the connective tissue. The severity of the lesions observed with histological analysis suggests that this *Minchinia* parasite has high pathogenicity.

P-073

# A COMPETITIVE REAL-TIME PCR FOR *MARTEILIA REFRINGENS* DETECTION AND DISCRIMINATION OF THE GENOTYPES "O" AND "M"

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Parasites belonging to the genus *Marteilia* are relevant shellfish pathogens with potential negative effects on the health status of several bivalve species. One of the parasite species, *Marteilia refringens*, is included in the list of OIE notifiable pathogens.Two genotypes have been described for *M. refringens*: the genotype "O", typically described in flat oysters and the genotype "M", typically described in mussels. Epidemiology of the pathogen related to genotype, host specificity, virulence and geographical distribution is no clear. On that sense, new tools are required to facilitate epidemiological data recollection. Pathogen detection for *M. refringens* is primarily carried out by histology which is laborious and requires skilled personel. Conventional PCRs for the detection of *Marteilia* species are available but presents different problems such as low sensitivity and in some assays unspecific amplification. In addition to this, a subsequent step of PCR-RFLP or sequencing has to be carried out for genotype characterization of the obtained PCR products. In the present work, a real-time PCR was developed for a rapid *M. refringens* detection with competitive TaqMan probes for discrimination between genotype "O" and genotype "M". This facilitates detection of *M. refringens* from large numbers of samples and the genotype discrimination giving relevant epidemiological information and thus, adding value to the obtained results.

# FIRST REPORT OF AN APICOMPLEXAN SPECIES PARASITIZING THE SEA SCALLOP *PLACOPECTEN MAGELLANICUS* IN CANADIAN WATERS

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Members of the phylum Apicomplexa are intracellular protozoan parasites. A previously unknown apicomplexan parasite, morphologically different from other apicomplexan species previously described from bivalves, was reported from three scallop species in Europe in 2011 (Krismundsson et al.). The parasite was found in various organs of the scallops, the adductor muscle was generally the most heavily infected organ. The aim the present study was to examine whether similar infection is found in the American sea scallop *Placopecten magellanicus* (Bivalvia).
Wild American sea scallops *P. magellanicus* (n= 25, mean height 11.8 cm) were collected at two sites in Bay of Fundy, East Canada in 2012. The adductor muscle was examined for the presence of apicomoplexan parasites in fresh mounts and by conventional histology. For comparison the gonads were also examined.
Apicomplexa zoite stages, i.e. sporozoites and/or merozoites, were found in 44% of the sea scallops. The parasite was detected in 36% of the adductor muscles and in 16% of the gonads examined. No other developmental stages were found.
The zoites were morphologically similar to the zoites reported from three species of scallops in Europe, slightly curved with a distinct and large nucleus. The zoites from *P. magellanicus* measured 17-19.5 x 6.5-8 µm, being similar to the size of zoites reported from European scallops. The intensity of infection was extremely low, in the majority of cases only 1-10 zoites were detected in fresh tissue mounts screened by microscopy. A cluster of zoites was encountered once, in the adductor muscle, counting approximately 13 zoites. There were no signs of disease associated with the infections.
The present work reports the first apicomplexan species found in the sea scallop *P. magellanicus*. The results suggest that the apicomplexan forms found in *P. magellanicus* are closely related to those recently described from European scallops.

**Reference**

Kristmundsson A, Helgason S, Bambir SH, Eydal M, Freeman MA (2011). Previously unknown apicomplexan species infecting Iceland scallop, *Chlamys islandica* (Müller, 1776), queen scallop, *Aequipecten opercularis* L., and king scallop, *Pecten maximus* L. Journal of Invertebrate Pathology 108, 147–155.

P-075

# *TRICHODINA* CILIATES IN THE SEA SCALLOP *PLACOPECTEN MAGELLANICUS*

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Ciliates of the genus *Trichodina* are well known ectocommensals/ectoparasites of fish but *Trichodina* of bivalves have not gained as much attention. The present study investigated the occurrance and prevalence of trichodinid ciliates in the American sea scallop *Placopecten magellanicus* (Bivalvia) and morphological characteristics of the ciliates.
Wild American sea scallops *P. magellanicus* (n=22, mean height 11.6 cm) were collected at two sites in Bay of Fundy, East Canada in 2012. Scrapings from the gills and from labial palps and mouth lips were examined for the presence of *Trichodina* ciliates. *Trichodina* ciliates were found in all of the *P. magellanicus* scallops. They were detected on 90% of the gills and on 90% of the labial palps and/or mouth lips. The intensity of the ciliates was not evaluated specifically, but infections were considered as low, although dozens of individuals were frequently seen by microscopy of the scrapings, both from gills and from labial palps/mouth lips. The *Trichodina* specimens were of medium size and their characteristics included: Body diameter 48-55 µm, adhesive disc diameter 42-48 µm, denticulate ring diameter 22-28 µm, denticle number 22-27, radial pins per denticle 9-10, central circle diameter 10-12 µm. Typically the *Trichodina* specimens had one or two very large contractile vacuoles. The macronucleus was C-shaped with an outer diameter of 41-57 µm and lenght of sector between terminations of macronucleus was 15-17 µm. The adoral ciliary spiral formed a turn of about 390°.
The findings in the present study demonstrate that *Trichodina* ciliates are prevalent in *P. magellanicus* sea scallops in Canadian waters and there is apparantly a single species involved, hitherto undescribed.

# SPECIES, THRESHOLDS AND SITE: THEIR IMPORTANCE TO MUSSEL HEALTH

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In Ireland, both wild and cultured *Mytilus* spp. are widely distributed on all coasts. *Mytilus edulis*, *Mytilus galloprovinicialis* and hybrids of both species are found on the west and south coast of Ireland while *M. edulis* is found on the east coast whereas in Wales, it is believed that only *M. edulis* is present. This study investigated the health status of these mussels. Samples of wild and cultured *Mytilus* spp. were collected from twenty-four sites encompassing all coasts of Ireland and the Welsh Coast over several years. 841 *Mytilus* spp. samples were screened using histology to determine health status and the presence of any potential pathogens/parasites and certain study sites a polymerase chain reaction (PCR) was carried out to differentiate which mytilid species were being screened. A further long term study was performed on *Mytilus edulis*. Two sites in Ireland (Flaxfort Strand and Bannow Bay), 215 km apart were chosen and mussel health was assessed across a range of parameters using histology and PCR: site, temperature, month, sex, and stage of gonadal development. The questions were posed "what is a normal or acceptable level of parasitism in a population?" and "does parasitism always result in a negative impact on the health of the individual". Despite parasites and morphological changes being present, mussels at both sites were robust, reproduced normally and no evidence of mortalities that might be above normal levels was observed. Interestingly despite having significantly greater prevalence and intensity of parasites, mussels from Flaxfort Strand were significantly greater in length and weight that those from Bannow Bay. This may show that parasitism does not equate to automatic ill health. The results reinforce the necessity for threshold levels of parasites to be present in the individual to induce negative impacts. External stressors will in turn impact upon these threshold levels that influence the animals at an individual, population, and community level.

P-077

# RISK FACTORS ASSOCIATED WITH INCREASED MORTALITY OF FARMED PACIFIC OYSTERS IN IRELAND DURING 2011

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The Pacific oyster, *Crassostrea gigas*, plays a significant role in the aquaculture industry in Ireland. Episodes of increased mortality in *C. gigas* have been described in many countries, and in Ireland since 2008. The cause of mortality events in *C. gigas* spat and larvae is multifactorial, with ostreid herpesvirus 1 (OsHV-1, in particular OsHV-1 µvar) considered a necessary, but not sufficient, cause. Other risk factors include an increase or a sudden change in the temperature, husbandry practices such as introduction of non-certified (possibly infected spat), and the movement and mixing of populations and age groups. The objectives of the current study were to describe mortality events that occurred in *C. gigas* in Ireland during the summer of 2011 and to identify any associated environmental, husbandry and oyster endogenous factors. A prospective cohort study was conducted in Ireland during 2010 to 2012. The cumulative batch-level mortality (%) from placement to first grading was used as the outcome of interest, and data were collected on a range of potential risk factors. Environmental data at high and low mortality sites were compared, and a risk factor analysis, using linear regression, was conducted. A total of 80 study batches, located at 24 sites within 17 bays were enrolled into the study. Cumulative batch mortality ranged from 2% to 100%, with a median of 16% and an interquartile range of between 10% and 34%. The final risk factor analysis model contained the variables hatchery (batches imported from French hatcheries had significantly lower mortalities than non-French hatcheries) and OsHV-1 µvar site status (negative sites had significantly lower mortalities than positive sites). There are several differences between the seed stocks from French and non-French hatcheries, including prior OsHV-1 µvar exposure and ploidy. The association with ploidy could be a consequence of confounding, as most of the French seed was triploid and most of the seed imported from non-French hatcheries was diploid. A range of risk factors relating to farm management and the local environment, such as water temperature, were also considered, but were not found significant. Work is underway to track seed from various hatchery sources under similar husbandry and environmental conditions. Further work is needed to elucidate the relative importance of prior OsHV-1 µvar infection and ploidy.

# INVESTIGATING MECHANISMS FOR VIBRIOS AND HERPES VIRUS (OSHV1) TO MAINTAIN THEMSELVES OUTSIDE THE PACIFIC OYSTER *CRASSOSTREA GIGAS*

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Abstract: The Pacific oyster, *Crassostrea gigas*, is one of the most important commercial species of bivalve mollusc cultured worldwide. However in recent years losses have occurred due to infection with pathogens such as the Ostreid *Herpes* virus (OSHV-1) and variants (OsHV-1 var, OSHV-1 µvar), and bacterial pathogens such as *Vibrio splendidus* and *Vibrio aestuarianus.* Together and separately these pathogens have been responsible for high mortalities in the Pacific oyster. It appears however that in a number of areas where mortalities have occurred that they are largely seasonal (mainly during the summer) and that a complex interaction of pathogen/environment and host may be responsible for these losses. As these pathogens can have a rapid impact on oyster stocks this project investigated the ability of these pathogens to maintain themselves outside of the host. A seasonal approach was taken to sampling in 2011, 2012 and 2013 to coincide with the period before, during and after the main mortality events. Screening at three oyster farms was conducted in Ireland. Samples comprised representatives from all available invertebrate species, water samples (plankton community), swab samples (biofilm) and sediment samples in and around the oyster culture area. During 2011 and 2012 oyster mortalities were low possibly due to low water temperatures. 30 different species of invertebrates, from each of the three sites were identified. DNA was extracted from a total of 1,028 samples and the presence of OSHV-1 was observed at a relatively low frequency with 23 positive samples. *Vibrio splendidus* was detected in a wide variety of the invertebrate community but further work will concentrate on determining the pathogenicity or not of these strains. No infection of *Vibrio aestuarianus* was detected.

P-079

# MOLECULAR CHARACTERISATION OF *MARTEILIA* SPECIES ISOLATED FROM MUSSELS (*MYTILUS GALLOPROVINCIALIS*) AND OYSTERS (*OSTREA EDULIS*) IN CROATIA

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European flat oyster (*Ostrea edulis*) and Mediterranean mussel (*Mytilus galloprovincialis*) are two molluscs species cultivated along the Croatian coast. The National surveyance programme regulating control of listed diseases was put in place in 2000. Samples of oysters and mussels are taken regularly in all production area with aim to control their health status with regard to parasites *Bonamia ostreae* and *Marteilia refringens*. *Marteilia* sp. was for the first time detected in *M. galloprovincialis* in the northern part of eastern Adriatic coast with prevalence of 5 %. There were several records of marteiliosis caused by *Marteilia refringens* type M in mussels with low prevalence (2 to 7 %) in all three cultivating areas since than. But although mussels and oysters are usually cultivated in same areas, until now were no marteiliosis in oysters. The sample of oysters submitted to health control last winter was positive on marteiliosis by means of tissue imprints and the positive finding was confirmed by PCR. (ITS-1) region from *Marteilia* spp. isolated from oyster was characterized by RFLP with endonuclease HhIal and sequenced. Phylogenetic affinity of the sequences revealed presence of *Marteilia refringens* type M in *O. edulis*.

# SEQUENCES OF SSU rDNA, ITS AND IGS REGIONS OF *MARTEILIA* SP. (CERCOZOA: PARAMYXIDAE) INFECTING COCKLES *CERASTODERMA EDULE* FROM GALICIA (NW SPAIN), AND A SPECIFIC PCR ASSAY FOR ITS DIAGNOSIS

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The genus *Marteilia* includes protistan parasites of marine molluscs. *Marteilia refringens*, has been blamed for mass mortalities of *Ostrea edulis* in Europe and infects other mollusc species as mussels *Mytilus edulis* and *Mytilus galloprovincialis*, striped Venus clams *Chamelea gallina* and razor clams *Solen marginatus*. *Marteilia sydneyi* is responsible for QX disease of *Saccostrea glomerata* in Australia. A parasite referred as *Marteilia* sp. was described from unburied cockles *Cerastoderma edule* from France (Comps et al. 1975). More recently, a parasite referred as *Marteilia* sp. type C was associated with cockle mortality in Catalonia (NE Spain) (Carrasco et al. 2012). In spring 2012, a *Marteilia*-like parasite caused cockle fishery collapse in ría de Arousa, the ría where cockle production used to be the highest in Galicia (NW Spain).
A molecular characterization involving sequencing of SSU rDNA, ITS and IGS regions of this *Marteilia*-like cockle parasite has been performed to state its taxonomic position. Additionally, a PCR diagnostic assay has been designed to distinguish this parasite from *Marteilia refringens*, which infects mussels *Mytilus galloprovincialis* in the Galician rías. The SSU rRNA gene was sequenced (1901 bp) and analysed by BLAST, showing a 99% of maximum identity with the *Marteilia* sp. type C infecting cockles in Catalonia, 98% with *M. refringens* infecting Galician mussels and lower identity values with other Paramyxidae parasites such as one infecting the amphipod *Echinogammarus marinus* (82%) or *Marteilioides chungmuensis* (79%). The ITS-1 fragment obtained of 657 bp showed a 99-100% of homology with *Marteilia* type C and 84-85% with *M. refringens.* A fragment of 712 bp of the IGS region showed 81% identity with *M. refringens*. Phylogenetic relationships of the cockle parasite from ría de Arousa support that it corresponds to a new species within the genus *Marteilia*.
A primer pair for specific PCR diagnosis was designed based on the ITS1 sequence; PCR assays showed that it gives rise to amplification in the case of the *Marteilia* sp. infecting Galician cockles but does not in the case of *M. refringens*. Lack of amplification for *M. sydneyi* and other Paramyxidae was deduced *in silico*.

P-081

# BIVALIFE, A EU FUNDED PROJET FOCUSING ON MANAGEMENT OF INFECTIOUS DISEASES IN OYSTERS AND MUSSELS IN EUROPE

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Bivalife is a 3 year EU funded project (FP7) including 12 participants representing 7 countries (France, Ireland, Italy, Spain, Netherlands, UK, Israel). The project focuses on 3 mollusc species, the Pacific oyster *Crassostrea gigas* and 2 mussel species *Mytilus edulis* and *M. galloprovincialis*. The targeted pathogens are the virus OsHV-1, *Vibrio* species including *V. splendidus* and *V.aestuarianus*, as well as the parasite *Marteilia refringens* and the bacterium *Nocardia crassostreae*.

The Bivalife project addresses the core objectives through 4 scientific work packages:

(i) detection and identification of relevant pathogens and associated risk factors,
(ii) mechanisms allowing concerned pathogens to survive outside the host,
(iii) relevant pathogens: identification of intrinsic virulence factors and effects on host defence mechanisms,
(iv) pathogen control and eradication with development of methods, field tests and recommendations.

During the 2 first year of the project

(i) techniques for pathogen (OsHV-1, *Vibrio splendidus*, *V. aestuarianus* and *Nocardia crassostreae*) detection have been transferred to the participating laboratories,
(ii) interlaboratory comparison assays have been carried out using the transferred techniques and reference biological materials,
(iii) the targeted pathogens have been sought in oyster and mussel samples collected in 2011 and 2012 at different times and location in Spain, France, Ireland, Italy and Netherlands,
(iv) 45 Pacific oyster families have been produced and their susceptibility to OsHV-1, *Vibrio splendidus*, *V. aestuarianus* and *V. harveyi* tested through experimental trials,
(v) finally, the efficacy of UV treatment on OsHV-1, *Vibrio splendidus*, *V. aestuarianus* and *Nocardia crassostreae* has been tested.

Further information can also be accessed at the project web site at http://www.bivalife.eu/

# DISEASE SURVEY OF BLUE MUSSELS (*MYTILUS EDULIS*) FROM SELECTED DANISH COASTAL WATERS

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Mollusc pathogens such as *Marteilia* spp., *Bonamia* spp. and Oyster Herpesvirus-1 (OsHV-1) have yet not been detected in Denmark despite being prevalent in adjacent countries. Danish flat oysters (*Ostrea edulis*) from Limfjorden are declared free for bonamiosis and marteiliosis, but diseases and pathogens present in Danish molluscs in the areas outside of Limfjorden have not been investigated. This lack of knowledge may have devastating consequences, if pathogens are introduced to naïve populations due to stock movements. Six hundred and twenty-six blue mussels (*Mytilus edulis*) from 12 different sites were microscopically examined for the presence of *Marteilia* spp. and other parasitic and bacterial infections. Digenean trematode infections were the most prevalent finding. Inflammatory lesions, typically of focal character and containing disintegrated parasitic matter, were observed in 61 mussels from nine different sites. Metacercariae were found in 17 mussels from three different sites. Bucephalid sporocysts were observed in nine mussels from two different sites. Copepod infections were relatively rare; present in six mussels from two different sites. The only bacterial infection detected was with *Rickettsia*-like organisms. These were observed in 30 mussels from ten different sites. No *Marteilia* spp. was detected in examined samples. Furthermore, the mussels will be examined for OsHV-1 with qPCR. Preliminary results will be presented, and it will be discussed how such results can aid in elucidating the risk of emergence of OsHV-1 due to settlement of Pacific oyster (*Crassostrea gigas*) in Danish waters.

# IS THE MARBELED CRAYFISH (*PROCAMBARUS FALLAX* FORMA *VIRGINALIS*) A POTENTIAL VECTOR FOR THE CRAYFISH PLAGUE PATHOGEN *APHANOMYCES ASTACI* ?

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The marbled crayfish, *Procambarus (P.) fallax* forma *virginalis*, was recently identified as the parthenogenetically reproducing form of *Procambarus fallax*, a North American species of *Decapoda*. Due to the fact that this crayfish reproduces asexually, even a single individual is able to establish a new population in the wild. Moreover, North American species are considered as vectors for the crayfish plague pathogen *Aphanomyces (A.) astaci*, which causes an infectious disease, which is lethal for European, Asian and Australian crayfish species. These facts make *P. fallax* forma *virginalis* potentially an extremely serious threat to the biodiversity in ecosystems harbouring crayfish plague susceptible *Decapoda* worldwide. Furthermore, wild populations of marbled crayfish in Europe were recorded recently. However, to date it was neither shown that marbled crayfish are susceptible for crayfish plague, nor that they are able to carry the pathogen and transmit it to susceptible crayfish species e.g. noble crayfish (*Astacus astacus*). Hence, in this experiment the susceptibility for crayfish plague and pathogen transmission to noble crayfish were studied under laboratory conditions.
For infection of crayfish suspensions of motile spores from the high virulent strain *A. astaci* PSI were used. During the experiment increased crayfish mortality among *Procambarus fallax* forma *virginalis* was observed directly after ecdysis. Autopsy was performed on dead crayfish and samples were analysed for presence of potential *A. astaci* hyphae using light microscopy. Samples from telson, pleopods, abdominal carapace, and melanised areas were analysed with PCR methods by Oidtmann *et al.* (2006), Vrålstad *et al.* (2009), and Hochwimmer *et al.* (2009).
Cohabitation of infected marbled crayfish with noble crayfish indicated that the marbled crayfish can also act as vector for transmission of crayfish plague. Moreover, we assume that *P. fallax* forma *virginalis* can develop crayfish plague similar to other North American crayfish species (e.g. signal crayfish) when the immune system is suppressed. This is concluded by detection of the pathogen by PCR, observation of clinical signs of crayfish plague, and increased mortality during moulting in infected marbled crayfish.

# OUTBREAK AND PROLIFERATION CHARACTERISTICS OF WHITE SPOT SYNDROME VIRUS (WSSV) IN RIDGETAIL WHITE PRAWN (*EXOPALAEMON CARINICAUDA*)

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The ridgetail white prawn, *Exopalaemon carinicauda*, has become one of the main species in mono- and polyculture systems in China. A serious disease accompanied by mass mortalities had previously occurred in pond-cultured ridgetail white prawn. Based on the results of the study, the white spot syndrome virus (WSSV) was identified as the causative pathogen. The ultrastructural examination showed that WSSV generally caused systemic infection with extensive tissue damage in ridgetail white prawn, similar to the proliferation of WSSV in penaeid shrimp. However, quantitative real-time PCR indicated that the number of virus particles in the gill tissue of ridgetail white prawn was 13.1 times as much as that in the muscle tissue, whereas that for penaeid shrimp is only 6.3 times; moreover, WSSV in ridgetail white prawn was particularly prevalent in the eyestalk tissue, suggesting that the eyestalk and gill tissue, especially the eyestalk, would be the best source for PCR template preparation in the virus surveillance of brooders. The artificial infection test further demonstrated that WSSV is highly pathogenic to the ridgetail white prawn species. To the best of our knowledge, this is the first study suggesting the possibility of WSSV becoming a severe new threat to the aquaculture of ridgetail white prawn.

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# *PERKINSUS CHESAPEAKI* OBSERVED IN A NEW HOST, THE EUROPEAN COMMON EDIBLE COCKLE *C. EDULE*, IN THE SPANISH MEDITERRANEAN COAST

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During 2012 a histopathological survey of the common edible cockle *Cerastoderma edule* populations from the Ebro Delta, in the south of Catalonia (Spain), was carried out in order to evaluated the health status of such populations after recent mortalities episodes. Histological observations showed the presence of a *Perkinsus* sp. parasite in *C. edule* tissues. *Perkinsus* sp. was observed by first time in cockles from the Spanish Mediterranean coast. ITS molecular characterization by PCR-RFLP and sequencing identified the parasite as *Perkinsus chesapeaki*, with a maximum identity of 99-100% with *P.chesapeaki* from France and 97% with *P. chesapeaki* sequences of North American origin in GenBank when BLAST analysis was carried out. Furthermore, phylogenetic studies placed the European cockle parasite in a well defined cluster together with the other European isolates. *P. chesapeaki* is observed in a new host, the cockle *C. edule.* The impact of the pathogen in *C. edule* populations needs to be evaluated.

# CHARACTERIZATION OF TYPE I INTERFERON THROUGH MODULATING MX AGAINST NODAVIRUS IN GROUPER

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The novel two-cysteine containing (2C) type I interferon (I-IFN) gene, termed 2C I-IFN, has recently been cloned and sequenced in the orange-spotted grouper (*Epinephelus coioides*). Grouper 2C I-IFN cDNA is composed of 769 base pairs and is translated into a protein of 172 amino acid residues. The grouper 2C I-IFN encodes a predicted signal peptide of 18 amino acid residues and contains two cysteins at amino acid residues (at positions 23 and 119) conserved among most of the mammalian IFN type I protein. Analysis of the homology between grouper 2C I-IFN and other known I-IFN and type II interferon (II-IFN) family members has revealed significant similarities to sea bass I-IFN. Phylogenetic analysis demonstrated that grouper 2C I-IFN clusters with I-IFN in teleosts, away from the other II-IFN family members. In addition, the gene structure for grouper 2C I-IFN is composed of 5 exons and 4 introns, a composition that is similar to that of the teleost I-IFN gene. mmunohistochemical (IHC) analysis was performed on section of normal healthy grouper that grouper 2C I-IFN expression is predominantly membrane-localized and demonstrated zonal distribution in normal healthy grouper. Here, grouper experimentally infected with nodavirus showed elevated transcript levels of grouper 2C I-IFN mainly at 72 hours. Next, an increase in the transcript level of grouper Mx was seen at 6 and 7 days. We also examined gene expression of the grouper Mx *in vitro* and *in vivo* following stimulation with lipopolysaccharide (LPS), poly I:C, or grouper recombinant 2C I-IFN. The results indicated that grouper recombinant 2C I-IFN was also able to activate grouper Mx, thereby leading to upregulated antiviral activity. The established grouper Mx promoter, two ISRE and one NFkB response element, was highly induced after treatment with grouper recombinant 2C I-IFN, comparing to poor LPS-inducible promoter activity. The present results suggest that the expression of grouper 2C I-IFN may participate in the immunologic barrier function of nodavirus.

P-087

# EVALUATION OF THE ABILITY OF VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS TO EVADE THE PROTECTIVE IMMUNE RESPONSE INDUCED IN RAINBOW TROUT BY DNA VACCINATION

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Viral haemorrhagic septicaemia virus, a negative strand RNA virus belonging to the genus *Novirhabdovirus* within the family *Rhabdoviridae*, is the causative agent of VHS, which is a serious disease in rainbow trout and other economically important fish species. The DNA vaccine encoding the viral glycoprotein, the only surface protein of the VHSV, has been successful as an experimental prophylactic treatment against this disease, because it induces a strong innate (interferon) and adaptive (cellular and humoral) immune response. However, since RNA viruses are known to posses high variability, this work aims to evaluate whether VHSV is able to evade the protective immune response induced by the DNA vaccination. Earlier studies have demonstrated that VHSV can evade the neutralizing effect of monoclonal antibodies by mutations in the glycoprotein gene. One approach (*in vitro* approach) of the present study is therefore to try to isolate VHSV variants which can escape the neutralizing activity of serum from fish immunized with the DNA vaccine. To do so, a highly pathogenic VHSV isolate (DK3592B) will be repeatedly passaged in fish cell cultures in the presence of neutralizing fish serum. Another approach (*in vivo* approach) comprises repeated passaging of VHSV in vaccinated fish at two different challenge times, 1 week and 6 weeks post-vaccination, aiming at isolation of virus variants able to evade the innate and adaptive immune response, respectively. After multiples passages in the *in vitro* approach, the virus has become more resistant to the treatment with neutralized serum from vaccinated fish. On the other hand, after the cycles of infection, in the *in vivo* approach, it has been possible to isolate virus from the survivors in every cycle but the vaccined fish have not shown clinical signs or increased the mortalities. The genetic characterization of the isolates will be done in further studies.

# DNA VACCINATION IN FISH PROMOTES AN EARLY CHEMOKINE-RELATED RECRUITMENT OF B CELLS TO THE MUSCLE

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In fish, intramuscular injection of plasmid DNA encoding viral proteins has proved as the most effective vaccination strategy against many viral pathogens. The efficacy of DNA vaccination in teleost fish is based on a high level of viral antigen expression in muscle cells inducing a strong and long-lasting protection. However, the mechanisms through which this protection is conferred in fish are still not understood. Moreover, similarities to mammalian models can not be established since DNA vaccination in mammals induces much lower responses. In this work, we have focused on the characterization of immune cells that infiltrate the muscle at the site of DNA delivery in vaccinated fish and the chemokines that may be involved in their infiltration. It was observed that B lymphocytes, both $IgM^+$ and $IgT^+$, represent a major infiltrating cell type in fish vaccinated with a viral hemorrhagic septicemia virus (VHSV) DNA vaccine, whereas in control fish injected with an oil adjuvant mainly granulocytes were attracted. While $IgM^+$ cells were the major B cell population at early time points post vaccination, $IgT^+$ cells represented the predominant cell type later on. Among twelve chemokine genes studied in the injected muscle tissues, only CXCL10, CK5B and CK6 were more strongly transcribed in DNA vaccinated fish compared to control fish injected with the corresponding vector backbone. *In vitro* tests performed with recombinant trout CK5B and CK6 revealed that these chemokines have chemotactic capacities which might explain the recruitment of immune cells to the site of DNA injection. Our results suggest that B cells are involved in the initial phase of the immune response to intramuscular DNA vaccination against VHSV. This appears to be a major difference to what we know from mammalian models where T cells play a major role.

P-089

# DIFFERENCES IN THE VIRAL INDUCTION OF THE SENEGALESE SOLE Mx PROTEIN IN RTG-2 AND CHSE-214 CELLS

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Interferons (IFNs) play an important role in the fish innate immune system against viral infections by stimulating the expression of genes encoding antiviral proteins, such as Mx. The aim of the current study is to characterize the induction of the Senegalese sole Mx (*Solea senegalensis*, SsMx) protein expression after infection with different fish viruses. In order to fulfill this objective, RTG-2 and CHSE-214 cells were transiently transfected with the luciferase reporter gene under the control of the SsMx promoter. Viruses considered in the present study have been: (i) Infectious Pancreatic Necrosis Virus (IPNV, A2 serotype), (ii) Viral Hemorrhagic Septicemia Virus (VHSV, Ip8 herring isolate, Baltic Sea) and (iii) Epizootic Hematopoitic Necrosis Virus (EHNV, *Perca fluviatilis* isolate).
The luciferase activity was measured and normalized to the green fluorescent protein (GFP) expression.
Transfected RTG-2 cells infected with VHSV showed significant induction of the luciferase reporter gene, compared to the control non-infected cells, at 24, 48 and 72 h post infection (p.i.). The maximum expression was recorded at 72 h p.i. (2.25 folds compared to the control cells). In these cells, the infection with IPNV and EHNV did not result in the luciferase expression at any time tested. In transfected CHSE-214 cells, EHNV stimulated luciferase expression at 24 h p.i. (2.17 folds compared to the control cells), whereas cells infected with IPNV and VHSV did not show luciferase activity at any time. The lack of induction of the SsMx promoter after VHSV infection in CHSE-214 cells, as well as after EHNV infection in RTG-2 cells may be caused by viral IFN-suppression mechanisms, as has been demonstrated for IPNV in previous studies. Furthermore, the different induction of the SsMx promoter observed in RTG-2 and CHSE-214 cells after infection with the same virus indicates that cellular specific factors are involved in the IFN-signaling response. Therefore, the use of two different cellular systems might be an interesting approach to identify such cellular factors.

Keywords: *Solea senegalensis*, Mx promoter, IFN, IPNV, VHSV, EHNV

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# ADAPTIVE IMMUNE RESPONSE IN YELLOWTAIL *SERIOLA QUINQUERADIATA* INDUCED BY FORMALIN-KILLED *MYCOBACTERIUM* SP. IN OIL-ADJUVANT

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*Mycobacterium* sp. is a Gram-positive, acid-fast intracellular bacterium that causes mycobacteriosis in cultured yellowtail *Seriola quinqueradiata*, which results in serious economic losses in aquaculture production in Japan. Therefore, an effective vaccine is urgently needed. In mammals, cell-mediated immunity plays an essential role in protection from infection by intracellular pathogens such as *Mycobacterium*. In this study, the adaptive immune response to a formalin-killed *Mycobacterium* sp. vaccine with oil adjuvant was evaluated in yellowtail. Fish were intraperitoneally injected with a formalin-killed cells (FKC) vaccine, an oil adjuvant vaccine, and PBS. Specific antibody titer and induction of delayed-type hyper sensitivity (DTH) to protein-purified derivative (PPD) extracted from *Mycobacterium* sp. were examined 30 days post immunization (dpi). In addition, the antibody titer and the expression of T-cell related genes (*CD4*, *CD8*, *T-bet*, and *GATA-3*) in vaccinated fish were analyzed at 10 days after infection with live *Mycobacterium* sp. An antibody titration assay showed that the FKC vaccine induced high levels of *Mycobacterium* sp.-specific antibodies. The adjuvant vaccine strongly induced DTH reaction against PPD in fish, but not the FKC vaccine. The expression levels of T-cell related genes were significantly upregulated in the kidney of oil adjuvant vaccine-injected fish compared with the expression in FKC vaccine- and PBS-injected fish. These results indicated that the FKC vaccine induces humoral immune response and promotes specific antibody production, whereas the oil adjuvant vaccine induces cell-mediated immune response. In addition, suppression of antibody production in oil adjuvant vaccine-injected fish suggested that the oil adjuvant vaccine promotes Th1 polarization in T helper cells. Moreover, the oil adjuvant vaccine initiates a strong secondary response against *Mycobacterium* sp. These results suggested that the oil adjuvant vaccine is effective against mycobacteriosis in yellowtail.

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# EFFECT OF FOUR IMMUNOSTIMULANT SUBSTANCES ON THE ACTIVITY OF ISOLATED COMMON CARP (*CYPRINUS CARPIO*) MACROPHAGES

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Many types of biological and synthetic compounds have been shown to enhance non-specific immune system of cultivated fish. Best-known immunostimulants are components of bacterial cell wall, like lipopolysaccharide (LPS) or glucans, but synthetic compounds, polysaccharides, animal and plant extracts or vitamins can enhance the non-specific immune response of fish. Many different immunostimulants are being tested in fish at the moment. Different immunostimulants were tested *in vitro*, on common carp head kidney macrophages under laboratory conditions (Research Institute for Fisheries, Aquaculture and Irrigation, Szarvas (HAKI)).
Four immunostimulant substances provided by BIOMIN GmbHG were used for the experiment: a phytogenic blend, a yeast extract, an algal extract and bacterial cell wall. These substances were dissolved in L-15 cell culture medium in 5.0, 1.0 and 0.1 µg/ml concentrations, except for the phytogenic blend, which was tested in 60.0, 30.0, 10.0, 5.0, 1.0 and 0.1 µg/ml concentrations. Lipopolysaccharide (LPS) in 50 µg/ml concentration was used as a positive control. Macrophages from dissected common carp head kidneys were isolated by centrifugation, and they were incubated with the experimental substances on 96-well microtiter plates for 72 hours. After the incubation period, respiratory burst activity and nitric oxide production of macrophages were measured.
Out of the four substances, three lower concentrations of the phytogenic blend, two higher concentrations of the yeast and algal extract significantly enhanced the nitric oxide production of carp macrophages. Respiratory burst activity was significantly enhanced by two lower concentrations of the phytogenic blend. The bacterial cell wall did not have a significant effect on either parameter.

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# CYTOKINES EXPRESSION IN GILTHEAD SEA BREAM (*SPARUS AURATA* L.) IN RESPONSE TO DIFFERENT IMMUNOSTIMULANTS FROM *VIBRIO ALGINOLYTICUS*

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Some viruses in replication produce double-stranded RNA (dsRNA) and it seems that fish have the ability to recognize these particles and to respond to them through their nonspecific immune system, activating the interferon (IFN) system. The dsRNA and synthetic dsRNA polyinosinic:polycytidylic acid (poly I:C) are two substances that are described as potent stimulators of IFN (Mx is an antiviral protein induced by IFN). Moreover some studies reported a regulation of some cytokines (TNF-α and IL-1β), IL-1 receptor (IL-1RII) and the enzyme Cox-2. Further, these can be stimulated with bacterial LPS and bacterial DNA. In the present work we have compared the fish response facing intraperitoneal inoculation by using different immunostimulants.
Gilthead sea bream, *Sparus aurata* L. were obtained from a commercial fish farm (ADSA S.A.), Spain. Fish were then acclimatized to laboratory conditions for 2 weeks at the Instituto Canario de Ciencias Marinas (ICCM), Spain. Fish were held in tanks with continuous running seawater and natural photoperiod. Three groups of fish were intraperitoneally injected with poly I:C, DNA or LPS of *Vibrio alginolyticus*. A group of fish was injected only with phosphate buffer saline (PBS). Animals were slaughtered in an anaesthesic bath at 0, 12, 24 and 72 hours, and 6 days post inoculation. We analyzed the expression of Mx, IL-1β, IL-1RII, TNF-α and Cox-2 genes in the liver. β-actin gene was used as housekeeping in real-time PCR. Our results indicate that fish inoculated with poly I:C presented elevated levels in liver of Mx gene at 12 hours post inoculation, IL-1β and IL-1RII genes at 24 hours post inoculation, TNF-α gene at 6 days post inoculation and Cox-2 gene at 72 hours post inoculation. Fish inoculated with DNA and LPS of *Vibrio alginolyticus* presented elevated levels in liver of Mx gene at 72 hours and 6 days post inoculation, respectively, IL-1β gene at 24 hours post inoculation and IL-1RII, TNF-α and Cox-2 genes at 6 days post inoculation.

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# EFFECTS OF ARSENIC ON SKIN MUCOSAL IMMUNITY AND GENE EXPRESSION OF GILTHEAD SEABREAM (*SPARUS AURATA* L.)

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The occurrence of heavy metals in aquatic environments influences the health and survival of fish by several mechanisms, including depression of the immune system. Arsenic (As) is associated with multitude of animal and human health problems; however, its impact on host immune system has not been extensively investigated. Fish assimilate As by ingestion of particulate material suspended in water, food ingestion, ion exchange of dissolved metals across lipophilic membranes (e.g., the skin and the gills) and adsorption by tissue and membrane surfaces. In the present study we have evaluated the effects of waterborne exposure (30 days) to sub-lethal concentrations of arsenic (1 ppm or 5 µM $As_2O_3$) in the teleost fish gilthead seabream (*Sparus aurata*), with special emphasis in the skin immunity. Specimens were sampled after 2, 10 or 30 exposure days. Previous results obtained in our laboratory demonstrated that under these conditions As alters seabream systemic immune system, being especially significant from the tenth day of exposure. Focusing on the immunological response in skin, mucus IgM and bactericidal activity (against *Vibrio anguillarum* and *Photobacterium damselae*) were not affected to a statistically significant extent due to the presence of As. However, the protease activity in mucus was significantly decreased and increased after 2 and 10 days of exposition, respectively, compared to the control fish. Furthermore, we have evaluated the expression of genes considered markers of pollution and cellular stress. Thus, the skin expression of cytochrome P4501A (*cyp1a*) and heat-shock protein-70 (*hsp-70*) was not affected by As exposure while the expression of metallothionein-A (*mta*) increased after 10 days of exposition. This indicates that skin cells are activated to clear the As effects. Further studies are needed to understand the immunotoxicological effects and mechanisms of action of As in the skin mucosal immunity of fish.

# DISTRIBUTION OF LYMPHOID TISSUE IN THE GILLS OF ATLANTIC SALMON (*SALMO SALAR*)

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Even though gills do not represent the anatomical equivalent to mammalian lungs, it represents the functional equivalent being responsible for gas exchange among others. This task necessitates a profound interaction with the surroundings, with waste amounts of water passing through the gills in order to fulfill metabolic requirements. This again makes the gills a strategic place for localization of lymphoid tissue, performing immune surveillance and representing the first line of defense against external environment. The immunological importance of the gill was further confirmed with the discovery of the interbranchial lymphoid tissue, an aggregation of intraepithelial lymphoid cells within gill epithelium at the caudal edge of interbranchial septum at the base of the gill filament. However, our work reveals that the interbranchial lymphoid tissue is not anatomical confined but rather spreads out in a continuous fashion throughout the gill filament, with the most profound aggregates found at the trailing edge of the filament.
In order to avoid potential effect by difference in salinity yet representing different life stages, smoltifying juvenils and mature adults, both sampled from freshwater, were chosen. Using different histological techniques and immunohistochemistry against different lymphoid markers, the lymphoid tissue of the gill were characterized. Besides underpinning finding from immunohistochemistry, gene expression analyses using qPCR further broadened the characterization beyond the limitation due to limited availability of appropriate cell makers. Vast amounts of T cells were discovered residing in cytokeratin positive epithelial cells stretching from the edge of the interbranchial septum along the trailing edge towards the gill tip, while clearly delineated from underlying connective tissue through a thick basement membrane. Other lymphoid markers were present in more scares amounts, indicating that this lymphoid tissue primarily consists of T cells. This was confirmed by qPCR analysis, which was used to further characterize the T cell population.
The lymphoid tissue in the gills of teleost, as exemplified by the Atlantic salmon, represents some unique features unparalleled by higher vertebrates. Our work represents the starting point of the characterization of the lymphoid tissue of the gills, which, ultimately, could offer a strategic and appealing target for mucosal delivered vaccines.

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# CD56- POSITIVE LEUCOCYTES POPULATIONS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) – MOLECULAR AND FUNCTIONAL CHARACTERIZATION

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Neural Cell Adhesion Molecule (NCAM) is a multifunctional member of Ig superfamily. In mammals, three alternatively spliced isoforms exists NCAM-120kDa (GPI anchored), NCAM-140kDa (short cytoplasmic domain) and NCAM-180kDa (long cytoplasmic domain). NCAM in mammals is involved in cell-cell interactions in the brain and in neuroectodermal/neuroendocrine differentiation, survival and outgrowth of neuronal cells. Functionally, the involvement of NCAM in synaptic plasticity, learning and memory is shown.
In the immune system, the 140 kDa transmembrane-anchored isoform of NCAM, called CD56, has been detected on the natural killer (NK) cells and 5% of peripheral blood T lymphocytes. CD56 was used for decades as a marker of the natural killer cells (NK). According to the expression level of CD56, NK cells were divided into CD56bright, responsible for NK cytokine production and CD56dim playing a key role in exocytosis-mediated cytotoxicity.
Only limited information about NCAM expression and function is available in fish. In rainbow trout, (*Oncorhynchus mykiss*), NCAM is detected only on the gene level and information about distribution and expression in leukocyte subsets is missing. In our study, we elucidate the expression of NCAN in tissues from healthy trout showing the highest expression of all NCAM isoforms in brain. While the NCAM-180 is detected exclusively in brain; NCAM-120 and NCAM-140 revealed high expression also in muscle. However, no muscle specific domain was detected. NCAM-140 is expressed in all lymphoid organs with the highest amount in head kidney and intestine. Furthermore, expression pattern of NCAM isoforms in different lymphocyte subpopulations (IgM+ or IgM- B-cells; CD8α+ or CD8α - Tcells, thrombocytes, myeloid cells) from naïve and from xenogenic stimulated fish has been analyzed. The presented study provides first hints about the expression of NCAM isoforms in rainbow trout leukocyte populations and opens field for future functional studies of NK cells in trout.

# ASSOCIATION OF MHC AND PROLIFERATIVE KIDNEY DISEASE (PKD) IN MULTIPLE NATURAL POPULATIONS OF BROWN TROUT

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Salmonid populations worldwide are threatened by various anthropogenic factors such as construction of dams, pollution and overfishing. During recent years, there is a rising concern that by producing suboptimal temperature conditions; global warming and construction of dams will increase fishes' susceptibility to disease and parasitic infections. Currently, one of the most serious parasitic diseases of salmonid fishes is caused by the myxozoan *Tetracapsuloides bryosalmonae* resulting in proliferative kidney disease (PKD). Importantly, *T. bryosalmonae* is expected to expand its distribution and increase in virulence in increasing temperatures.This parasite infects the kidney of juvenile fish (0+) causing strong inflammatory response, anemia and kidney hypertrophy in water temperature above 16° C. An important question is whether the host populations can adapt to increased pathogen load in the face of elevated temperature regimes fast enough to avoid going extinct? If yes, which host genes are involved? This scientific work aims to address these questions in the context of host-parasite co-evolution by investigating the variation at the major histocompatibility complex (MHC) genome in relation to PKD resistance in multiple natural populations of brown trout. Evaluation of the fitness consequences of allelic variation at immune relevant candidate loci is expected to provide rare insights into the strength of selection.

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# MOLECULAR CLONING AND EXPRESSION ANALYSIS OF THE ASC GENE FROM JAPANESE FLOUNDER (*PARALICHTHYS OLIVACEUS*)

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Pathogenic DNA recognition receptors such as DExD family play an important role in the immune innate response among organisms. DDX41, one of the receptors belonging to the DExD family of helicase, had been known to recognize microbial nucleic acids in the cytoplasm and enhances antiviral response in host cells. However, its mechanism and structure in *Paralichthys olivaceus*, Japanese flounder, remain poorly understood. Here, we aim to investigate the role of DDX41 in eliciting immune response genes in Japanese flounder. In this study, the full-length cDNA of DDX41 in Japanese flounder was cloned and sequenced and it was found to have 1845 nucleotides with 155 bp 5'-UTR and 269 bp 3'-UTR which encodes 615 amino acids residues. The DDX41 gene was determined and was found to have 5786 bp which includes 17 exons and 16 introns. At the amino acid sequence level, the full-length Japanese Flounder DDX41 showed highest similarity to the DDX41s of medaka and tilapia with 97% identity. The Q-PCR analysis showed that DDX41 mRNA is distinctly expressed in the brain, eyes, gill, head kidney, trunk kidney, spleen, liver, skin, stomach, intestine, muscle, heart of the fish. To evaluate the antiviral activity and the activation of immune response genes of the flounder DDX41, the plasmid construct containing full-length DDX41 was transfected into the hirame natural embryo (HINAE) cell line. Reporter assay results showed that mRNA expression levels of type I IFN were increased in the HINAE cells treated with DDX41. The results suggest that Japanese flounder DDX41 plays an important role in the recognition of cytoplasmic DNA to induce the antiviral activity by the production of IFN and regulate proinflammatory molecules-stimulated protein.

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# EFFECT OF BLACK MUSTARD, *BRASSICA NIGRA* (SEEDS AND EXTRACT) ON ENHANCEMENT THE IMMUNE RESPONSE OF NILE TILAPIA, *OREOCHROMIS NILOTICUS* AGAINST AQUATIC POLLUTANTS

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Aquatic pollutants are responsible about alterations in the fish immune system and consequently incidence of infectious diseases. Our study was design to examine the efficacy of dietary supplement of black mustard, *Brassica nigra* (seeds and extract) on enhancement the immune response of Nile tilapia, *Oreochromis niloticus* against Benzo-a-pyrene (hydrocarbones). Fish were divided into 3 groups before being fed for 28 days with 30% of black mustard seeds, 1% extraxt and with unsupplemented commercial diet as the control. At the end of experiment, fish were exposed to 1mg/L of Benzo-a-pyrene for 24hr. Humoral immune parameters including lysozyme, antiprotease and total protein were investigated. Results recorded highly significant difference ($p < 0.05$) at antiprotease and total protein in groups fed with 1% extract followed by crude seeds as compared with control. The highest significant lysozyme activity ($p < 0.05$) was recorded in group fed with 1% extract as compared with other groups. Therefore, the results suggest that using these dietary supplements can increase the immune function and reduce the harmful effect of aquatic pollutants on Nile tilapia.

# VIRAL AND BACTERIAL INFECTIONS STIMULATE THE EXPRESSION OF PACAP SPLICING VARIANTS IN BROWN TROUT IMMUNE ORGANS

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Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) is found in two biologically active forms, PACAP and PACAP-Related Peptide (PRP) encoded in the same precursor protein. Exerting their pleiotropic biological activities through G-protein coupled receptors (PAC1, VPAC1 and VPAC2), their transcription has been detected not only in the brain but also in a wide range of peripheral tissues, including organs of the immune system. Recent findings have added PACAP and its receptors to the growing list of mediators that allow cross-talk between the nervous, endocrine and immune systems in fish. However, experimental evidence is still required to fully characterize their role during an effective immune response against pathogens. The expression of genes encoding for PACAP and PRP, as well as for their receptors, was studied in laboratory-reared brown trout (*Salmo trutta*) after infection with Viral Haemorrhagic Septicemia, an OIE notifiable listed disease caused by a *Novirhabdovirus*, and Enteric Red Mouth disease caused by the Gram negative bacterium *Yersinia ruckeri*. Kidney and spleen, major lymphopoietic organs, were sampled at different timings post-infection and RT-qPCR analysis used to determined gene expression levels. PACAP and PRP expression in each organ was positively correlated with the respective pathogen burden, assessed by PCR detection of the VHSV-glycoprotein and *Y. ruckeri* 16S rRNA. Although PACAP had higher levels of expression, both splicing variants were found to be consistently and strongly induced, peaking during the early stages of the bacterial infection, with a predominant induction in the spleen. During the viral infection, PRP was found to be less highly induced and was induced later, while PACAP was already up-regulated at day 1 post-infection in both kidney and spleen, reaching a maximal induction 3 days post-infection in the spleen. However, only irregular expression patterns were observed for the receptors. These gene expression results from brown trout provide more clues as to how the PACAP system is modulated and confirm an involvement during active immune responses elicited by both viral and bacterial aetiological agents.

**These authors have contributed equally to this work.*

# THE EFFECT OF β-GLUCAN ON NEUTROPHIL EXTRACELLULAR TRAPS IN COMMON CARP

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A novel innate immune defence mech[illegible]nism a[illegible]ai[illegible]t inv[illegible]ding pathogens, namely the formation of neutrophil extracellu[illegible] traps (NETs), has recently been described in fish. These NETs are defined as DNA fibre[illegible]entwined with antimicrobial peptides, which ar[illegible]able [illegible] entra[illegible] and kill bacteria. Here we characterised the function of carp [illegible]onephro[illegible] and kidney derived NETs against the opportunistic fish pathogen *Ae[illegible]monas h[illegible]drophila* in response to the addition of the feed additive β-glucan (MacroC[illegible]rd®). Firstly a time kinetic of NET formation in response to β-glucan-t[illegible]atment was performed. Therefore, common carp (*Cyprinus carpio*) pronephros and kidney derived cells-each consisting of approximately 45% neutrophils- were isolated, stimulated with 0, 2, 20 or 200 µg/ml β-glucan over 15, 30, 60, 120 and 240 min and subsequently NET-formation was analysed by immunofluorescence microscopy. The results show that NET production occurred very rapid with NETs observed after just 15 min of β-glucan treatment. Furthermore, a significantly higher percentage of kidney-derived cells produced NETs relative to pronephros-derived neutrophils. Secondly the effect of β-glucan on bacterial entrapment and killing by NETs was investigated. The results show that carp NETs are able to entrap *A. hydrophila*, and the addition of β-glucan increased the percentage of entrapped bacteria. However no bacterial killing was observed. Thirdly, host-evasion strategies utilised by *A. hydrophila* to degrade NETs were investigated. For this, carp derived pronephros cells were seeded with or without the addition of β-glucan and *A. hydrophila* was added at a multiplicity of infection of 1 bacterium per cell. The results showed that *A. hydrophila* was able to degrade NETs, which was attributed to the secretion of DNA degrading nucleases by *A. hydrophila*. Interestingly, the addition of β-glucan significantly protected the NETs against bacteria-mediated degradation. The findings of this work shows that carp derived neutrophils are able to produce NETs, which are able to entrap, but not kill *A. hydrophila*. Interestingly the addition of β-glucan significantly stimulated NET production over time and protected the NETs against host evasion strategies utilised by *A. hydrophila* that degrade NETs.

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# ACTIVITY OF HEMOLYMPH AGGLUTINATION FACTORS OF *MODIOLUS MODIOLUS* (MOLLUSCA: BIVALVIA) FROM DIFFERENT WATER AREAS OF THE SEA OF JAPAN

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The activity of agglutination factors (AAF) in hemolymph of *Modiolus modiolus* (Mollusca: Bivalvia) from the Sea of Japan was studied by means of hemagglutination (HA) assay in relation to some influences. HA was carried out with the cell-free hemolymph of modiolus and human erythrocytes (ER) of different blood groups (0, A, B, AB). The index of HA was calculated as – $\log_2$(titer of agglutinins) and the nonparametric statistic methods were applied. Also, the inhibition of HA was performed with 10 mM solutions of 23 carbohydrates to determine the specificity of modiolus agglutinins.
The results didn't reveal any predominate affinity of agglutinins to ER of any particular blood group. Comparison of AAF in mollusks from three non-impacted water areas (Kievka Bay, Troitsa Bay and Vostok Bay) and the impacted one (Sportivnaya Harbor) indicate no significant differences at $p>0.05$. General variation limits of HA indexes were 2-11; 75% of values were limited by quartiles (25-75%) varying from 4 to 7. The measurement of HA indexes in season dynamics showed a significant ($p>0.05$) increase of AAF from March to May, and the high values were maintained until November.
The changes of AAF level in response to injection of heat-inactivated bacteria *Staphylococcus aureus* were evaluated. The measurement of AAF was performed after 3 h, 6 h, 12 h, 1 day, 1,5 days, 2 days, 2,5 days, 3 days, 7 days, 14 days. The results of measurements taken on the interval from 3 h to 3 days showed significant ($p<0.05$) increase of HA indexes in individuals analyzed after injection in comparison with the ones in the same individuals analyzed before injection. On the interval from 7 days to 14 days no differences were found.
In the present study, the carbohydrates affinity described earlier for some lectins of *M. modiolus* from North Atlantic (modiolins E and H) was detected. For the first time the affinity of hemolymph agglutinins to D-glucuronic acid and D-galacturonic acid was revealed. These results should be considered as the finding of a new lectin or a group of lectins in hemolymph of *M. modiolus.*

# PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST TO VARIABLE LYMPHOCYTE RECEPTORS (VLR) IN HAGFISH (*EPTATRETUS BURGERI*)

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**Introduction:** Hagfish, under class Myxini, are eel-shaped slime-producing marine animals which are Jawless and basal to vertebrates in evolutionary column.
Recently, it was revealed that jawless vertebrates possess a unique or alternative form of immune receptor system called Variable Lymphocyte Receptors (VLRs) that function in a way reminiscent of the adaptive immune system wherein receptor variants are generated to recognize and eliminate specific antigen. These characteristics make VLRs potential non-immunoglobulin-based antibodies which can be used as agents that can recognize antigens that elude or are invisible to immunoglobulin-based antibodies in higher invertebrates including mammals.
In this study, the production of mAbs against VLR in hagfish that can be rearranged to respond or bind to specific antigens or antigenic determinants make it a good candidate for antibody therapies and should be applicable to treating various diseases.
**Method:** We produced monoclonal antibodies against purified VLR from hagfish serum by mice immunization. The antibodies screening were investigated using ELISA, western blotting and mass spectrometry (MALDI-TOF MS/MS).
**Result and conclusion:** Sixty-one (61) positive clones in 1098 hybridomas showed the highest antibody titer and 15 of 61 clones reacted with single protein band with 37kDa in immnublot assay. This protein band was identified as VLR and its amino acid sequence was confirmed by MALDI-TOF MS/MS.
Specifically, 5 clones reacted with Hen Egg Lysozyme (HEL) in ELISA and immunoblot assay which have epitope on HEL, and it can recognize one or more specific antigenic determinant.
In the aquaculture setting, VLR-specific antibodies can be used as biomarkers to detect the presence of potential antigens and reveal the health status of commercially important fish species. And this system can be applied in the development of diagnosis tool and antibody therapy in various fields.

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# CYTOKINE HOMOLOGUE GENES IN KURUMA SHRIMP *MARSUPENAEUS JAPONICUS*: GENE EXPRESSION ANALYSES, GENE KNOCKDOWN AND PREDICTION OF PROTEIN HIGHER-ORDER STRUCTURE

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Cytokines are small cell-signaling protein molecules for intercellular communication. The vascular endothelial growth factor (VEGF) is known as cytokine which promotes angiogenesis, chemotaxis for macrophages and granulocytes, and lymphangiogenesis in vertebrates. The macrophage migration inhibitory factor (MIF) is known as an inflammatory multi-functional cytokine and plays significant role as the regulator of innate and adaptive immunity. On the other hand, the astakine is known as the invertebrate cytokine which can induce the hematopoietic stem cell differentiation in freshwater crayfish, *Pacifastacus leniusculus* in invertebrate.
In this study, we report the identification and characterization of genes of two types of VEGF, MIF and astakine from kuruma shrimp, *Marsupenaeus japonicus*. The full-length cDNA sequence of the *M. japonicus* VEGF (*Mj*VEGF and *Mj*VEGF2), MIF (*Mj*MIF) and astakine (*Mj*Astakine) genes were 845 bp, 1,170 bp, 894 bp and 1,589 bp. In prediction of higher-order structure, *Mj*VEGF and *Mj*MIF formed dimer and trimer, respectively. The bioinfomatics analyses such as domain, homology and phylogenetic analyses, gene expression analysis and gene knockdown by specific double-stranded RNA injection of *Mj*VEGF, *Mj*VEGF2, *Mj*MIF and *Mj*Astakine were performed. The details of results will be discussed. These data suggested that *Mj*VEGF, *Mj*VEGF2, *Mj*MIF and *Mj*Astakine are important in innate immunity and homeostasis in kuruma shrimp. This study is the first report about shrimp cytokine genes homologous to vertebrate VEGF and MIF.

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# FREE RADICAL GENERATION ENZYME GENES IN KURUMA SHRIMP *MARSUPENAEUS JAPONICUS*: GENE EXPRESSION ANALYSIS DURING INFECTION OF *VIBRIO PENAEICIDA* OR WSSV AND GENE KNOCKDOWN

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In many physiological processes, including the innate immune system, free radicals such as nitric oxide (NO) and reactive oxygen species (ROS) play significant roles in signal transduction and biological defense system.
In mammals, nitric oxide synthase (NOS) generating nitric oxide (NO) and NADPH oxidase (Nox), Dual oxidases (Duox) and aldehyde oxidase (Aox) generating ROS are known as free radical-generating enzymes. In this study, in order to clarify the integrative biological control mechanism including biological defense and bioregulation in shrimp from the aspect of free radicals, we identified free radical-generating enzyme genes. Additionally, we performed gene expression analyses using *Vibrio penaeicida*, the causative agent of Vibriosis and white spot syndrome virus. Furthermore, gene knockdown of free radical-generating enzymes using RNA interference clarified the function of genes in biological defense system.
The free radical-generating enzyme genes in kuruma shrimp found in this research were NOS; 4,616 bp, Nox; 4,216 bp, Duox; 4,695 bp, Aox; 4,417 bp and estimated mass of these enzymes were 134kDa, 146kDa, 173kDa and146kDa, respectively. The expression of the *Mj*Duox and *Mj*Nox genes increased at 24 and 48 h after WSSV injection, respectively. In order to confirm the effect of gene knockdown effect of *Mj*Nox gene in biological defense system, WSSV was injected in shrimp after the *Mj*Nox gene knockdown treatment. The survival rate decreased 3-10 days after WSSV injection in *Mj*Nox gene knockdown treatment group. In conclusion, NO and ROS are important in redox homeostasis control and are essential to retain the biological defense system and bioregulation in kuruma shrimp. It was also suggested that *Mj*Nox and *Mj*Duox were the adequate marker genes to detect the virfal infection in shrimp because these genes markedly increased in the early phase of viral infection.

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# THE INTESTINAL MICROBIOTA IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) IS INFLUENCED BY DIET TYPE AND *YERSINIA RUCKERI* CHALLENGE

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In recent years it has become more and more evident that the bacterial flora in the gut of warm-blooded animals modulates physiological processes and the immunological status of the host. Besides effects on growth parameters, commensal intestinal bacteria balance the immune system and prevent colonization of pathogenic bacteria. The question is if the gut microbiota is also important in lower vertebrates such as fish? Is the microbiota related to the diet type and does it play a protective role in connection to pathogenic challenge? To examine these questions rainbow trout fry were fed two different diets of either a marine or vegetable origin from first feeding and onwards. At a size of about four gram the fish were bath challenged by *Yersinia ruckeri* serotype O1 and intestines were then sampled 5 days post challenge for subsequent metagenomic examination. Next-generation sequencing was applied for the metagenomic studies using the Illumina HiSeq 2000 platform. The results showed two distinctly different microbial patterns in the intestines dependent on the diet type. Fish fed a marine based diet overall had a significantly higher amount of the class β-proteobacteria, while the amount of reads belonging to phylum Firmicutes were significantly higher in the intestines of vegetable fed fish. The genera within phylum Firmicutes present in significantly higher amounts in vegetable fed fish were *Weissella*, *Leuconostoc* and *Streptococcus*. Genus *Aeromonas* from the γ-proteobacteria class was also present in significantly higher amounts in the vegetable fed fish. When challenged with *Yersinia ruckeri*, fish with a high amount of sequence reads belonging to genus *Yersinia* had a significantly lower amount of reads from the order Burkholderiales relative to non-infected control fish and fish with a low amount of *Yersinia* specific sequences. Further, these infected fish further clustered separately when analyzing the bacterial community on a PCA plot. The immunological examinations using RT-qPCR showed similar constitutive expression between the two diet groups, but the response differed between the two diet groups in challenged fish. Here, the general pattern was a pro-inflammatory response in the intestine of marine fed fish challenged with *Yersinia ruckeri* relative to non-infected control fish, while several immune genes were down-regulated in vegetable fed fish relative to non-infected control fish. Overall, the results indicate that the gut microbiota in rainbow trout is highly plastic according to the type of diet and does further seem to be involved in the immunological response in connection to pathogenic challenge.

# CHANGES IN SKIN MUCUS PROTEIN COMPOSITION PROFILES OF JAPANESE FLOUNDER *PARALICHTHYS OLIVACEUS* FED DIETS SUPPLEMENTED WITH HIGH-CONCENTRATION ASCORBIC ACID

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Ascorbic acid (AsA), commonly known as vitamin C, is an essential vitamin for normal growth and physiological function in fish. This dietary material is known to be beneficial for immune responses in fish, and increased disease resistance in fish fed elevated levels of AsA has been demonstrated in many fish species. However, knowledge concerning the beneficial effects of AsA supplementation with high-concentration AsA in regard to the innate immune system of mucosal tissue is scarce. Therefore, the present study examined two-dimensional gel electrophoresis (2D-PAGE) profiles of skin mucus in Japanese flounder *Paralichthys olivaceus* fed commercial diets supplemented with high-concentration AsA (2000 mg/kg diet) for 7 days. Approximately 300 protein spots were detected from the skin mucus sample when applying a 20-μg protein 2D-PAGE gel in the pH range 4–7. Compared to a control sample, significant changes were visualized in 50 protein spots for fish fed a diet supplemented with AsA, and the collected spots were further identified by LC-MS/MS analysis and a database search. Most of the protein spots specific to AsA administration were identified as transferrin, serotransferrin, and complement component C3, which are considered immune competent molecules. In conclusion, this research presents protein composition profiles of skin mucus and provides information demonstrating improvement of the mucosal immune system following mega-dose AsA supplementation in fish.

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# RAINBOW TROUT THROMBOCYTES ARE INVOLVED IN MHC II DEPENDENT ANTIGEN PRESENTATION.

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Antigen presentation involves highly specialized cells that take up antigen, process it and activate the helper T-cells by presenting digested foreign peptides. Those cell types are well described as myeloid cells, B-cells, dendritic cells. Additionally, in higher vertebrates like birds the thrombocytes are also considered as effective phagocytic cells. Unfortunately, the information whether thrombocytes in lower vertebrates may belong also to true phagocytic cells is incomplete and confusing. There are indications that they may contribute to the stimulation process by antigen uptake and presentation in carp, turbot or turtle. However, this function has never been clearly investigated in trout. Thus, neither any functional and molecular data related to MHC II restricted presentation nor the ability to engulf, kill and digest the antigen has been shown.
In this study, we aimed to specify the role of trout thrombocytes in the process of antigen presentation. First indication is the increased MHC II mRNA expression in the magnetically sorted thrombocytes from immunized and stimulated rainbow trout. As it is the key molecule of antigen presenting cells we raised questions about expression profile of cytokines IL-6 , IL-10, chemokines IL-8, IL-22 and genes from TLR family found in rainbow trout. Similar cell stimulation was done using the pathogen A. salmonicida in vitro. Additionally, the ability of the thrombocytes to interfere with the bacterial growth has been investigated by co-cultivation experiments. Results will be discussed.

# SERUM INTERFERON GAMMA LEVELS ARE AFFECTED BY HIGH-CONCENTRATION ASCORBIC ACID SUPPLEMENTATION IN THE RAINBOW TROUT *ONCORHYNCHUS MYKISS*

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Ascorbic acid (AsA) is an essential vitamin for normal growth and is known to be beneficial for immune responses in fish. Numerous studies have shown a diet supplemented with high-concentration AsA of at least 1000 mg/kg protects fish against several viral infections. However, since knowledge concerning the mechanism of action of AsA is limited, we focused on the serum level of interferon gamma (IFNγ). IFNγ is a cytokine that enhances antiviral activity and macrophage activity in fish and mammals. This study examined the effects of high-concentration AsA (5000 mg/kg diet) on serum IFNγ levels in the rainbow trout *Oncorhynchus mykiss*. IFNγ levels were quantified using indirect ELISA, which was constructed using polyclonal antibodies produced from recombinant rainbow trout IFNγ (rIFNγ). Additionally, the detection of IFNγ-producing cells was achieved by immunostaining (IS). Serum IFNγ levels of fish fed diets supplemented with high-concentration AsA were statistically higher than those of fish fed a non-supplemented diet, and a correlation was found between serum IFNγ levels and AsA contents in liver. IFNγ-positive cells were confirmed in gill and spleen tissues in both groups by IS, and the number and intensity of positive cells tended to increase, especially in fish fed AsA-supplemented diets. Our results and previous studies suggest that high-concentration AsA supplementation in fish induces activation of IFNγ-producing cells as part of the modulation of innate immunity against viral infections..

# THE EFFECTS OF BETA GLUCAN STRUCTURE AND FORM ON IMMUNOMODULATOR PROPERTIES IN COMMON CARP (*CYPRINUS CARPIO*)

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Fish diseases have become one of the most important challenges facing the expansion and development of the aquaculture industry. In recent years there has been considerable interest in the use of natural products such as β-glucan in improving immune status and disease protection in humans and animals including fish. Different immunomodulatory effects have been observed from several polysaccharides which have different structure and originate from different sources.
In this investigation the immunomodulatory effect of structure and form of carbohydrates on fish immune cell activity were studied. Initial observations established the methodology and techniques through using different parameters i.e. β- glucan sources and concentrations, cell activity assays, and incubation periods. The Carp Leucocyte Cell line (CLC) was exposed to different carbohydrate concentrations (50µg/ml and 5µg/ml) and molecular forms which had been either obtained from natural sources e.g. MacroGard® or specifically manufactured. The effects on cell activity were monitored utilising the NBT assay. There was a significant increase ($P \leq 0.05$) in phagocytic activity in 9 of the 35 carbohydrates utilised. These positive inducers of phagocytosis had a range of significant increases ($P \leq 0.05$) in relation to their concentrations. Future studies will establish if, and to what effect, these novel carbohydrates affect the activity of pronephric cells (macrophages), utilising other assays such as the BrdU technique to determine proliferation in immune cells.
Once these affects *in vitro* have been ascertained to induce an immunostimulatory response *in vivo* studies using common carp will be carried out.

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# TECHNIQUES TO DETERMINE THE CELLULAR ACTIVITY OF DIFFERENT FORMS OF B GLUCAN; AN IMMUNOSTIMULANT USED IN FISH PRODUCTION

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The aim of this study was to establish an *in vitro* system utilising fish immune cells and cell lines i.e. carp leukocyte cell line (CLC) and the Epithelioma papulosum cyprini cell line (EPC) to determine the effect of natural and formulated β-glucan e.g. MacroGard®, sulphated MacroGard®, zymosan on cell activity. Different assays were utilised to measure mitochondrial activity i.e. MTT and MTS, and phagocytic activity with the NBT.
Comparative studies highlighted that the optimisation of the assays required consideration of several parameters e.g. cell density, culture medium, dye concentration, exposure time to the assay products, nutrient deletion by using spent medium, and the metabolic activity of the cells.
The MTS was not as sensitive as the MTT in the detection of cell proliferation. For example, the sulphated MacroGard® affected significantly ($p \leq 0.05$) cell proliferation at concentrations 2, 2.5, 10µg/ ml after 24h incubation and 1, 1.5, 2, 2.5µg/ ml after 48 and 72h incubation, whereas there was no significant effect in cell proliferation in MTS after 24 and 48h incubation. NBT was successfully applied to investigate the effect of carbohydrates such as sulphated MacroGard® on phagocytic activity on the CLC cell line, where after 24 hours incubation at concentration 50µg/ ml a significant increase in cell activity was noted.
In contrast, the EPC cell line appeared to be unsuitable to monitor carbohydrate induced activity as highlighted utilising the MTS assay, where proliferation did not occur in cells treated with sulphated MacroGard® at concentrations greater than 10µg/ml compared to control at 24h incubation, and at concentrations greater than 2µg/ml at 48h incubation.
In conclusion, different cell types were adapted to determine the immunomodulatory effect of β-glucan, and both of pronephric cells and CLC cell lines are appropriate cell types to examine β- glucan effects. Additionally, two different assays were employed to determine the immunomodulator effect of β-glucan: (i) MTT assay for cell activity and proliferation; (ii) NBT assay for macrophages phagocytic activity. Moreover, it appears that low doses of β-glucan more effective on immune cell activity under the experimental conditions utilised.

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# MELANIN AND MELANOMACROPHAGES: A ROLE IN FISH IMMUNITY?

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Melanin comprises a complex group of pigmented polymers with functions in particular being attributed to dermal solar protection, but increasing information also suggests a prominent role in innate immunity. In ectothermic vertebrates, melanogenesis may occur in leukocyte populations. The presence of melanin and melanomacrophages in muscle fillets of farmed salmon represents a considerable quality problem for the salmon industry with major economic concerns. We have addressed melanin, melanomacrophages and pigmented spots in musculature of Atlantic salmon (*Salmo salar*) in a number of ways including cloning of genes responsible for the melanogenesis followed by expressional and morphoglogical investigations of tissues and pathologlogical changes and also in vivo and in vitro experiments. In this presentation, we focus on the regulation of melanogenesis in a cell line consisting of pigment-producing salmon leukocytes, the SHK-1 cell line. At 10°C cultivation, tyrosinase and dopachrome tautomerase remained unregulated. At 15°C, a weak but significant up-regulation was induced, while at 20°C, these genes were up-regulated in an exponential manner over time. Temperature did not affect the transcription of the immune-related genes. Virus infections, poly I:C, or bacterin in form of formalin-inactivated *Aliivibrio salmonicida*, had no influence on the transcription of the melanogenesis-related genes. Our findings suggest that elevated temperature may contribute to melanization in salmon. Together with other resent results, our results indicate that melanin in fish melanomacrophages has a role in anti-oxidation processes that may be indirectly linked to immunity.

# MOLECULAR CHARACTERIZATION, PHYLOGENY, AND EXPRESSION PATTERN OF C-TYPE LYSOZYME IN TAIMEN (*HUCHO TAIMEN*, PALLAS)

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Lysozymes are important defense proteins of the innate immune system and possess high antibacterial activities. In the present study, a full-length c-type lysozyme cDNA (HtLysC) was cloned and characterized by RT-PCR and RACE techniques from taimen (*Hucho taimen*, Pallas). The cDNA contains an open reading frame (ORF) of 432 bp encoding 144 amino acid (aa), with 129 bp located in the 5' untranslated region (UTR) and 228 bp in the 3' UTR. The deduced amino acid sequence of HtLysC had a signal peptide at the N-terminal sequence and was predicted to localize extracellularly, with a predicted molecular mass of 14.3 kDa and a theoretical pI of 8.66. The aa sequence possessed a LYZ1 domain (16-140 aa) which contained two conserved residues (Glu 50 and Asp 67), eight conserved cysteine residues and a calcium binding site. Pair-wise alignments showed a higher identity (greater than 90%) with several species in salmon families (Salmonidae family) while a lower identity (37.4%) with *Danio rerio*. Quantitative RT-PCR analysis indicated that the mRNA levels of *HtLysC* were detected in the liver, intestine, blood, spleen, kidney, and gill and up-regulated in the liver when challenged with *Y. ruckeri*. This is the first study to report the genetics and functions of the c-type lysozyme in taimen; our results suggest that HtLysC may play a role in the innate immune system. A further study on antibacterial property of recombinant HtLysC should be performed to help us better understand the function of c-type lysozyme in fish.

# IMMUNE RESPONSE AND PHAGOCYTOSIS OF WHITE SHRIMP *LITOPENAEUS VANNAMEI* THAT RECEIVED HEAT-KILLED *VIBRIO ALGINOLYTICUS*

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Immune parameters of white shrimp *Litopenaeus vannamei* that received live *Vibrio alginolyticus* (LVa), and heat-killed *V. alginolyticus* (HVa) were examined at days 0.5~7, and haematopoietic tissues (HPTs) were examined at 5 days after PE. Immune parameters of shrimp with no treatment served as background values. Immune parameters of LVa-received shrimp were lower than those of control shrimp at 0.5~7 days. HVa-received shrimp showed earlier (day 1) increases in immune parameters, and these gradually decreased to background values after 7 days. HVa-received shrimp showed higher proliferation and mitotic index of HPTs after 5 days. In another experiment, control shrimp, and HVa-received shrimp at 7 days were challenged with *V. alginolyticu*s, and the survival rate, immune parameters, and HPT were respectively examined at 0.5~7, 0.5~7, and 3 days. Survival rates were higher in HVa-received shrimp at 4~7 days. Immune parameters of control shrimp and 7-day-HVa-received shrimp remained lower than background values at 1~7 days after challenge with *V. alginolyticu*s. Phagocytosis and clearance of shrimp that had received HVa were much higher than those of control shrimp over 7~28 days. It was concluded that shrimp that received HVa could induce earlier immune response, and show phagocytosis and clearance to LVa.

# EFFECT OF MIXTURE COMPOSED OF β GLUCANES AND MOS ON THE IMMUNOLOGICAL PARAMETERS IN THE REARED EUROPEAN SEA BASS

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We used a mixture composed of yeast (*Saccharomyces cerevisiae*) that contains a blend of beta 1.3 and 1.6 glucans and MOS (mannan oligosaccharide), suggested to have beneficial effect when administered in fish feed at a rate of 0.1%. To our knowledge there are no studies that assessed the physiological response of the reared European sea bass (*Dicentrarchus labrax*) to the supplementation of feed with such immunostimulant mixture (IM). Moreover, practical trials on the farm have suggested that fish indeed exhibit better growth rate and resistance to the farm strains of Tenacibaculum maritimum. This for we have fed sea bass (N=60 per trial) for 4 weeks by 4 different feeding regimes: 1.) IM administered daily in feed by 0.1%; 2.) IM administered in feed by 0.1% on every 4th day of feeding; 3.) IM administered daily in feed by 0.1% for 2 weeks, followed by regular feed the next 2 weeks; 4.) control fish fed by regular feed without IM. Prior to the feeding trial (day 0) and further on 1st, 4th, 7th, 14th, 21st and 28th day of trial, different physiological parameters were sampled: fish length and weight; serum and whole blood for lysozyme activity and macrophage respiratory burst; gills, gut and hematopoietic organs for histological analysis as well as for expression of interleukin-10. Our preliminary results show that although IM did not significantly affect fish growth, different feeding regimes induced different levels of innate immunity reaction.

Keywords: European sea bass, *Dicentrarchus labrax*, immunostimulation

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# CHARACTERIZATION OF THREE MAJOR CYTOKINES: IL1, TNF1 AND TNF2 IN REARED ATLANTIC BLUEFIN TUNA *THUNNUS THYNNUS*

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Atlantic bluefin tuna (*Thunnus thynnus*) (BFT) is of great economic significance for Croatian aquaculture and therefore it is necessary to ensure optimal and sustainable conditions during its farming process. Intensive culture of BFT is limited by infectious diseases that, beside the stress, cause heavy losses negatively affecting an individual's immunocompetence and growth performance. However, to date there are no reports of cloning and expression analysis of any major immunity gene of BFT, therefore in this study we have characterized the first cytokine molecules in Atlantic bluefin tuna, through: 1) Isolation of full-length cDNA and gene sequences of Atlantic BFT TNFα1, TNFα2, IL1β and their comparison to known sequences in other vertebrates, especially teleost fish; 2) Designing of three-dimensional models for three BFT cytokines based on homology modeling; 3) Quantification of in vivo tissue specific expression of chosen cytokines in reared BFT over the duration (two years) of the farming process, in order to evaluate their importance as a putative health biomarkers within tuna aquaculture. Understanding of how the innate immunity response is functioning in this bluefin species will allow us to study immune regulation more detailed.

Keywords: tuna, immunity, cytokines, cloning, expression

# IMMUNOSTIMULATION WITH RECOMBINANT INTERFERON GAMMA ENHANCES RESISTANCE OF GINBUNA CRUCIAN CARP *CARASSIUS AURATUS LANGSDORFII* TO THE INTRACELLULAR PARASITIC BACTRIUM *EDWARDSIELLA TARDA*

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Interferon gamma (IFNγ) is an antiviral and immunoregulatory cytokine that is essential for cellular defense against a variety of infectious agents in mammals. However, knowledge concerning the underlying mechanism of the immune response of IFNγ in fish is scarce. Therefore, in the present study we examined the *in vivo* immunostimulatory effects of a recombinant crucian carp (*Carassius auratus langsdorfii*) interferon gamma (rIFNγ). Mature rIFNγ, expressed and purified from *Escherichia coli*, was administered to ginbuna crucian carp *Carassius auratus langsdorfii* via intraperitoneal injection. Injections of rIFNγ at 0.1 and 0.01 μg/fish (about 7 g in size) resulted in significantly greater protection than a phosphate buffered saline (PBS) injection against a lethal challenge involving the intracellular parasitic bacterium *Edwardsiella tarda*, showing a 40% relative percentage of survival. The colony forming unit (CFU) in each tissue at 24 hours after the challenge did not differ among groups, while that of the liver and kidney after 48 hours was significantly lower in the rIFNγ-injected group than the PBS-injected group, and this was correlated with fish survival rates in the challenge test. At 6 hours after the challenge, fish injected with rIFNγ showed a significantly higher number of lymphocytes in head kidney leucocytes compared to fish injected with PBS. Histopathological characteristics observed in the kidneys of infected fish included haemorrhage and necrosis of hematopoietic cells, and haemorrhages were more extensive in PBS-injected fish than rIFNγ-injected fish. Therefore, fish rIFNγ can modulate the innate immune response and mediate early antibacterial protection against *E. tarda* infection.

# A MONOCLONAL ANTIBODY RECOGNIZING A SURFACE MARKER ON OLIVER FLOUNDER *(PARALICHTHYS OLIVACEUS)* T LYMPHOCYTE

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Despite the great advance in recent years, much information on the fish immune system has been limited to B lymphocyte lineage. The lack of marker-specific antibodies to recognize the T lymphocyte makes the comprehensive understanding of immune response difficult. In this study, we described the preparation and characterization of a monoclonal antibody (mAb) specific for T lymphocyte of *Paralichthys olivaceus*, olive flounder. The results from these methods will enable us to understand the mechanism of immune reaction from infected fish and ultimately find a way to protect them.
The antibody was obtained by fusing B cell derived from a mouse immunized with olive flounder leukocyte and mouse myeloma cell. After fusion, the cultured hybridomas were screened by ELISA, indirect immunofluorescence (IIF) and flow cytometry using olive flounder leukocyte. From three repetitive B cell - myeloma fusions we obtained a total of 1017 hybridomas, all of which were screened through IIF and flow cytometry on live leukocyte. Among the obtained hybridomas, 21 resulted positive at various levels, and then from that we chose the one showing the highest staining level to T lymphocyte.
The selected monoclonal antibody which recognizes the marker of T lymphocyte can be used as a probe to identify the role of T lymphocyte in immune mechanism of fish. The findings in this study will lead us in the development of reliable agents for the identification of lymphocyte subpopulations as well as in the production of vaccines for fish. Lastly, this study will give us a wider knowledge regarding the immune response mechanism between mammal and fish on the evolutionary level.

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# EFFECT OF *VAGOCOCCUS FLUVIALIS* ON CYTOKINE EXPRESSION OF EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*): PRELIMINARY STUDY

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In vitro dynamic of the expression of immune-related genes in sea bass after incubation with live and inactivated (heat and Uv-light) probiotic *Vagococcus fluvialis* L-21 at different times (T1, T12, T24, T48) was investigated by real-time PCR . The immune associated genes, interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin 10 (IL-10), Tumor necrosis factor-α (TNF- α), ciclo-oxigenase-2 (COX-2), caspase-3 (Casp-3) and Mx were studied in head-kidney (HK) leucocytes of sea bass after incubation with this probiotic.
Transcript of pro-inflammatory cytokines (IL-1β, TNF-α, and COX-2) was highly up-regulated after 1 h of incubation with the probiotic strain *V. fluvialis* L-21. We found statistically significant difference in pick of expression of TNF-α after 1 h of incubation with Uv-light inactivated probiotic strain. The COX-2 expression was highly up-regulated at all times studied, with the exception of 12 and 24 h post incubation for the Uv-light inactivated bacteria. Transcript of IL-10 and Casp-3 showed the higher statistically significant differences of expression after 48 h post incubation with live bacteria. In the contrast, sea bass HK leucocytes expressed Mx at 12 and 48 h without statistically differences among treatments. Our results suggest that *V. fluvialis* L-21 is able to stimulate *in vitro* some immune-related genes associated with the early inflammatory response. Future studies *in vivo* are necessary to clarify this process in sea bass.

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# EFFECT OF DIETARY 1.3-1.6 β-GLUCAN (BIOLEX, LEIBER) ON THE INNATE IMMUNE RESPONSES AND DISEASE RESISTANCE IN INTENSIVE CULTURE SYSTEM OF THE EUROPEAN EEL (*ANGUILLA ANGUILLA*)

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The intensification of commercial production of eel species may negatively affect the fish immune system, exposing animals for polyetiological stress what, in turn, can increase susceptibility of fish to infectious diseases. Because fish depend more strongly on nonspecific defence mechanisms than mammals, immunostimulants might play a significant role in control the disease outbreaks. The aim of the present study was to determine the influence of different doses of 1,3-1,6-β-D-glucan (BIOLEX, Leiber GmbH Germany) on the innate immunity parameters and mortality rate in eel challenged with *Aeromonas hydrophila*. The gut mucous membrane forms a direct protective barrier against pathogenic microorganisms. 1,3-1,6-β-D-glucan molecules are able to pass through the barriers provided by the mucous membrane and the gut's protective epithelia. Specific surface structures of the β-glucans (epitopes) are identified as alien by macrophages, lymphocyte B and other immunomocompetence cells of the lymphoid tissue. For this study 200 healthy European eel with average weight of 25 g were used. The fish were fed commercial pellets containing BIOLEX E at dose 200 mg per kg of feed and BIOLEX A at dose 500 mg per kg of feed. The control group was fed commercial pellets without immunostimulant. Four weeks after, blood, spleen and headkidney samples were taken for immunological study. The metabolic activity and potential killing activity of spleen phagocytes were examined. The proliferative response of headkidney lymphocytes stimulated by mitogens ConA or LPS were determined by the MTT colorimetric assay. Also the lysozyme and ceruloplasmine activities in plasma and total immunoglobulin levels in serum were examined by spectrophotometric assay. The results showed that BIOLEX E and BIOLEX A statistically significantlly the innate immunity parameters in eel. The higher cell-mediated and humoral-mediated immunity was observed in eel fed with BIOLEX A (at dose 500 mg per kg of feed), as compared to the lower dose group. The lover mortality after challenge test with *Aeromonas hydrophila* was observed in eel fed with BIOLEX A and E, compared to the control group. Based on the results of our study, the use of natural immunomodulators for the prevention of eel diseases seems to hold great promise.

# INNATE IMMUNITY IN HUCHEN (*HUCHO HUCHO*) GROWING IN NATURAL CONDITIONS AND IN INTENSIVE CULTURE SYSTEM: A COMPARATIVE STUDY

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Cellular and humoral factors of innate immunity are a very important part of immunological mechanisms and perform a key role in the regulation of immune response in fish. The aim of this study was to examin the cell-mediated and humoral-mediated defence mechanisms of huchen (*Hucho hucho*) grown in a pond system of culture and in natural conditions. In order to determine selected nonspecific immune parameters in pond culture condition, 20 healthy huchen of approximately 30 - 50 g were examined and to determine these parameters in natural conditions, 20 healthy huchen of approximately 30 - 50 g (obtained from Dunajec river) were examined. Fish were anaesthetised in Propiscin (IFI, Poland) and blood samples were taken. Also the spleen and pronephros were removed for cells separation. The metabolic activity of spleen phagocytes by their respiratory burst activity (RBA) and potential killing activity (PKA) were measured by spectrophotometric assay. The pronephros lymphocytes proliferation (LP) was determined by the MTT colorimetric assay. Lymphocytes were stimulated by concanavaline A (ConA) or lipopolisaccharide (LPS). The lysozyme activity in the plasma was measured using turbidimetric assay. Ceruloplasmine activity in the plasma was determined by spectrophotometric micro-methods. The total protein and immunoglobulin (Ig) levels in the serum were measured by spectrophotometric methods. The data were statistically evaluated with the Student's t-test, and the results are presented as mean and standard deviations (SD). For all calculations $P<0.05$ was assumed as significant. This basic examination provided very important information about physiological levels of nonspecific humoral and cellular parameters in huchen at different environmental conditions. Results showed that, the level of all measured parameters excluding total protein in serum were statistically significantly higher ($P < 0.05$) in huchen from river, compared to fish from pond culture. Basic information regarding cellular and humoral defence mechanisms in healthy huchen reared in pond culture and natural condition are very important in monitoring of huchen health and in the early diagnosis of infection diseases in different system of culture.

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# HOST INNATE IMMUNE MECHANISMS INVOLVED IN THE RESOLUTION OF SVCV INFECTION

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Spring viraemia of carp virus (SVCV) is a rhabdovirus associated with systemic illness and mortality in cyprinids. In this work, we have used the zebrafish (*Danio rerio*) larvae to study the importance of the innate immunity against SVCV systemic infection. Injection of this pathogen in zebrafish larvae caused a high and rapid mortality. Most of the fish died with visible head hemorrhages and after the loss of the tail circulation. Also, the transparency of zebrafish at this age allows us to observe the contribution of different immune cell types to the virus progression.

We also used different zebrafish transgenic lines and the knockdown of the mammalian transcription factor SPI-1 (PU.1) to shed light on the events that occurs immediately after the virus entry in the host.

This work will contribute to further increase the knowledge about the importance of the early innate immune response in the resolution of rhabdovirus infection.

# BIOTYPING AND PFGE GENOTYPING OF *YERSINIA RUCKERI* ISOLATES FROM FARMED STURGEON IN FINLAND

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In Finland sturgeon (*Acipencer sp.)* is farmed in three recirculating aquaculture system (RAS) farms A, B and C of two companies. The original fish material has been imported from several European countries. During 2002-2012 *Yersinia ruckeri*, the causative agent of enteric red mouth disease, was isolated five times in Evira laboratories from diseased sturgeons: twice from farms A and C and once from farm B. In this study biotypes and PFGE profiles of the isolates were determined and compared with *Y.ruckeri* isolates from other fish species in Finland.

The biotypes of the five sturgeon *Y. ruckeri* isolates were determined by motility test on motility agar and by the ability to hydrolyse Tween 80 on Shotts-Waltman agar. The PFGE profiles were produced and analysed using NotI enzyme restriction of whole genome DNA. The biotypes and PFGE profiles of the isolates were compared with the previously typed 16 *Y. ruckeri* strains isolated from other fish species during 1986-2007.

All the five sturgeon *Y. ruckeri* isolates were motile and Tween 80 positive, thus biotype 1. The PFGE characterization of them resulted in four clearly different profiles. The two strains isolated two months apart from farm C in 2011 were identical. All the PFGE profiles of the sturgeon isolates differed also from the profiles of the heterogeneous biotype 1 isolates and from the more homogenous biotype 2 isolates from other fish species.

The biotype 1 isolates were genetically heterogeneous regardless of the fish species. The origin of the *Y. ruckeri* infections in the sturgeon farms remains unclear. The results of the analysis of farm C isolates indicated that a *Y. ruckeri* strain, once introduced into a fish farm, may survive in the farm for months and cause disease repeatedly. In RAS it is crucial to prevent introduction of pathogens into the farm. Once introduced, elaborate and expensive emptying and sanitation of the recirculation system may be the only successful measure to avoid recurrent infections and excessive use of antibiotics.

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# EUROPEAN HERRING SHOWS HIGH MORTALITY RATE IN BATH CHALLENGE WITH VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS (VHSV)

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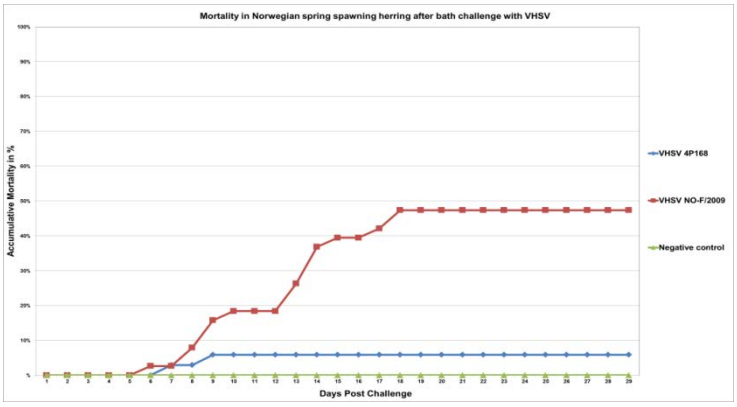
Earlier investigations have demonstrated the presence of viral haemorrhagic septicaemia virus (VHSV) in European herring *Clupea harengus* in the North Sea, Baltic Sea, English Channel, Skagerrak and Kattegat. Most of the approximately 100 VHSV isolates included in the www.fishpathogen.eu database are of genotype Ib, four are of genotype II and one is of genotype III (4P-168). VHSV is also well known in Pacific herring *Clupea pallasii* where genotype IV have been detected both in diseased and healthy wild herring. Challenge trials with genotype IV isolates in Pacific herring in the US have shown high mortality rates, while no VHS challenge trials have earlier been conducted on European herring.
High prevalence of VHSV genotype Ib was detected in Norwegian spring spawning herring during the spawning season (Johansen et al 2013). The sampled herring showed no signs of disease and were caught by commercial trawl boats. High amounts of virus was found in internal organs (heart, kidney, spleen and brain) showing a septicaemia. How this virus affects the herring stock is unknown. A bath challenge trial with one of these isolates from Norway (NO-F/2009) was conducted on Atlantic herring of approximately 3 gram. In addition herring was challenged with a genotype III isolate from herring (4p168).




The Norwegian Ib isolate from herring gave an accumulated mortality of 47% compared to 6% morality with the genotype III isolate. Results from histopathology, immunohistochemistry, RT-PCR and cell culture will be presented. How severely VHSV affects wild stocks of herring in the Northern European waters, and which threat VHSV in wild fish represents to farmed fish are two of the main questions that need to be further investigated.

Johansen et al. 2013 "High prevalence og viral haemorrhagic septicaemia virus (VHSV) in Norwegian spring-spawning herring" Marine Ecology Progress Series 478: 223-230

# THE MAIN STURGEON DISEASES IN AQUACULTURE OF SOUTH RUSSIA

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Not long ago the Azov Sea was characterized by the high level of fishery and natural reproduction of valuable commercial fish species, to which sturgeons belong at the first turn. At the middle of the XIX$^{th}$ century, the catch of sturgeons at the Azov Sea basin reached its pike. At that time, annual catch was 10-14 thousands of tons. By the end of the 90-es of the last century, official amount of harvesting decreased 7 times, and by the beginning of two thousandths – 70 times. During recent years, the official catch of sturgeons was carried out only for scientific purposes and for reproduction at hatcheries and in 2009 was below 2 tons.

More then 5000 specimens of sturgeons of different ages which belong to 6 species of pure lines and their hybrids (great sturgeon, Russian sturgeon, starred sturgeon, Siberian sturgeon, paddlefish, and hybrids of sturgeons) were taken for parasitological, bacteriological and hematological studies.

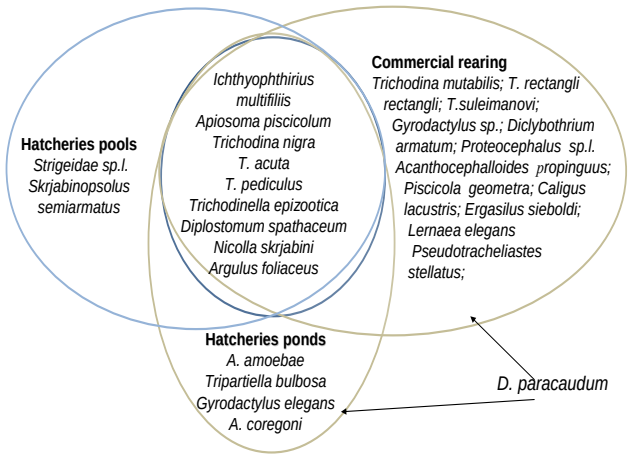


Figure 1. Biodiversity of sturgeon parasite fauna in aquaculture

The analysis of biodiversity of sturgeon parasites in southern Russia allowed us to determine the causative agents of the diseases which caused mass fish mortality in natural waters and aquaculture. It is necessary to have comprehensive information about the composition of species, the hosts and geographical distribution of sturgeon parasites to prevent and control the diseases.There are two directions in commercial rearing of sturgeons. The first is the incubation of eggs, larvae rearing in the hatcheries and further their release into the sea. The second is the commercial rearing of sturgeons in fish farms by the usage of different methods. These two directions are popular in southern Russia. It was noted that there are common parasite species which form the core (fig. 1) of the parasite fauna of sturgeons. Mostly, there are widespread species often with direct life cycle. In addition to them, there were registered parasite species (*Diplostomum spathaceum*, *Nicolla skrjabini*) with complex life cycle which includes mollusks in their development. According to our data the environmental conditions influence on the structure of parasite fauna of sturgeons reared in aquaculture.

# NEGATIVE EFFECTS OF A NOVEL *KUDOA* SPECIES ON AQUACULTURE AND WILD FISHERIES.

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Myxosporeans are a diverse group of parasites commonly infecting fish. Although the life cycles of many species are not described, known life cycles require an alternate host; this is typically an annelid worm. Myxosporeans from the family Kudoidae are mostly histozoic in muscular tissues of fish. About 90 species of *Kudoa* have been described to date, but no life cycle data exists for this group. They are generally considered non-pathogenic to fish, however a number of species have been shown to cause great economic losses in both commercial fisheries and aquaculture, due to post mortem proteolysis causing muscle liquefaction. Furthermore, recent studies have demonstrated that fish infected with *Kudoa* can cause gastrointestinal problems in humans after consumption.
We report *Kudoa* infections from three different fish hosts: spotted wolffish *Anarhichas minor,* Atlantic wolffish *A. lupus* and Atlantic lumpfish *Cyclopterus lumpus*, all of which are commercially valuable fish species in the North Atlantic. In 2001, experimental rearing of spotted wolffish began in Iceland. Subsequent health surveillance revealed persistent, extensive and highly prevalent *Kudoa* infections of trunk muscles. Although not pathogenic to the fish, the infections caused serious financial losses due to severe post mortem myoliquefaction that rendered significant parts of the fillets from harvested fish unsuitable for human consumption. After 5 years, the rearing of spotted wolffish came to an end, largely due to problems with *Kudoa*.
Large female lumpfish are traditionally air dried for human consumption after removal of the valuable eggs. During this process, some fish wither and contain little muscle after drying compared to other fish and are consequently discarded. To examine the etiological agent responsible, fifteen fish, 5 of each species, caught off the west coast of Iceland were examined for the presence of *Kudoa* using histological, morphological and molecular methods. Most examined fish, regardless of fish species, were found to be infected with a single novel species of *Kudoa*.
Post mortem myoliquefaction due to *Kudoa* infections has been a concern for years, both in aquaculture and commercial fisheries. In aquaculture, problems due to *Kudoa* have been restricted to fish held in sea pens that are consequently fully exposed to pathogens in the wild environment. However, the farming of the spotted wolffish in Iceland was carried out in land based facilities, which raises questions regarding the life cycle of *Kudoa*.
This novel *Kudoa* causes economical loss to lumpfish products and is not host specific, which is a concern as lumpfish are increasingly used as cleaner fish in salmonid culture.

# *IN VITRO* PASSAGES IMPACT ON VIRULENCE OF *SAPROLEGNIA PARASITICA* TO ATLANTIC SALMON (*SALMO SALAR* L.) PARR

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The effect of serial *in vitro* sub-culturing on three pathogenic strains of *Saprolegnia parasitica*
VIO 5337, VIO 2736 and VIO 5708 was investigated. The isolates were passed through Atlantic salmon (*Salmo salar* L.) parr, and then re-isolated as single spore colonies. All strains, VIO 5337, VIO 2736 and VIO 5708 produced infection and the isolate obtained from diseased fish served as a virulent reference culture and was designated "AP" ("activated through passage"). Successive sub-culturing was performed by obtaining an inoculum from AP to produce the 2nd sub-culture, then passaged to the 3rd sub-culture (from the 2nd), until the 15th passage was obtained. This was achieved by inducing asexual spore reproduction in Glucose-Yeast agar and broth. Spores used to produce storage cultures were collected at passages 5, 10 and 15. The different passages of each strain were used to artificially infect Atlantic salmon parr to demonstrate and compare levels of attenuation between passages within each strain. Morphological characterization of growth patterns was performed to observe differences, if any, occurring as a result of serial *in vitro* sub-culturing. VIO 5337 and VIO 2736 declined in virulence after 15 successive *in vitro* sub-cultures whereas VIO 5708 did not. This study is the first to investigate attenuation of virulence in *Saprolegnia*, and whether or not isolates of *S. parasitica* should be passed through the fish host prior to challenge experiments, and it reveals that some strains of *S. parasitica* clearly degenerate more rapidly than others when subjected to successive *in vitro* sub-culturing on Glucose-Yeast extract.

# *GLYPHIDOHAPTOR* AND *TETRANCISTRUM* (MONOGENEA: DACTYLOGYRIDAE) FROM *SIGANUS CANALICULATUS* (PARK, 1797) AND *SIGANUS SUTOR* (VALENCIENNES, 1835), OFF OMANI WATERS, WITH RE-DESCRIPTION OF *TETRANCISTRUM INDICUM* PAPERNA, 1972

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A survey of the parasite fauna of the Whitespotted rabbitfish, *Siganus canaliculatus* and shoemaker rabbitfish *Siganus sutor* (Siganidae) along the coasts of the Sultanate of Oman (Sea of Oman and The Arabian Sea) revealed two new species of dactylogyrids, *Glyphidohaptor omanii* and *Tetrancistrum spiralis* on the gills. So far, three species were previously reported within the genus *Glyphidohator* from Australia and Egypt. *Glyphidohaptor omanii* **n.sp.** is the first report of this genus from the Sultanate of Oman. The genus is characterised by the comparative morphology of the haptoral armament, the haptor and in the relative position of the gonads (Kritsky *et al*., 2007a). The new species differs from its congeners by the shape of the male copulatory organ and its larger body size. The genus *Tetrancistrum* has 16 valid species (Kritsky *et al*., 2007b) *Tetrancistrum spiralis* **n.sp.** can be differentiated from its congeners by its distinctive highly sclerotized spiral maze-like vaginal vestibule. This specie is the third member of *Tetrancistrum* known from *S. canaliculatus* and the second from *S. sutor*. In addition, *Tetrancistrum indicum* is re-described from a new host and new locality.

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Kritsky D.C., Galli P. and Tingbao Y. (2007b) Dactylogyrids (Monogenoidea) parasitizing the gills of Spinefoots (Teleostei, Siganidae): revision of Tetrancistrum Goto and Kikuchi, 1917, with descriptions of two new species from Siganus spp. Of the Red Sea and Celebes

# ICHTHYOFAUNA AND PARASITE FAUNA OF COMMON MINNOW *PHOXINUS PHOXINUS* IN THE UNGRA AND CHULMAN RIVERS IN SOUTHERN YAKUTIA, REPUBLIK SAKHA (YAKUTIA)

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The common minnow *Phoxinus phoxinus,* which has a palaeartic distribution, hosts 36 species of parasites in the Chulman and Ungra rivers in Yakutia.
The graylings, salmonids, whitefishes and cyprinid fishes form the composition of ichthyofauna in the Yakutian rivers. The predominate fishes in the Ungra river (the conservation «Ungra») are lenok, East Siberian grayling, common minnow, spotted sculpin, pike, Siberian dace, bearded stone loach, river perch. There are Yakutian crucian carp, Siberian spiny loach and burbot in this river. The rare fishes are presented by taimen, Siberian whitefish and round whitefish. In the Chulman river (technogenous zona) grayling, spotted sculpin and bearded stone loach are found, but only minnow is abundant.
Among the most diverse group of parasites of common minnow are infusoria which inhabit the Chulman and Ungra rivers (14 species). Myxosporea are represented by 8 species and monogenean – by 7 species. Specific parasites of minnows are: *Myxobolus mongolicus, Apiosoma phoxini, Epistylis phoxini, Trichodina mira, Paratrichodina phoxini, Gyrodactylus konovalovi, G. laevis, G. limneus, G. macronychus, G. magnificus, Diplostomum phoxini.* Widely distributed parasites among the cyprinid are: *Zschokkella nova, Chloromyxum carassii* (*cristatum*), *Myxobolus dogieli, M. ellipsoides, M. macrocapsularis, M. muelleri, M. musculi, Apiosoma campanulatum, A. piscicolum, Paratrichodina incisa, Cleidodiscus brachus, Raphidascaris acus, Ergasilus briani, E. sieboldi.*
The specific composition of parasite faunas of minnow in Chulman and Ungra rivers is almost identical, but some differences in the intensity of fish invasion are related with the size of the population of intermediate hosts (gastropod mollusks, oligochaetes).
The parasite faunas of common minnow in different reservoirs of Siberia and Far East of Russia is similar in general. The similarity index of Czekanovski-Serensen in parasite faunas between fish from Southern Yakutia and from Primorie region is 37,9%. The parasites which inhabit both regions are: *A. piscicolum, G. konovalovi, G. limneus, G. macronychus, C. bracus, R. acus.* Index of similarity between fish from Southern Yakutia and the basin of Severnaya Dvina is 26,9%. *M. muelleri, A. piscicolum, G. laevis, G. limneus, G. macronychus, D. phoxini* are found as in the Ungra and Chulman rivers as in the basin of Severnaya Dvina. Index of similarity between fish from Southern Yakutia and the Kolyma river is only 17,9%. The common parasites are: *M. muelleri, A. piscicolum, D. phoxini* and *R. acus.*

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# PREVALENCE OF SALMONID ALPHAVIRUS IN COMMON DAB *LIMANDA LIMANDA* WITH EMPHASIS ON VIRUS CULTURE AND SEQUENCING

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Common dab, *Limanda limanda* from Scottish and international waters were examined by quantitative real-time RT-PCR for evidence of viral RNA consistent with salmonid alphavirus (SAV). SAV prevalence in heart tissue varied between sampling sites and reached up to 17 % from fish collected near the Shetland Islands, Scotland. Phylogenetic analysis, inferred from the partial E2 gene sequence dataset, identified the virus as belonging to SAV subtypes I, II and V, however subtypes I and II were only from single fish. Positive material (subtype V) revealed Ct values ranging from 26.45 to 39.50. Sequencing of the partial E2 gene indicated a 99.5% similarity between dab-derived SAV and isolates previously recovered from farmed salmon. The qRT-PCR amplification of SAV RNA from common dab in areas remote from fish farming would suggest these fish are natural carriers of the virus rather than the result of fish farming activity and identifies a wild reservoir for this pathogen. There was no link between positive fish, length and sex of the dab, water depth or health status as recorded using International Council for the Exploration of the SEA (ICES) guidelines. For first time, this study demonstrates that a common dab-derived SAV V can be successfully cultured on CHSE-214 cell line.

# EXPERIMENTAL TRANSMISSION OF *GYRODACTYLUS MARINUS* TO JUVENILE ATLANTIC COD (*GADUS MORHUA*)

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[2]*Saint Mary's University, Halifax, Nova Scotia, Canada*
[3]*University of New Brunswick, Fredericton, New Brunswick, Canada*

Flatworms of the genus *Gyrodactylus* (Monogenea) are ectoparasites of fish. They have a direct life cycle requiring no intermediate host. Six *Gyrodactylus* species are known to infect Atlantic cod. Four of them have been identidied on cod in Icelandic waters. The species primarily found on the gill filaments is *Gyrodactylus marinus*. The aim of the present study was to transmit *Gyrodactylus* worms from gills of wild cod to juvenile cod and monitor the progress of infection. Four groups of disease free juvenile cod (1-6 g) were exposed for 30-50 minutes to different number of *Gyrodactylus* worms which were collected from the gills of wild adult cod from Icelandic waters. After exposure, the juveniles were moved to tanks and kept there for 3-5 weeks at 9˚C. At weekly intervals the gills, body and fins of a sample of juveniles were examined for the presence of *Gyrodactylus* worms. The transmission was successful. One week after exposure the prevalence of infection in the four groups was 80%, 20%, 7% and 0 respectively. Prevalence in each group remained virtually stable until the end of the trial when it decreased suddenly in the most heavily infected group from 80% to 9% and was low or zero in the other groups. All worms were found solely on the gill filaments and belonged to the species *G. marinus* except for a single *G. callariatis* worm. The number of worms on the gills per fish ranged from 1-5 during the trial, highest numbers were found on fish which had been exposed to the highest number of *Gyrodactylus* worms. No pathological changes were observed associated with the infections. Transmitted *G. marinus* apparently attach to the skin of the cod and then move to the preferred site, the gill filaments. No *Gyrodactylus* worms were found on juvenile cod in control groups. It remains unexplained why there was no increase in the number of worms or prevalence of infection during the course of the experiment. Experimental transmission of *Gyrodactylus* to cod has seemingly not been reported before.

*This study was supported by the Natural Sciences and Engineering Research Council of Canada.*

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# MYCOBACTERIAL INFECTIONS IN AQUARIUM FISH IN SWEDISH PET-SHOPS

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In a health survey of aquarium fish from 24 Swedish pet-shops, carried out 2006 to 2007, one of the most common causes of diseases in the fish was infection with acid-fast bacteria (Hongslo & Jansson, 2009). Thorough microscopic investigation of 120 aquarium fish with signs of illness identified 28 (23%) aquarium fish with probable or established infection with acid-fast bacteria. The aim of this study was to identify these bacteria to the species level by molecular genetic technique. Tissue samples from the 28 fish were homogenized with a BeadBeater (Biospec. Products), followed by DNA extraction from the homogenized tissue samples, using QIAamp DNA Mini Kit (Qiagen). In fish there no bacterial DNA was detected in homogenized tissue samples (n=10), tissue samples were also cultivated on agar and DNA was extracted from growing bacterial colonies. The DNA samples were amplified by PCR and the 16S rRNA gene was sequenced with BigDye terminator v3.1 (Applied Biosystems). Thereafter, the consensus sequence was matched against known sequences of different mycobacteria species, using the software Ribosomal Database Project (http://www.msu.edu). The results showed presence of *Mycobacterium (M.) marinum* in 14 (50%) of the 28 fish, *M. chelonae* in 2 (7%), *M. haemophilum* in 2 (7%), *M. fortuitum* in 1 (4%) and *M. sp.* in 1 (4%). In 3 (11%) of the 28 fish, a combination of mycobacteria species were present, whereas in 5 (18%) no mycobacteria could be found. In summary, 20 (17%) of 120 aquarium fish in Swedish pet-shops with signs of illness showed presence of *M. marinum, M. chelonae, M. fortuitum* or *M. haemophilum,* which all are mycobacteria species known not only to be fish pathogenic agents, but also to be pathogenic to humans, usually causing local skin infections on the hands and upper extremities, but in immunocompromised individuals even systemic infections.

# NEW MONOGENEAN IN THE PARASITE FAUNA OF SANTER BREAM *CHEMIERIUS NUFAR* VALENCIENNES, 1830 FROM OMAN WATERS



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*Fishery Quality Control Center of Ministry of Agriculture & Fisheries Wealth of Sultanate of Oman*

Starting from November 2012 a profound investigation on parasite of *C. nufar* was commenced. Samples were collected along the Southeastern coasts of the Arabian Sea, Sultanate of Oman. Different parasites were found to infect the gills of *C. nufar*, among them a Monogenean parasite that has the characteristic features of the genus *Lamellodiscus* Johnston et Tiegs, 1922 (fam. Diplectanidae) was frequently recovered. The analysis of the structure of the haptor in particular, lamellodiscs and MCO states that this species belongs to "ignoratus" group of *Lamellodiscus* (Diamanka et al., 2011). It is known that only 6 *Lamellodiscus spp.* are representatives of this group, which has specificity to fishes of the family Sparidae. Based on the morphological information obtained in this study we assume that this parasite could be the seventh species in group "ignoratus". In the present a detailed description of this new species will be given based on deferential morphological and morphometric analysis with previously reported *Lamellodiscus*. Biological investigation on the infection rate of this parasite showed that the infection intensities were often high and the infection prevalence reached 100% throughout the duration of the study. Infection intensity appeared to be affected by seasonal changes and varied from 5 up to 97 parasite/fish. Studying of the biology and distribution of this monogenean has the an important practical value, as it is known that some monogenea can cause epizootics in open waters cage culture such as the infection of meager *Argyrosomus regius* (Sciaenidae) in floating cages located in north-eastern Sardinia (western Mediterranean Sea) due to the infection by the monogenean *Sciaenacotyle panceri* (Microcotylidae) (Merella et al., 2009). The data obtained in this study can help in the development of preventive measures against this parasite in future aquaculture industry in the country.

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# DETECTION OF SALMON ALPHAVIRUS RNA IN CELTIC AND IRISH SEA GROUNDFISH

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Pancreas disease (PD) caused by the salmonid alphavirus (SAV) is an established and persistent cause of disease in farmed Atlantic salmon (*Salmo salar* L.) and has been the most significant cause of mortalities in Irish farmed salmon over the past decade. SAV is a single strand sense RNA virus, originally thought to be unique to salmonid fish, but has recently been detected using qRT-PCR in a number of wild non salmonid fish. In the present report, wild groundfish consisting of dab (*Limanda limanda*), plaice (*Pleuronectes platessa*) and megrim (*Lepidorhombus whiffiagonis*) were caught from the Irish and Celtic Seas and screened for SAV using qRT-PCR and sequencing. SAV positive samples were detected in both the Irish and Celtic Seas. Generally a very low prevalence was recorded in dab and plaice and all megrim tested negative. However, one haul in Dublin Bay had a high prevalence where 42% of dab were SAV positive. Sequence analysis showed that all positive samples formed a single grouping consistent with SAV subtype I, the predominant subtype found in farmed salmon in Ireland. The significance of these findings in relation to farmed salmon will be discussed.

# A CASE OF A FUNGAL GRANULOMA IN THE MOUTH OF A LACED MORAY *GYMNOTHORAX FAVAGINEUS* REARED IN A PUBLIC AQUARIUM: DIAGNOSIS, CHIRURGICAL EXCISION AND POST-TREATMENT.

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In 2011, a specimen of Laced moray *Gymnothorax favagineus* in exhibition in one the tropical aquaria of the L'Aquàrium of Barcelona progressively developed an external tumour-like growth on the dorsal side of the mouth. The fish was transferred into an isolation tank in the quarantine area. Although a preliminary tentative diagnosis of cutaneous tumour associated to retrovirus was done, due to a very fast growth and expansion of this lesion, a biopsy using slight sedation and immobilization of the fish was taken in order to evaluate the nature of the lesion and the potential treatment approach. The histopathological study revealed the presence of chronic granulomatous inflammatory reaction as the main pathological finding but no specific pathogens could be detected at this moment.
In June 2012, as the moray eel stopped feeding and displayed discomfort and pain associated to the lesion, a surgical excision of the lesion was recommended. Anesthesia/analgesia was performed using MS-222 and also an intramuscular dose of butorphanol. The body of the fish was hold into the anaesthesia tank with aeration and only the head was maintained outside the tank as the lesion was located in the mouth. The lesion was progressively excised using a bipolar electrocautery until all of the granulomatous tissue was removed from the area. The excised tissues were immediately fixed in 10% formalin for further studies. Bleeding was not excessive as vascularization of the lesion was not high.
The cavity was cleaned with gauzes with diluted povidone-iodine solution. Wound closure was performed with synthetic absorbable material. After surgery, the specimen received two extra butorphanol doses to avoid postsurgical pain and also ceftazidime (Fortam ®) IM to cover potential secondary bacterial infections. Recovery was excellent. Fish started feeding again after few days and skin re-epitelialization was faster than expected. In less than one month, the specimen was re-introduced again in its original tank, showing a good adaptation and displaying a normal behaviour.
Histopathological studies of the excised tissues clearly indicate that all the affected tissues correspond to granulomatous inflammatory lesions where in some areas, fungal hyphae were clearly evident with PAS and Grocott´s stains. Unfortunately, no mycological culture was performed.

# PATHOGENS IN ANADROMOUS ARCTIC CHARR (*SALVELINUS ALPINUS*) AND SEA TROUT (*SALMO TRUTTA*) IN NORTH NORWEGIAN FJORDS

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Arctic charr (*Salvelinus alpinus*) has a circumpolar distribution and is an important and unique resource in Northern Norway and Svalbard. The population of this fish species has decreased dramatically over the last decade. The cause(s) are unknown although there are indications that climate change and human activity are affecting wild ecosystems. Pathogens might be another cause of the decline and the present study therefore wanted to gain knowledge about the health situation of wild anadromous fish in Northern Norway with focus on Arctic charr. Twenty-six anadromous Arctic charr and fourteen Sea trout (*Salmo trutta*) were sampled at three different fjords in Northern-Norway during summer 2012. Autopsy was performed in addition to histology of gills, pseudobranch, heart, skin, muscle, liver, spleen, gastrointestinal tract, pancreas and kidney. Screening using real time PCR was done to detect the following pathogens known to cause disease in farmed salmonids in Northern Norway: Infection salmon anemia-virus (ISAV), Pancreas disease-virus (SAV), Infectious pancreas necrosis-virus (IPNV), Piscine reovirus (PRV), Piscine myocarditis-virus (PMCV), *Parvicapsula pseudobranchicola* (Myxozoa) and *Desmozoon lepeophtherii* (Microsporidia). Standard parasitological examination of the gastrointestinal tract was performed and all parasites were identified using morphological and molecular methods. Results from the screening will be presented.

*This study was part of the project "Anadromous Arctic charr (Salvelinus alpinus) in Northern Norway-migration, habitat, use and effects of climate changes" financed by Fram centre, Tromsø.*

# MORPHOLOGYICAL AND MOLECULAR CHARACTERIZATION OF *HENNEGUYA TUNISIENSIS* (MYXOSPOREA, BIVALVULIDA) INFECTING THE GILLS OF *SYMPHODUS TINCA* (L.) (TELEOSTEI: LABRIDAE) OFF TUNISIA

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The East Atlantic Peacock wrasse, *Symphodus* (*Crenilabrus*) *tinca* is the most common fish in the family Labridae in Tunisia. The spawning period of this species is from April to June. In Kerkennah Islands (34°37'57''N, 11°02'44''E), *Symphodus tinca* is mainly caught in spring by nets or fixed fisheries called "Charfia or Chrafi" and is very appreciated by the consumers.
During a parasitological survey of *S. tinca* caught in Kerkennah, big white plasmodia were observed in the gill arches. On dissection, they were found to be plasmodia of a myxosporean species belonging to the genus *Henneguya*.
The proposed name for this new parasite is *Henneguya tunisiensis.* It is characterized by the presence of elongated white plasmodia of 1–1.5×1.5–2 mm. Development was asynchronous. Plasmodia in advanced stages contained mature and immature spores. The mature spores have a total length of 41.8 ± 3.6 (38.0-50.0) µm; the length of spore body 13.1 ± 0.5 (13.0-14.0) µm, width 9.1 ± 0.2 (9.0-10.0) µm and thickness 8 ± 0.1 (7.4-8.6) µm. The two polar capsules were pyriform and equal in size, 4 ± 0.2 (3.5-4.0) µm long and 2 ± 0.1 (1.8-2.0) µm wide. Polar filaments coiled with 4-5 turns, situated perpendicularly to longitudinal axis of polar capsule. The caudal processes were 28.4 (25.0-32.0) µm in length; they fold up at the final portion.
Both light and electron microscopy data showed that this species differs in several morphological features from all previously described *Henneguya* spp. The molecular analysis indicates that *Henneguya tunisiensis* is well distinguishable from other myxozoan DNA sequences in GenBank. Phylogenetically, the new species is placed in the marine *Henneguya* clade, which is a sister group of marine *Myxobolus* spp. from perciform fish in Tunisia. In addition, *Henneguya tunisiensis* was genetically the most similar to *H. pagri*, *H. akule* and *H. lateolabracis*, which parasitize the bulbus arterious of marine fishes.

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# TAXONOMY AND ULTRASTRUCTURAL DESCRIPTION OF *CERATOMYXA AEGYPTIACA* (MYXOZOA: BIVALVULIDA) PARASITE OF *SOLEA AEGYPTIACA* (PLEURONECTIFORMES) FROM TUNISIAN LAGOON

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Recently, a new myxosporean species, *Ceratomyxa aegyptiaca* is found in the gallbladder of *Solea aegyptiaca* collected from the Ghar El Melh lagoon in northeastern Tunisia. According to the previously described *Ceratomyxa* species worldwide, no *Ceratomyxa* species has been reported from soleid fish up to date. Mature spores are elongate and crescent shaped, measuring 8–11 µm in length and 48–58 µm in width. The polar capsules are spherical, measuring 3.2–4 µm in diameter and equal in size. Trophozoites are polysporous, almost spherical in shape with 40–48 µm in diameter.
A total of 1,690 bp long, 18S rDNA sequence was generated from *Ceratomyxa aegyptiaca.* Morphology as well as molecular analysis based on 18S rDNA sequence indicates that the examined species differs from all previously *Ceratomyxa* sequences available in GenBank.
The infection with *Ceratomyxa aegyptiaca* was detected only in spring and summer, with a maximum prevalence occurring in summer (10%). An influence of the host size on the infection prevalence was observed. Generally, infection shows a tendency for decreasing prevalence with increasing size of the fish. Ultrastructural observations revealed the presence of young trophozoites with various shape and size, free in the lumen or attached to the epithelium of the gall-bladder. In mature spores, polar capsules were formed within the capsulogenic cells. The polar filament coils 6 times within the polar capsule. Polar capsules with an electron-dense matrix were surrounded by a fibrous coat. The apical channel for the discharge of the polar filament exhibited a close contact with the valves. The sporoplasm in the posterior end of the spore occupying most of spore volume contained 2 nuclei close to each other. Valvogenic cells were joined in suture line and occupied an external position relative to other sporogenic cells. Concerning the pathology effects of this new species, ultrathin sections of the infected tissues demonstrated different degrees of epithelium alterations. Usually, necrotic cells with mitochondrial degeneration were observed. In advanced stages of the infection, epithelial cells were completely degenerated and no cellular structure could be distinguished.

# EFFECTS OF TEMPERATURE ON MYXOZOAN DISEASE: HOW A CHANGING CLIMATE MAY ALTER *CERATOMYXA SHASTA* DYNAMICS

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Diseases of fish are often more severe at elevated water temperatures, thus climate warming threatens the performance of species like salmon that already contend with habitat degradation and impaired water quality. Warming trends of stream temperatures can cause salmon parasites to result in faster development rates, earlier reproduction, and more generations per year. Here, we developed a biological model of the *Ceratomyxa shasta* life cycle, identifying the thermal requirements (i.e., degree-days) for development rates in Chinook (*Oncorhynchus tshawytscha*) and coho (*O. kisutch*) salmon and the invertebrate host (*Manayunkia speciosa*).

In the salmon hosts, we saw differential effects of temperature. For both Chinook and coho salmon, elevated water temperatures consistently resulted in higher and more rapid mortality and thus more rapid parasite maturation. However, accumulation of degree-days to death was consistent among a range of rearing temperatures (13-21°C). Coho salmon appear more sensitive to increased temperatures, but the thermal constant for parasite development was 324 DD, compared with 283 DD in Chinook salmon. We used observations of seasonal *C. shasta* patterns to corroborate the development time in the polychaete (652 DD), and then applied the model to projected Klamath River temperatures to forecast the potential change in annual *C. shasta* generations and seasonal onset of *C. shasta* abundance in response to climate change. Additionally, we examined the effect of temperature on the myxospore stage to determine how longevity of the different free-living stages could affect transmission. Myxospores were robust to temperature effects, surviving 49-175 days at temperatures 7-23°C, compared to actinospores which typically survive less than one weed. Our degree-day model estimates an increase in the number of annual *C. shasta* life cycles from 3.00 to 4.29 over the next 50 years in addition to an advance of seasonal parasite onset of 7.5-8.4 days/decade.

As models for predicting future stream temperatures are refined, disease models may be useful for predicting changes in parasite development and phenology. The complicated interactions between disease severity and temperature suggest that management of rivers to decrease disease effects will need to consider a variety of approaches, including river restoration to protect habitats that provide thermal refuge.

# MORPHOLOGICAL, HISTOPATHOLOGICAL AND ULTRASTRUCTURAL ANALYSIS OF *MYXOBOLUS* SP. INFECTING *BRYCON HILARII* FROM A FISH FARM IN SÃO PAULO STATE, BRAZIL

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*Brycon hilarii* is a characiform of the Bryconidae family, popularly known as *piraputanga*. It is endemic of the Paraguay River basin, and can reach approximately 50cm in length and weigh up to 3.4kg. It is widely found in fish farms in Brazil. In the natural environment and in fish farms fish are hosts to various parasite organisms, such as the myxosporeans, which are highly specialized endoparasites, characterized by multicellular spores and polar capsules. In the present study, are presented morphologic, histopathologic and ultrastructural data of an unknown *Myxobolus* species found in kidney of *piraputanga* from a fish farm in São Paulo state, Brazil. Of the 13 specimens examined, 100% had rounded and white plasmodia in the kidney, which contained round spores measuring 11.7 ± 0.8 µm in length, 10.7 ± 0.9 µm in width and 6.9 ± 0.3 µm in thickness. The polar capsules were elongated and of equal size, measuring 6.1 ± 0.4 µm in length and 4.0 ± 0.2 µm in width. Previously only *Myxobolus oliverai* (Milanin et al., 2010) and *Myxobolus brycon* (Azevedo et al., 2011) has been found to infect *B. hilarii*, taken from the Pantanal Wetland area in Brazil. These are the only myxosporeans species found in fish of the *Brycon* genus. These two *Myxobolus* species were found infecting the gills, while the species analyzed herein occurred in the kidney, and differed in morphology and in size from the two species previously reported. Histological analysis revealed the presence of several small plasmodia developing between the tubule cells, causing destruction of the affected cells and compression and deformation of the tubule structures. No inflammatory reaction was observed at the site of infection. Ultrastructural analysis allowed a single wall membrane in direct contact with the host tissue, and numerous mitochondria in plasmodial ectoplasm, to be seen. Plasmodial development was asynchronous, with generative cells and spores in the young developmental stages in the periphery, and mature spores in the central portion of the plasmodium. In immature spores it was possible to visualize structures such as polar capsule, polar filament with 5-7 turns, binucleate esporoplasm and esporoplasmosomes.

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# CELLULAR IMMUNE RESPONSE OF GILTHEAD SEA BREAM (*SPARUS AURATA* L.)TO *ENTEROMYXUM LEEI* (MYXOZOA)

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Enteromyxosis in gilthead sea bream (GSB) (*Sparus aurata*) consists of severe chronic catharral enteritis causing a cachectic syndrome and death. The ethiologic agent is the intestinal parasite *Enteromyxum leei*, which penetrates and proliferates in the paracellular space between enterocytes. The parasite disrupts the epithelial organization and provokes epithelial desquamation triggering an intense inflammatory response locally. By means of light microscopy and immunohistochemistry, the distribution pattern of some leukocytic populations was studied at intestinal and haematopoietic levels in GSB anally intubated with *E. leei* (R, recipient).

Tissue sections of Bouin fixed and paraffin embedded portions of anterior intestine (Ai), posterior intestine (Pi), head kidney (Hk), spleen (Sp) and thymus (Th) were taken at 15 and 40 days post inoculum (d.p.i.). For immunohistochemistry, the G7 monoclonal antibody (Mab) against GSB acidophilic granulocytes (AGs), a polyclonal antibody (Pab) against histamine, which is stored in mast cell (MC) granules, and a Pab against GSB IgM labeling plasma cells and B cells (PCs/BCs) were applied. In splenic sections, melanomacrophage centres (MMCs) and their sizes were quantified.

PCs/BCs and MCs increased significantly, whereas AGs decreased in the inflammatory infiltrates of parasitized (PAR) intestinal sections, compared to intestines of non-parasitized (NON-PAR) and unexposed (CTRL) fish. These differences were stronger at the Pi section, the main target of the parasite, and at 40 d.p.i. In Hk and Sp of R fish at 40 d.p.i., PCs/BCs and MCs also increased, whereas AGs decreased. No differences were found in the Th. In NON-PAR GSB (*vs*. PAR and CTRL groups), the number of MMCs and the percentage of splenic surface occupied by MMCs increased, though only significantly for the latter.

To conclude, during enteromyxosis in GSB, PCs/BCs, MCs and MMCs seem to proliferate in haematopoietic tissues and PCs/BCs and MCs are recruited to the site of infection, while AGs show an overall depletion.

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# ANALYSIS OF MITOCHONDRIAL GENOMIC DATA REVEALS THE PHYLOGENETIC PLACEMENT OF MYXOZOA AND *POLYPODIUM HYDRIFORME* WITHIN THE MEDUSOZOAN CNIDARIANS

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The phylogenetic position of the parasitic Myxozoa and *Polypodium hydriforme* within the Cnidaria is still not entirely clear. The SSU rDNA sequences of both Myxozoa and *P. hydriforme* are fast evolving and give artificial results in phylogenetic analyses: Myxozoa are placed as close relatives to bilaterians and *P. hydriforme* as a sister taxon to the Myxozoa. Recent analyses of protein coding genes have suggested Myxozoa as a sister lineage to the Medusozoa (Cnidaria), however, these analyses suffer of poor taxon sampling of cnidarians and rely solely on the phylogenetic analysis of the DNA and/or AA data.
The analyses of mitochondrial genomes have become an effective tool in resolving the phylogenetic placements of taxa within metazoans. The medusozoan genome is unique within all Metazoa by having a linear character, instead of typical circular one, that can be fragmented to two (some Hydrozoa) or several (Cubozoa) „chromosomes“. Gene order and presence or absence of polB ORF and/or cox1 duplications are other specific features of particular medusozoan lineages.
We obtained the complete mitochondrial genome of *P. hydriforme* and a partial genome of the Myxozoa. The phylogenetic analysis of mitochondrial sequence data revealed that myxozoans are closely related to box jellyfish (Cubozoa) whereas *P. hydriforme* clusters within the Hydrozoa, with a group of parasitic cnidarians, the Narcomedusae. The mitochondrial genome of *P. hydriforme* contains an ORF homologous to the polB gene at the beginning of the molecule as well as a cox1 gene duplication. This character fits into the evolutionary scheme of Cnidarians, which gradually loose its ancestral ORF sequences replacing them with a cox1 pseudogene. The revealed specific order of mitochondrial genes: polB - rnl - cox1 pseudogene at the opposite end of the molecule proved that *P. hydriforme* is closely related to freshwater jellyfish (Limnomedusae), sister group of the Narcomedusae, for which mitochondrial genomes are not available. Five mitochondrial genes, i. e. cox1, nadh1, nadh4, rns and rnl, were sequenced in the Myxozoa. These five genes are likely to be mapped on the three short linear mitochondrial molecules, as was recently detected in the Cubozoa, which linear genome is fragmented into eight chromosomes. Therefore, the partial mitochondrial genome data of Myxozoa suggest a close relationship with box jellyfish.

# ASSESSING THE RISK OF AN EMERGING SALMONID DISEASE

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Proliferative kidney disease (PKD) is an emerging disease that is linked with environmental change (rising temperatures, eutrophication) and causes mortality of both wild and farmed salmonids across Europe and North America.
The causative agent of PKD is the myxozoan parasite *Tetracapsuloides bryosalmonae* (*Tb*) which exploits the freshwater bryozoan *Fredericella sultana* (*Fs*) as primary hosts. *Fs* is a colonial invertebrate that reproduces asexually through budding and by the production of "statoblasts" (over-wintering dormant stages). In bryozoans the parasite cycles between overt and covert infection stages, the former being constituted by sacs filled with spores infective to fish and the latter by single cells infecting the bryozoan body wall. This project focuses on the development of *Tb* in bryozoans to assess the risk of PKD. The objectives of this research programme are to: 1) characterise bryozoan population dynamics and the dynamics of *Tb* development within this host; 2) establish risk factors associated with *Tb* prevalence and burden in bryozoans and subsequent transmission to fish hosts; 3) create a protocol to sample bryozoans as a surrogate for sampling of wild fish in order to assess and monitor parasite levels and to produce a PKD risk map. Results presented here will include: levels of vertical transmission in bryozoan hosts (through fragmentation of bryozoan colonies and via statoblasts); presence of infectious spores in water samples; and the prevalences of overt/covert/uninfected bryozoans in populations sampled every 45 days from two rivers in southern England. A major aim of the research is to reduce direct sampling of valuable fish stocks for disease detection and to help conserve the health and diversity of wild fish populations.

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# GENETIC DIVERSITY OF *MYXOBOLUS PSEUDODISPAR* (MYXOZOA) ISOLATES: AN EXAMPLE OF CRYPTIC SPECIES COMPLEX?

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*Myxobolus pseudodispar* Gorbunova, 1936 is a common parasite of cyprinids that is easy to obtain from natural habitats all around Europe. Its host range is wide, myxospores develop intracellularly in the skeletal muscle of roach (*Rutilus rutilus*), white bream (*Abramis bjoerkna*), common bream (*Abramis brama*), rudd (*Scardinius erythrophthalmus*) and bleak (*Alburnus alburnus*). Previous studies have already indicated that the SSU rDNA of *M. pseudodispar* isolates from different fish host species may differ up to 5% from each other. Since intraspecific divergence in most myxozoan species rarely exceeds 1%, the high genetic variability among *M. pseudodispar* isolates raises the question whether it is a cryptic species complex. Considering the biological species concept and the present understanding that myxozoans are strictly host specific parasites, the barrier between separate species and a genetic lineage can be established by cross-infection experiments. To that end, *in vivo* exposures were performed to examine whether different *M. pseudodispar* lineages were able to infect closely related cyprinid fishes and develop mature spores in them. The laboratory cultures of *M. pseudodispar* have been maintained in our laboratory for several years, and they supplied sufficient amount of fish-infecting triactinomyxons for the trials. SPF roach, rudd and common bream were exposed individually and dissected 3 months p.i. Infection intensity was detected by counting myxospores.
As significant differences were detected in the myxospore numbers among fish species, our findings suggest that *M. pseudodispar* has already reached the level of speciation where the genetic lineages are not able to recombine anymore. Although the morphological appearance and tissue preference of the lineages are identical, their genetic variability (even in the conserved SSU rDNA) and their segregated host range suggest that *M. pseudodispar* is a cryptic species complex and its taxonomic revision should be considered.

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# LONG-TERM MONITORING OF A MYXOZOAN PARASITE, *CERATOMYXA SHASTA,* BY QUANTIFICATION OF WATERBORNE STAGES

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*Ceratomyxa shasta* causes enteronecrosis in juvenile salmon and trout in the Pacific Northwest of North America and is limiting their recovery in the Klamath River. It is a freshwater parasite that has two waterborne stages: actinospores released from freshwater polychaete worms develop into myxospores in salmonid fishes. In response to the high prevalence and severity of *C. shasta* infection in Klamath River salmonids, we developed a parasite monitoring program that included river water sampling.

In 2006, we established 5 index sites in the mainstem, which spanned 212 river kilometres, and 4 sites in tributaries. Weekly, automatic samplers collected and pooled 1L of river water every 2h for 24h. Replicate 1L samples from the pool were filtered to concentrate waterborne material, from which DNA was extracted. Total *C. shasta* was quantified using a TaqMan qPCR that targeted the SSU rRNA gene. Parasite genotypes were determined using a SYTO9 qPCR and Sanger sequencing. We assayed >5000 samples collected over 7 years. We genotyped a subset of 278 samples, which comprised weekly samples for one index site from 2006-2011, and all samples available for the other 4 index sites in 2 years of high total parasite density (2007 and 2009).

The river water samples yielded spatial and temporal data of parasite density and genetic diversity across high-impact and low-impact years. The parasite was detected at all mainstem sites, but levels differed among sites. Tributaries did not contribute significant numbers of parasites to the mainstem. Typically, parasite density increased early spring (when salmonids are out-migrating) and peaked in late spring/early summer. Levels then decreased, but increased again to a lower second peak in late summer/early autumn. Not all 4 genotypes were detected at all sites in all years, but genotype I was dominant in all years. We are now exploring relationships among parasite occurrence, invertebrate and vertebrate host life histories, and water temperature and flow. These data influence management practices and inform epidemiological models and risk assessments.

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# THE DIMENSIONS OF THE SPORES OF SOME MARINE MYXOSPOREA DESCRIBED FROM NORWEGIAN MARINE FISHES BY M. AUERBACH 1909-1910

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During several visits to Norway 1908-1909 the German zoologist M. Auerbach collected material used to describe 8 new species of Myxosporea from marine fishes. We collected new material of all these from their type hosts in the type locality (Bergen) and from other areas along western and northern Norway. Careful measurements from fresh spores in the bile show that the dimensions of the spores are smaller than those reported by Auerbach. The largest material at hand is from three species, *Myxidium bergense, Ceratomyxa informis* and *Ceratomyxa macrospora*. The range of major dimensions such as spore length (*Myxidium*) or thickness (*Ceratomyxa*) given by Auerbach does not include the means of the present measurements. There is an apparent systematic difference in all spore dimensions, my measurements being ca. 89% of Auerbach's. Similar differences have been noted also in *Myxidium inflatum, Myxidium procerum, Ceratomyxa longipes, Zschokkella hildae* and *Sphaeromyxa hellandi*.
Auerbach measured fresh spores, as is the recommended method also at present. There is no known type material. The cause of these differences in dimensions cannot be known, a possibility is erroneous microscope calibration. Myxosporean spore dimensions reported from Auerbach's 1911 survey in Norway do not show this systematic deviation.
The consequences of reporting erroneous dimensions are large. Other researchers encountering Auerbach's myxosporeans in their typical hosts have identified them with other, smaller species, or described them as new. On the other hand species with larger spores from other hosts, worldwide, has in some cases been identified with the species described by Auerbach. Adding to this confusion is the deposition in GenBank of sequences of Auerbach's species under different names, and deposition of sequences of different species with Auerbach's names.
The evidence suggesting these dimension-differences will be presented, with examples of the consequences in myxosporean taxonomy. A redescription and revision of the 8 myxosporeans described by Auerbach (1909-10) from Norway is urgently needed in order to avoid further confusion. With the exception of *M. inflatum*, all species have been sequenced and morphological characterization is ongoing.

# *PRISTINA AMERICANA* (OLIGOCHAETA: NAIDIDAE) INVERTEBRATE HOST OF MYXOZOA IN FISH FARMS IN THE SOUTHEAST OF BRAZIL

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Parasites of the phylum Myxozoa are important pathogens of fish.
The actinosporo phase of these parasites have been intensively studied since 1984, when Wolf and Markiw demonstrated that *Myxobolus cerebralis* occurs in an actinosporea phase in the oligochaete *Tubifex tubifex.* This paper presents preliminary data of the life cycle of myxosporean parasites found in fish farms in Brazil. Sediment samples containing oligochaetes were collected from a fish farm in the municipal district of Porto Ferreira, Brazil, and taken to the laboratory for the study of oligochaetes and actinospores. The oligochaetes found were identified as *Pristina Americana* through morphological study. A total of 144 oligochaetes were examined, of which five (3.5%) were positive for actinosporos. Two distinct morphotypes were observed, both of which belonged to the Aurantiactinomyxon type. Morphotype 1 infected 1 (0.7%) specimen of *P. Americana* and morphotype 2 was found in 4 (2.8%) specimens.
The morphological difference between the two morphotypes is related to the diameter of the body of the spores and the size of the caudal processes.
The diameter of morphotype 1 was 10.8µm (11.05 to 11.51) and the caudal processes were 18.58µm (18.62 to 20.01) long and 9.0µm (9:33 to 9:37) wide. Distance between the caudal processes was 39.08µm (39.08 to 39.75).
The diameter of morphotype 2 was 7.10µm (7.38 to 8.41) and the caudal processes were 16.23µm (15.03 to 16.14) long and 5.10µm (4.73 to 5.52) wide, while the distance between the caudal processes was 30.25µm (28.23 to 30.91).
Molecular analysis of the 18S rDNA gene confirmed that these two actinospores are from two different species of Myxozoa. The genetic distance between the two morphotypes was 24%. Blast analysis revealed that the sequences did not match any of those myxoxoporean sequences deposited in GenBank.

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# MORPHOLOGY, ULTRASTRUCTURE AND MOLECULAR DATA OF *HENNEGUYA* SP. INFECTING *SALMINUS BRASILIENSIS* FROM MOGI-GUAÇU RIVER, SÃO PAULO STATE, BRAZIL

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The characin dourado (*Salminus brasiliensis* Cuvier, 1816) occurs throughout the La Plata river basin (Brazil, Uruguay, Paraguay and Bolivia). It is an important species in the fishing economy of the region, valued for its meat and by sports fisherman. It can reach over 100 cm in length and weigh 31.4 kg. In recent decades, it has been successfully introduced into fish farms. Until now no evidence has been found of the *Henneguya* species infecting *S. brasiliensis*. From April 2011 to August 2012, seventeen *S. brasiliensis* specimens were examined to evaluate the presence of myxosporean parasites. The fish were taken from the Mogi-Guaçu river, near the Cachoeira de Emas waterfall, in the municipal district of Pirassununga, state of São Paulo, Brazil. Morphological, ultrastructural and molecular analysis showed the occurrence of unknown *Henneguya* sp. infecting the fins and the surface of the gill arch of *S. brasiliensis.* Elongated plasmodia were observed in the fins and spherical plasmodia were found in the gill arch. Prevalence was 11.7% in fins and 5.9% in the gill arch. Morphological analysis showed that the parasite had the same morphological characteristics in both infection sites. The dimensions were 23.6 ± 1.1 µm in total length, 5.6 ± 0.2 µm in spore body length, 16.4 ± 1.2 µm of caudal process length and 3.7 ± 0.1 µm in width. The polar capsule was of equal size, with a length of 3.4 ± 0.2 µm and width of 1.8 ± 0.1. Ultrastructural analysis revealed that the plasmodia had asynchronous development, being the initial phase of sporogenesis in the periphery and mature spores found in the central region of the plasmodia. The ectoplasm of the plasmodia showed numerous pinocytosis channels, and there were conspicuous vesicular structures containing amorphous material in the innermost layer, which is believed to be debris of esporogonic cells that did not develop. Analysis of 18S rDNA gene sequencing revealed that samples obtained from the fins and gill arch were 100% similar. Phylogenetic analysis using maximum parsimony and maximum likelihood methods showed *Henneguya* sp. clustering with other myxosporean species parasites of characiform fish.

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# MOLECULAR AND ULTRASTRUCTURAL ANALYSIS OF TWO *MYXOBOLUS* SPECIES INFECTING *SALMINUS FRANCISCANUS* FROM THE SÃO FRANCISCO RIVER, BRAZIL

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*Salminus franciscanus* is a characiform of the Bryconidae family, popularly known as *dourado* in Portuguese, *dorado* in Spanish and Jaw characin in English. It is endemic to the San Francisco river basin in the northeast of Brazil and can reach over 1 m in length. It is an important species in the fishing economy of the region, valued for its meat and by sports fisherman. The aim of the present study was to analyze the myxosporean parasites of fish of the San Francisco River. A total of 51 specimens of *dourado* were collected. Parasitological analysis showed that the *dourado* were infected by two unknown *Myxobolus* species. Morphological and 18S rDNA sequencing data showed that these two species were distinct both from each other and from other previously identified *Myxobolus* species. *Myxobolus* sp. 1 was observed in the form of white plasmodia in the liver of 41.2% of the specimens examined. Its mature spores were oval in the frontal view and biconvex in the lateral view (7.6±0.4 µm in length, 4.8±0.5 µm in width). *Myxobolus* sp. 2 occurred in the fins of 29.4% of the specimens examined. White plasmodia harbored mature spores which were round in the frontal view and biconvex in the lateral view (10.7±0.4 µm in length, 8.1±0.5 µm in width, 5.4±0.1 µm in in thickness). Using 18S rDNA sequencing, the distance between *Myxobolus* sp. 1 and *Myxobolus* sp. 2 was found to be 14.4%. The closest myxosporean parasite species of South American freshwater fish to these species was *Henneguya eirasi*, a parasite of the pimelodid *Pseudoplatystoma corruscans*, with a distance of 18% to *Myxobolus* sp. 1 and 18.9% to *Myxobolus* sp. 2. Ultrastructural analysis of both species revealed an asynchronous sporogenesis process, with germinative cells and young developmental stage spores in the periphery of the plasmodium. The walls of the plasmodia of both *Myxobolus* species were formed by a single membrane. In *Myxobolus* sp. 1 this was in direct contact with the host tissue, but in *Myxobolus* sp. 2 a layer of fibroblasts was observed surrounding the plasmodium, which emits projections that reach the membrane of the plasmodium.

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# *THELOHANELLUS NIKOLSKII* INFECTION OF THE SCALES AND SKIN IN COMMON CARP (*CYPRINUS CARPIO*)

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*Thelohanellus nikolskii* Achmerov, 1955. manifests itself in two forms. The first form occurs on fins in one-year-old carp fingerlings and the second form occurs on scales and skin in two-year-old, three-year-old and older categories of common carp (*Cyprinus carpio*). This investigations were conducted between 2008 and 2012 and covered 22 fish ponds, 18 of which were located in Serbia, and 4 were located in Bosnia and Herzegovina. By monitoring epizootic distribution, it has been concluded that both forms of disease were present in all researched fish ponds. Thelohanellosis prevalence in the scale and skin ranged between 2% and 75%, and the infection intensity between 2 and 206 cysts per individual, while the same values for fin thelohanellosis were 3-30% and 2-84 cysts per individual respectively. Changes on scales and skin were present during April and May, and changes on fins during July and August. After determination of morphological characteristics of spores from plasmodia located on fins and scales, and after measuring them, no significant differences were noticed between them, indicating them as the same species. BLAST search of amplified sequence (which originated from spores taken from scales of two-year-old carp), against GenBank database revealed 95.2% similarity to the 18 SSU rDNA gene of *Thelohanellus nikolskii*. This method confirmed that the etiological cause of *Thelohanellus nikolskii* Achmerov, 1955. is the same for both, fin disease, and scales as well as skin disease. Spreading of thelohanellosis is made possible by movement of fry between fish ponds where this disease was not taken into account. The second important factor is hydrographical connection between fish ponds, where water circulation enables transmission of the causative agent. Given the fact that the distance between fish ponds is small, fish eating birds are also an important factor in disease spreading. Since there is no adequate therapeutic measure, the control of thelohanellosis is still based on compliance with basic sanitary-prophylactic measures such as drying of ponds, freezing, mechanical cleaning and disinfection with lime.

# NEW DATA OF PARASITIC FISH MYXOZOA (MYXOBOLIDAE) OF MALAYSIAN BIOTOPES

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There are only few data on the occurrence of parasitic myxozoans on Malaysian fishes. Research up to this time has been concentrated parasites of freshwater fishes (Molnár et al. 2006a,b; Székely et al. 2009a,b). Most recently a species, *Myxobolus tambroides* was reported from the gills of an appreciated cyprinid, *Tor tambroides* collected from the Lake Kenyir Water-reservoir (Székely et al., 2012). In this study, we report on collection of new myxosporean species between 2010 and 2013. Three of them infected freshwater fishes, one however was collected from an estuarine fish host species. Besides morphological characterization of the species found, we made a molecular analysis on their 18S rDNA.
*Myxobolus* sp. I. (15% prevalence) was found in the muscle tissue of a *Labiobarbus* sp. (Cyprinidae). The spores showed up an ellipsoidal to elongate ellipsoidal shape in frontal view, measured 12.2 $\pm$ 0.85 (10.91-13.64 μm) in length and 6.7 $\pm$ 0.96 (5.45-8.18 μm) in width. *Myxobolus* sp. II (10% prevalence) was found in *Ophiocara porocephala* (Gobiidae) collected from Merang Estuarine, Kuala Terengganu. The spores of this *Myxobolus* sp. were roundish or circular shape, 10.3 $\pm$ 0.43 (9.3-10.6 μm) in length and 8.6 $\pm$ 0.38 (7.98-9.26 μm) in width in valvular view, and biconvex in sutural view. *Myxidium* sp. I (66.7% prevalence) was found in the gall bladder of *Notopterus notopterus* (Notopteridae). The spores of this species showed ellipsoidal to elongate ovoid shape, 14.7 $\pm$ 0.6 (13.8-16.03 μm) in length and 6.34 $\pm$ 0.59 (5.45-7.73 μm) in width in frontal view. *Myxidium* sp. II (15.4% prevalence) was found in the gall bladder of *Tor tambroides* (Cyprinidae). Spores of *Myxidium* sp. II had an oblong to elongate ovoid shape, and measured 24.1 $\pm$ 1.0 (23.9-25.6 μm) in length and 10.6 $\pm$ 1.0 (9.6-13.2 μm) in width in frontal view.
Sequences of *Myxobolus* sp. I resembled to *M. cyprini* with 94.6% similarity; while *Myxobolus* sp. II showed closest similarity with 78.4% to *M. nagaraensis* respectively. For the *Myxidium* sp. I, partial sequences of the specimen showed 90.6% similarity to *M. cuneiforme*; while complete sequences of *Myxidium* sp. II specimen collected from *T. tambroides* showed 87.2% identity to *M. anatidum*. Phylogenetic analyses of the 18S rDNA were performed to estimate and analyze relationships between myxozoan entities.

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# MONITORING THE HEALTH STATUS OF SOME FISHES EXPOSED TO ENVIRONMENTAL HEAVY METALS POLLUTION

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In this work, the environmental pollution by heavy metals (lead, cadmium & mercury) in water and fish (*Orechromis spp., C. garipenus*) is studied in 3 localities at River Nile (Helwan, El-Hawameia, El-Warak) and also we studied the relationship between the physicochemical parameters of water and the accumulation of these metals in fish flesh, and it is concluded that the highest value of lead in water was recorded in Helwan at summer, while the highest value of cadmium in water was in El-Warak at spring and finally the highest value of mercury was in Helwan at spring. Regarding fish, the highest value of lead was recorded in *C. garipenus* in Helwan at summer, while the highest value of cadmium was in *Oreochromis* in El-Warak at autumn, finally the highest value of mercury was in *C. garipenus* in El-Warak at summer. Moreover, the effect of heavy metals on healthy status of *O. niloticus* also was studied and the results recorded that the $LC_{50}$ of lead, cadmium and mercury for *O. niloticus* was 3.5, 21, 0.72 mg/L, respectively. It is also noticed reduction in RBCs count, Hb content, PCV% in *O. niloticus* exposed to those metals and there were elevation in serum urea and creatinien as well as liver enzymes. Augmentation these results was by the histopathological studies, which revealed changes in liver, kidney, gills and spleen. It could be concluded that the liver is the primary organ of the accumulation of these metals in *O. niloticus*.

# BUTYLTIN COMPOUNDS IN FISHES, BIVALVES, GASTROPODS, SHRIMPS AND CUTTLEFISH COLLECTED FROM THE BIZERTA LAGOON (NORTH OF TUNISIA)

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Organotins, especially tributyltins (TBT) are highly toxic to many marine organisms. These compounds are introduced in marine waters especially by antifouling compounds used to provide protection from fouling to ship hulls. Marine organisms are easily prone to organotins contamination. In this study, levels of TBT and its degradation products, mono (MBT) and dibutyltin (DBT), were monitored in seven species of fish (*Boops boops*, *Trachurus mediterraneus*, *Sarpa salpa*, *Diplodus vulgaris*, *Spicara smaris*, *Serranus scriba* and *Trachinus draco*), two species of gastropods (*Bolinus brandaris*, *Hexaplex trunculus*), five species of bivalves (*Mytilus galloprovincialis*, *Ruditapes decussatus*, *Cerastoderma glaucum*, *Pinna nobilis*, *Chlamys flexuosa*), one species of cuttlefish (*Sepia officinalis*) and one species of shrimps (*Penaeus notialis*) commonly consumed in Tunisia. Samples were fished from the lagoon of Bizerta during March 2012 and butyltin levels were evaluated using isotope dilution GC–MS. TBT, DBT and MBT were detected in the muscle of these fishes with lowers levels. In bivalves, TBT concentration varied between 13.40 and 21.45 ng $Sn.g^{-1}$ dw. Gastropods contained TBT levels higher than the concentration known to induce imposex in these species. In cuttlefish and shrimps species, TBT levels were of 1.85 ng $Sn.g^{-1}$. The intakes of butyltins by humans via consumption of fish, Mollusks, cuttlefish and shrimps in this area were lower than the tolerable daily intake. The highest values were observed in bivalves which live in the sediment and in Gastropods that accumulate high levels of butyltins in their body through food chains because our two gastropod species are predator of all the five bivalves. The levels of butyltins suggest that the consumption of all organisms doesn't pose health problems to humans. To our knowledge, this is the first study reporting on butyltin pollution in fish cuttlefish and shrimps from the southern Mediterranean coast.

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# STUDY OF UV-B IMPACTS ON MORPHOLOGY AND RETINA OF *ONCORHYNCHUS MYKISS* LARVAE

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The harmful effects of ultraviolet radiation on aquatic animals, due to ozone layer reduction, have been long studied in recent years (Huntsman 1924; Klugh 1930; Thomasson 1956; Kevin 1994; Meyer-Rochow 2000; Sinha and Häder 2002; Palancar and Toselli 2004; Häder et al., 2007; Ghanizadeh Kazerouni and Khodabandeh 2010). The exposure of Rainbow Trout larvae (*Oncorhynchus mykiss*) to Ultraviolet-B radiation (UV-B) at different doses (68.75 µw/cm$^2$ and 94.83 µw/cm$^2$ as the minimum and maximum dose of UV-B in natural environment respectively) for 15 minutes once a day in dark condition in comparison to control group (without any solar or UV) showed a wide variety of body abnormalities and eye damages. Body curvature, yolk sac edema, fin blistering, dwarfism, eye and head abnormalities as morphological malformations were revealed during the experiment, none of the malformations were observed in control group. Histopathological changes in retina such as; irregular and discontinues pigmented epithelium, necrosis of photoreceptors and degeneration of nucleus layers confirmed the destructive effects of UV-B radiation in the eyes of Rainbow Trout. Such changes in larvae can be valid as bio-indicator for pollution and UV radiation and also introduce fishes as model for toxicological studies.

# HISTOPATHOLOGY OF SOME IMPORTANT ORGANS OF SALMON (*SALMO TRUTTA CASPIUS*) IN SOUTH PART OF CASPIAN SEA WITH EMPHASIZE ON POLLUTANTS

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This investigation was conducted to study the histopathology of some of important organs of Salmon (*Salmo trutta caspius*) in the south part of the Caspian Sea with emphasize on pollutants. Twenty salmon were captured from the south region of the Caspian Sea, then immediately samples from the liver, kidney, gills, gonads and muscles were obtained and fixed in %10 buffer formalin. Microscopic slides were prepared from the fixed samples using standard method of histology, and then studied by light microscope. Swelling, hypertrophy and hyperplasia, fusion of secondary lamellae and also congestion, hemorrhage and necrosis of the lamellae were observed in the gill of salmon. Congestion, hemorrhage, degeneration and necrosis of the tubules, glomeruli and interstitial tissue of the kidney of salmon were the most important signs which were observed. Histopathologic signs such as congestion and hemorrhage of the blood vessels, dilation and congestion of the sinusoids, hypertrophy, degeneration and vacuolation of hepatocytes and also necrosis of the liver tissue were the most obvious signs in the liver of the examined salmons. The results of this study showed that the important organs of this fish have serious disorders and abnormal conditions, which could be due to the long term exposing fishes to the toxic materials. In point of view of public health, if such fishes have more than standard amounts of pollutants in their body, utilizing them could be dangerous to the consumers.

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# STUDY OF ENDOCRINE DISRUPTION IN MULLETS (*CHELON LABROSUS*) FROM THE BASQUE COAST (BAY OF BISCAY) APPLYING A BIOMARKER BASED APPROACH

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Biomonitoring programs are essential tools to evaluate the biological quality status of aquatic environments. Recently, several compounds with endocrine disruption ability have been included in the list of priority substances and therefore, implementation of biomarkers assessing the presence and the effects of such substances are required. In the present work we applied a battery of chemical and biological markers in order to study effects of endocrine disruptors in four thicklip grey mullet (*Chelon labrosus*) populations from the Basque Country (Bay of Biscay) inhabiting differently polluted estuaries: Arriluze and Pasaia are marinas located in highly industrialized and densely populated areas, Plentzia is a leisure and touristic town and Gernika is located in the Biosphere Reserve of Urdaibai. Chemical analyses of fish bile were performed in order to determine the uptake of endocrine disruptors. Liver, gonad and brain samples were collected for the study of expression levels of genes associated with reproduction and development such as *vtg*, *cyp19a1* and *a2*, *er* and *rxr*. Histological analysis of gonads was performed to identify possible gametogenic alterations such as intersex gonads. Results indicated clear pollution dependent responses among four estuaries. Endocrine disruption effects were very marked in mullets from Gernika and Plentzia, these two populations showed high *vtg* gene expression levels in male mullets together with high alkylphenol metabolites in the bile. Bisphenol A was also present at high concentration in mullets from Gernika. *cyp19a2* was upregulated in male mullets from Plentzia. Intersex fish were found in Gernika and Pasaia, the last ones showing high hormone metabolites in bile.
The combination of chemical and biomarker approach in biomonitoring programmes can be a valuable tool to be implemented within the framework of the new water policies.

Keywords: Water pollution, endocrine disrupting compounds, Mullet.

# EFFECTS OF *IN VIVO* CHRONIC EXPOSURE TO PENDIMETHALIN ON EROD ACTIVITY AND ANTIOXIDANT DEFENSES IN RAINBOW TROUT *(ONCORHYNCHUS MYKISS)*

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Following a previous study demonstrating the presence of the active substance of pendimethalin in fish muscle after *in vivo* chronic exposure to pesticide, the detoxification process and the antioxidant defense system were assessed in rainbow trout, *Oncorhynchus mykiss*. Four nominal exposure conditions were tested: control (C), 500 ng $L^{-1}$ (P500), 800 ng $L^{-1}$ (P800) and the commercial formulation Prowl® at 500 ng $L^{-1}$ (Pw500). After a 28 day exposure period (D28), 10 fish were sampled for each condition, and then 10 more fish were collected after a 15 day recovery period in clean fresh water (D43). The pendimethalin concentration in bile and the liver ethoxyresorufin-O-deethylase (EROD) activity were monitored. In addition, non-enzymatic (glutathione content (GSH)) and enzymatic (superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT)) antioxidant parameters were measured in gills and liver tissues in trout.
At D28, EROD activity was not activated in liver in spite of the pendimethalin uptake in fish. At D43, EROD activity in fish exposed to Pw500 was lower than in control fish, which may be explained by the high presence of herbicide in fish ($613 \pm 163$ ng g $bile^{-1}$) and an impairment of cellular protein synthesis. Furthermore, antioxidant defense responses were set up by trout in gills and liver following chronic exposure to the highest pendimethalin concentration. While the GSH content decreased in gills, it increased in liver associated with higher activities of GPx and SOD. These disturbances could lead to reactive oxygen species production and oxidative stress in the vital organs in fish. After 15 days in clean water, while the SOD activity was restored, there were still significant differences between treatments in other antioxidant defenses parameters measured, attesting to the irreversibility of the effects.

Key words: pendimethalin; *Oncorhynchus mykiss*; EROD; GSH; SOD; GPx; CAT.

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# ANTIFUNGAL ACTIVITY OF *ORIGANUM* ESSENTIAL OIL ON HATCHING RATE IN RAINBOW TROUT

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One of the most important causes of economic losses in the rainbow trout farming industry is saprolegniasis, caused by species in the genus *Saprolegnia*. The therapeutic control (e.g. formalin, malachite green and etc.) of Saprolegniasis is difficult because the most effective products have been limited due to their toxicity and persistence in the environment. Especially malachite green was the most effective fungicide used for many years in aquaculture. But based on toxicologic and teratogenic effects of malachite green, using this chemical agent in aquaculture has been banned. Therefore the use of these chemicals stimulate the research for new constituents of natural origin, such as plant essential oils as antimicrobials.
In the present study, the chemical composition and the antifungal properties of the essential oils of oregano (*Origanum onites* L.) collected from Aegan region of Turkey were evaluated. The composition of oils was analysed by gas chromatography/mass spectrometry (GC/MS). Effective doses of essential oils for control of *experimentally induced* Saprolegniasis in *rainbow trout* and theirs eggs were investigated. For this aim, antifungal activities of essential oils in *Oncorhynchus mykiss* eggs and its effects on hatching rate in comparison with formaldehyte were detected. Infected eggs treated with four concentration of the essential oil (2,5, 5, 10, 25 and 50 ppm) and formaldehyte (positive control, 0,5 ml/lt) for 15 days after fertilization incubation period. In the end of hatching rate, the mold infection and hatching rate were calculated. The results reveased significant antifungal effects of essential oils on fish eggs so that it could increase hatching rate on concentration 5 and 10 ppm at *O. onites* and *T. spicata*, 50 ppm at *S. tymbra* ($p<0.05$).
In conclusion it can be claim that essential oil derived from *Origanum* species could be used as natural antifungal agents for control of Saprolegniasis.

# EXPRESSED SEQUENCE TAQS (ESTS) ANALYSIS OF *NEOCARIDINA DENTICULATA DENTICULATA* FOLLOWING SHORT-TERM EXPOSURE TO COPPER

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Small freshwater shrimp, *Neocaridina denticulata denticulata* (De Haan, 1844) inhabits lentic and lotic waters of the Asia and Pacific. Several characteristics of *N. denticulata denticulata* make this shrimp a good aquatic indicator for assessing environmental pollution: small size (2~3 cm), spontaneous interbreeding, and the absence of a metamorphosis stage during development. In this study, we constructed a cDNA library using mRNA extracted from the whole body of fresh shrimp (*N. denticulata denticulata*) and determined its EST sequence. This is the first report profiling the transcriptome in fresh shrimp (*N. denticulata denticulata*). Of the 1,296 clones sequenced, a total of 1,247 high-quality ESTs were obtained with a 96.2% sequencing success rate. These pre-processed ESTs ranged from 100 bp to 844 bp in length with a mean length of 695±97 bp.
The clustering analysis of those 1,247 ESTs yielded 603 unique sequences, of which 205 and 398 were contigs and singletons, respectively, with an average length of 726 bp. The top 43 cluster with the largest number of ESTs are summarized. Of these, the most abundantly sequenced EST cluster, CL 1 (Arginine kinase gene), accounted for 3.9% of all sequenced ESTs.
To analyze expression patterns of various genes after heavy metal stress, we treated freshwater shrimp with varying concentrations of copper. After treatment with different concentrations of copper, gene expression patterns of the putative biomarker were verified by semi-quantitative RT–PCR. The results showed that EST cluster, CL1 and CL7(Cytochrome c oxidase subunit I gene), increased highly expression level under different concentrations of copper. Especially, the high expression of the genes encoding Arginine kinase and Cytochrome c oxidase subunit I implied that these two enzymes might play key roles in biological biomarker by the Copper exposure. These results suggested that cytochrome P450 related protein and arginine kinase pathway might be a very important role in detoxicfication system of hosts.

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# IMPACT OF PARASITISM ON THE ACCUMULATION OF POLLUTANTS IN *VENERUPIS DECUSSATA* FROM TUNISIA

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Among benthic organisms, the clam *Venerupis decussata* is often used as bioindicator of environmental pollution because of its bioaccumulation capacity for trace metals. This mollusc is known also to shelter a very diversified parasities which can influence the accumulation of pollutants. The research carried out on mollusc parasites showed that the infesting stages of parasites and in particular the digenea penetrate in the host by digging galleries. These perforations open a way of contamination for the pollutants; so their accumulation would be different according to the presence or the absence of digenea in examined mollusc. In order to confirm whether digenea can facilitate metal contamination, we conducted this study.

During our research, 11 species of digenea (*Bucephalus labracis, Caecincola parvulus, Acanthparyphium sp., Curtuteria australis, Cercaria lata, Robphildollfusium fractum, Gymnophallus fossarum, Gymnophallus rebecqui, Lepocreadium pegorchis*, *Psilostomum brevicolle,* and *Parazoogonus sp*, Looss, 1901) were found in 2523 clams sampled from the lagoon of Tunis and Rades stations. Among the collected parasites, *C. lata* and *Curtuteria australis* are the most frequent throughout the year.

The comparison of the bioaccumulation of trace metals within uninfected and infected mollusks, shows that the infected clams accumulate less cadmium and zinc compared to healthy uninfected specimen while for lead the accumulation is similar.

Contamination of parasitized clams by trace metals varies according to the season and the parasite species. In Rades station and during winter, individuals infected by *C. lata* accumulate more the three metals than those parasitized by *C. australis*. Furthermore, molluscs infected by *C. australis* accumulate more in spring than in winter, whatever the metal.

In the north lake of Tunis station, the presence of *C. lata* in clams collected in spring increases the bioaccumulation of the two respectively metals: zinc and copper.

# MONITORING THE HEALTH STATUS OF SOME FISHES EXPOSED TO ENVIRONMENTAL HEAVY METALS POLLUTION

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In this work, the environmental pollution by heavy metals (lead, cadmium & mercury) in water and fish (*Orechromis spp., C. garipenus*) is studied in 3 localities at River Nile (Helwan, El-Hawameia, El-Warak) and also we studied the relationship between the physicochemical parameters of water and the accumulation of these metals in fish flesh, and it is concluded that the highest value of lead in water was recorded in Helwan at summer, while the highest value of cadmium in water was in El-Warak at spring and finally the highest value of mercury was in Helwan at spring. Regarding fish, the highest value of lead was recorded in *C. garipenus* in Helwan at summer, while the highest value of cadmium was in *Oreochromis* in El-Warak at autumn, finally the highest value of mercury was in *C. garipenus* in El-Warak at summer. Moreover, the effect of heavy metals on healthy status of *O. niloticus* also was studied and the results recorded that the $LC_{50}$ of lead, cadmium and mercury for *O. niloticus* was 3.5, 21, 0.72 mg/L, respectively. It is also noticed reduction in RBCs count, Hb content, PCV% in *O. niloticus* exposed to those metals and there were elevation in serum urea and creatinien as well as liver enzymes. Augmentation these results was by the histopathological studies, which revealed changes in liver, kidney, gills and spleen. It could be concluded that the liver is the primary organ of the accumulation of these metals in *O. niloticus*.

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# OUTCOMES OF COMBINED EXPOSURE OF CARP (*CYPRINUS CARPIO* L.) TO CYANOBACTERIAL BIOMASS AND SPRING VIRAEMIA OF CARP

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Under environmental conditions, fish can be exposed to multiple stressors including natural toxins and infectious agents at the same time. This study brings new knowledge on the effects of controlled exposure to multiple stressors in fish. The aim of this study was to test the hypothesis that influence of cyanobacterial biomass and infection agent represented by the virus of spring viraemia of carp can combine to enhance the joint effects on fish. For this purpose we compared the effects of single and combined exposures and evaluated the clinical signs, mortality, biochemistry, haematology, histopathology, body weight and toxin (microcystin) accumulation. Total leukocyte counts, leukograms, phagocytic activity, total immunoglobulins and titters of specific antibodies were evaluated as selected immunological parameters. In short time after application of the virus, patho-anatomic changes appeared. These changes responded to acute form of spring viraemia of carp and disappeared within relatively short time also with respect to higher water temperature and active defence of fish. This fact also correlates with increased specific antibody titter of infected fish. Statistical evaluation of results in experimental groups demonstrated different modulation of parameters monitored, depending on the duration of the experiment. The most significant changes were observed in the evaluation of total immunoglobulin levels, which were significantly elevated in the combined exposure of fish throughout the experiment. The exposure to cyanobacteria was manifested by accumulation of microcystins in hepatopancreas of fish. Higher concentrations were found in groups exposed only to cyanobacteria without co-exposition with the virus. The study indicates clear interaction between both studied factors, i.e. chemical stressor (toxic cyanobacterial biomass) and model viral infection.

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# PATHOPHYSIOLOGICAL EFFECTS OF HARMFUL ALGAE AND JELLYFISH ON ATLANTIC SALMON *SALMO SALAR* L. GILLS IN NORWEGIAN WATERS (JELLYTOX)

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There is a recognized concern that the number of jellyfish blooms are increasing as a consequence of anthropogenic activities such as aquaculture. Jellyfish can either pass through the cage nets or become fractured into smaller pieces that become inhaled or come into contact with fish epithelia like the skin or the gills. Cases of acute trauma occur during exposure of fish to harmful algal blooms (usually diatoms) resulting in episodes of mortality in aquaculture. The diatoms bloom usually involves physical penetration of the gill epithelium by the silicious and calcerous spines of the diatom, the resulting pathology of haemorrhage, epithelial hyperplasia and excessive mucus production causing acute hypoxemia similar to that seen with jellyfish intoxication. The present study (JELLYTOX) is an on-going investigation into the effects of different jellyfish (eg *Aurelia aurita*, *Periphylla periphylla* and *Cyanea capillata*) and harmful algal blooms (eg *Chaetoceros* sp.) on the respiratory epithelium and physiology of Atlantic salmon and was initiated in 2013 to begin to understand the potential effects of phyto and zooplankton on gill health of farmed salmonids. In this respect, the respiratory and cardiovascular pathology is investigated, along with the partial characterization of potential toxins and their effects on cultured gill cell lines (RTgill-W1). Since some species of zooplankton have been associated with fish pathogens, the presence of *Tenacibaculum maritimum*, *Vibrio anguillarum*, *Aeromonas salmonicida*, *Pisckirittesia salmonis*, *Desmozoon lepeoptherii*, *Branchiomonas cysticola* and *Neoparmoeba peruans* are investigated using by qPCR.

# IS STRESS IN FARMED SALMON (*SALMO SALAR*) AN IMPORTANT FACTOR FOR SAPROLEGNIOSIS?

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Saprolegniosis is a major concern to fish farms worldwide, leading to heavy losses due to infection by a filamentous oomycete pathogen. *Saprolegnia* infections are often observed on fish farms after procedures that involve handling or movement of the fish, which most likely induce significant stress to the fish. Fish farms employ unavoidable practices, which might cause significant stress, involving handling and/or crowding of the fish. Vaccination by intraperitoneal injection is one such stress factor. Especially after vaccination, fish show signs of Saprolegniosis, if not given preventative treatment such as Pyceze and/or formalin. Stress, measured by an increased release of cortisol, has been shown to have adverse effects on long term fish health. The expression of the genes steroidogenic acute regulatory protein (StAR) and cytochrome P450side-chain-cleavage, responsible for the regulation of steroidogenesis, and glucocorticoid receptor (GR) as well as heat shock proteins 90 and 70 (HSP90/70) can be used as a proxy for stress effects at the gene level. First data corroborates observations of prolonged susceptibility of salmon to *Saprolegnia* after vaccination. Direct measurements of blood cortisol and glucose concentration are also used to determine stress level. In this study we aim to link stress marker gene expression to immune gene expression, with major changes in the latter seen during infection of salmon with *Saprolegnia*.

# ACUTE EXPOSURE TO HEAVY METALS AND CONTAMINATED SEDIMENTS DOWN-REGULATE THE GENE EXPRESSION OF POLLUTION AND STRESS BIOMARKERS IN THE GILTHEAD SEABREAM (*Sparus aurata*)

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Gilthead seabream (*Sparus aurata*) is a teleost fish of crucial importance for the human diet in the Mediterranean region. Unfortunately, the location of some aquaculture industries in waters receiving pollutants form industrial or mining areas could lead to bioaccumulation and adverse effects to the human consumers. Thus, safer waters and food controls are needed for aquaculture business. Several studies have related the effects of pollutants on fish homeostasis, including the immune response. Thus, contamination causes stress in fish and therefore the immune response and disease resistance is altered. Unfortunately, little is known about the effects of pollutants in the gilthead seabream and very few have focused on the immunity. Thus, in this study, we have exposed seabream specimens to 1 ppm waterborne cadmium ($CdCl_2$) or arsenic ($As_2O_3$) in one trial and to highly-contaminated sediments in another experiment. Afterwards, the expression of metallothionein-A (*mta*) and heat-shock protein-70 (*hsp-70*) genes as heavy metal contamination and stress biomarkers, respectively, was evaluated by real-time PCR in liver and head-kidney tissues.

Strikingly, results showed that, in liver, the *mta* gene expression was down-regulated after exposure to low waterborne Cd and As (58% and 13% respectively). Moreover, in the sediment-exposed group, this gene was 4-fold down-regulated compared to the control. Besides, a decrease in the expression of *hsp-70 gene* was registered after exposure to Cd, As and sediment. In head-kidney, both genes were also down-regulated by exposure to Cd, As and sediments being this effect much higher in the case of Cd. Further studies are needed to understand this effects since the same fish showed stress and decreased immune response.

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# ULCERATIVE DERMAL NECROSIS AND OTHER SKIN CONDITIONS AFFECTING WILD ATLANTIC SALMON *SALMO SALAR*

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Ulcerative Dermal necrosis (UDN) is a skin condition of unknown aetiology affecting wild Atlantic salmon *Salmo salar*. UDN was first reported in the 19$^{th}$ century and remained until beginning of the 20$^{th}$ century. Subsequently the condition re-emerged in southwest Ireland in the 1960s and spread to almost all rivers of the British Isles, but seemed to disappear again in the mid 1970s.
The skin lesions are normally restricted to the head and externally, they range from early small grey-white coloured superficial lesions above the eye, opercula or along the snout, to more advance stages where ulcers develop.
The histopathology assessment (currently the only diagnostic tool available) of early lesions reveals swelling and degeneration of the malpighian cells, spongiosis and presence of pemphigoid-like lesions in the epidermis. More advance lesions can appear ulcerated and show dermal necrosis, infiltration and secondary fungal infections.
Wild Atlantic salmon is an important symbol of Scottish waters and of a great environmental and economic value. In the summer of 2012, an increased observation of returning adult salmon showing skin lesions was raised by individual anglers and fisheries boards. Samples were taken by Marine Scotland Science and processed for histology and bacteriology. At least in one of the cases, UDN-like lesions were observed by histology, raising again the concern and awareness for this condition. Previous studies have ruled out the involvement of an infectious agent as the primary cause of the condition. Alternative hypothesis include solar radiation, nutritional and hormonal imbalances, but none of these have been proven.
The absence of frequent cases, pathognomic signs or complementary diagnostic tests can complicate the achievement of a conclusive diagnosis for this condition. We describe the histopathology findings from the 2012 cases in the scope to discuss and compare other skin conditions affecting wild Atlantic salmon and to highlight the need of an updated UDN case definition.

# CYSTS OF UNKNOWN ETIOLOGY IN MEDITERRANEAN WILD SEA FISHES: AN OVERVIEW

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Cysts of unknown etiology (CUE) have been described in the gill filaments of marine and fresh water fishes throughout the world. The nature of these cysts is presently undetermined. However, its presence has been suggested as potential marker for stock discrimination in long lived deepwater species. Samples of 19 species of Mediterranean fishes were obtained from the platform and continental slope of the western Mediterranean Sea during 2007 and 2009. Gill samples were processed by routine histological methods and electron microscopy techniques and different histochemical stains were used in order to elucidate the nature of these alterations. Macroscopically, rosaceous cysts with low opacity and elastic consistency, ranged from 125µm to 350µm, were detected in the gills filaments. Histological sections of these CUE revealed the presence of four concentric acellular layers: (1) a thick and pale external layer; (2) a thinnerunderlying layer; (3) a thicker layer composed by basophilic material; and (4) a large homogeneous core of, amorphous and eosinophilic material. Connective tissue and capillary vessels, sometimes dilated, were usually observed surrounding the cyst. These cysts where located mainly on the primary filament but also on the secondary ones. Ultrastructurally, the outer thinner layer was composed of multiple small electron dense granules and aggregates of glycogen and lipid droplets appear to be present in the core and the adjacent layer. Of all species analyzed, only two fish species of the platform (*Citharus linguatula* and *Trachinus draco*) and three of the slope (*Helicolenus dactilopterus, Hymenocephalus italicus* and *Notacanthus bonaparte*) did not show CUEs in the gills. All gadiform fishes (except macrourids and *Mora moro*) had prevalences higher than 40%, being *Phycis blennoides, Micromesistius poutassou* and *Merluccius merluccius* those presenting the highest values (75%, 70% and 68%, respectively). Seasonal significant differences in prevalence were observed in five species (*Merluccius merluccius, Mullus barbatus, Pagellus erythrinus, Lepidion lepidion, Nezumia aequalis* and *Trachyrincus scabrus*). Significant differences between localities and depths in prevalence were also found for *Lepidion lepidion* and *Phycis blennoides*.

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# HISTOLOGICAL OBSERVATIONS ON SKIN PAPILLOMA IN WILD RED HALIBUT (*HIPPOGOSSOIDES DUBIUS*)

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Histopathological observations on epidermal papilloma of wild red halibut (*Hippogossoides dubius*) inhabited the sea area between Eochung and Gogunsan Islands in Korea were performed. The papilloma formed on the dorsal fin was the same color with body on the ocular side (pigmented side) but black color on the non-ocular side (unpigmented side). Under the light microscopy, the tumor on the skin was very similar to epidermal papillomas supported by dermal connective tissue, the stroma. The epithelial cells of the tumor were dispersed with no orderly arrangement. The disordered epithelial cells showed central-located enlarged nuclei in eosinophilc cytoplasm which are supported by connective tissue with black melanin pigments. However, over-sized nucleoli in the nuclei of tumor cells which are characteristic symptoms in X-cells were indistinct.

# STUDY ON THE MAID (*LIZA KLUNZINGERI)* MORTALITY USING HISTOPATHOLOGICAL AND IMMUNOFLUORESCENCE ANTIBODY TEST (FAT) IN PERSIAN GULF COASTLINE

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An unknown mortality was occurred in some (maid) *Liza klunzingeri* in south of country in Bandar Abbas during 2009-2010. The range of length fish was between 8 to 18 cm and the weight between 15 to 200 gr. Moribund fishes revealed clinical signs such as changing in body coloration, abnormal swimming behavior, belly up, disorientation, ventral and operculum haemorrhage. Histological study of the brain and retina showed severe vacuolation in these tissues. The lesions were detected in different parts of the brain, especially in the granular layer. Necrotic nerve cells in the brain showed pyknotic and marginated nuclei. The inflammatory process and hyperemia was detected in all layers of the brain. Necrotic lesions seem much less severe in the eye compared to the brain and less in vacuolation. The vacuolation was also observed in the optic nerve. The immunofluorescence antibody test (FAT) was done against Viral Nervous Necrosis (VNN) virus antigen but FAT findings were negative and showed no viral antigen and apparently all slides were without any antibody- antigen complex. Only one slide seems positive which was not enough to definite Viral Nervous Necrosis (VNN). In pathogenicity test challenging with the brain-homogenate was carried out on Guppy *(Poecilia reticulata)* as susceptible fish. A few challenged fishes showed the same clinical and behavioral signs with infected Maids, and mortality was low. In some fishes abnormal and neural behaviors were observed and some of them showed ventral swelling. In histopathological study, brain lesions (necrosis and vacuolation) were detected. Vacuolation was also observed in granular layer of retina in some cases. Despite the negative result of the IFAT test, pathological observations showed VNN symptoms. All observed vacuolations in these fishes and Maids were similar and further vacuolation in the granular layer of the brain and retina is pathogenomic and a typical symptom of VNN disease. Finally, these results showed that VNN disease could be one of the important probably reasons for recent acute mortality in *L. klunzingeri* and it would be approved with comprehensive studies and more investigations in future cases.

Key words: *Liza klunzingeri*, viral nervous necrosis, Immunofluorescence, vacuolation, Iran

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# THE PYLORIC CAECA AREA IS A MAJOR SITE FOR B CELL RECRUITMENT IN RESPONSE TO ORAL IPNV VACCINATION

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Although previous studies have characterized some aspects of the immune response of the teleost gut in response to diverse pathogens or stimuli, most studies have focused on the posterior segments exclusively. However, there are still many details of how teleost intestinal immunity is regulated that remain unsolved, including the location of $IgM^+$ and $IgT^+$ B cells along the digestive tract and their role during the course of a local stimulus. Thus, in the current work, we have studied the B cell response in five different segments of the rainbow trout (*Oncorhynchus mykiss)* digestive tract in both naïve fish and fish orally vaccinated with an alginate-encapsulated DNA vaccine against infectious pancreatic necrosis virus (IPNV). $IgM^+$ and $IgT^+$ cells were identified all along the tract with the exception of the stomach in naïve fish. While $IgM^+$ cells were mostly located in the lamina propria (LP), $IgT^+$ cells were primarily localized as intraepithelial lymphocytes (IELs). Scattered $IgM^+$ IELs were only detected in the pyloric caeca. In response to oral vaccination, the pyloric caeca region was the area of the digestive tract in which a major recruitment of B cells was demonstrated through both real time PCR and immunohistochemistry, observing an important increase in the number of both $IgM^+$ and $IgT^+$ IELs. Our findings demonstrate that both $IgM^+$ and $IgT^+$ respond to oral stimulation and challenge the paradigm that teleost IELs are exclusively T cells.

# PATHOGEN HAZARDS ASSOCIATED WITH THE IMPORT OF PREDATOR BAITS TO THE UK

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The use of angling bait is recognised as a potential pathway for the introduction of pathogens and parasites into aquatic environments but the bait industry is largely unregulated. Fish baits, both marine and freshwater species, are popularly used in angling for large predators. The use of predator baits is potentially an important route of disease introduction as the fish are often used whole and come into direct contact with susceptible species. Businesses supplying predator baits were contacted and a list of bait species compiled. The most popular bait species are lamprey, smelt, mackerel, sprat and herring. Freshwater species such as eel, perch, roach and trout are also used. One supplier of predator baits advertised eels as coming from sustainable sources, as they are mortalities from aquaculture.
In addition, rainbow trout intended for human consumption may be diverted for use in freshwater angling.
Disease hazards (for freshwater fish species in the UK) associated with imported bait species used were identified, i.e. pathogens or parasites that are absent from the UK or with limited distribution, OIE listed or a significant threat, and able to withstand freezing (predator baits are sold frozen). The most important hazards identified were viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV) and eels pathogens (eel herpesvirus and rhabdovirus). The persistence of VHSV in the tissues of infected trout has been demonstrated. Whilst the major pathogens of eels have been detected in the UK, their distribution may be restricted. The use of imported fish as bait in freshwater angling is a potentially important route of disease spread since it creates direct routes for the exposure of susceptible wild populations. The use of mortalities from aquaculture is particularly worrying because the animals are highly likely to be infected, and it provides a route for establishment and spread of new pathogens. The introduction of VHSV and IHNV (through diversion of imported trout for use as bait) and eel pathogens need to be investigated further in an import risk analysis to support strengthened biosecurity.

# TROUT FARMS IN THE NORTH-EAST OF ITALY: EXPLORATORY STUDY ON FARMING FEATURES AND RISK FACTORS ASSOCIATED WITH DISEASE EXPOSURE

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More than 65% of the freshwater salmonid production yields in the north-east of Italy, with about 200 facilities - mainly trout farms for human consumption (THC farms) and a few others keeping their own brood stock for egg production. This paper aims to describe the main management characteristics of THC farms in this area and the risk factors related to diseases transmission. An exploratory study has been conducted in 37 randomly selected THC farms among the 168 present in the north-east of Italy (Friuli Venezia Giulia, Veneto and Trentino regions).
A standardized questionnaire was used to collect data on farm characteristics, management and risk factors related to VHS introduction and spread, according to Decision 2008/896/EC. A descriptive analysis was performed to evaluate either farm management profiles or risk factors related to introduction and spread of diseases. Eight of the 37 evaluated farms (8/37) have the whole production cycle, keeping their own brood stocks, while twenty-nine (29/37) buy mainly eggs or fingerlings from other farms. Considering the type of production, twenty-two (22/37) farms grow fish for human consumption eleven (11/37) produce fish for human consumption and for restocking of put and take fisheries and four (4/37) release fish into the wild for restocking purposes. The average production is of 242 tons a year. The main risk factors considered were: type of production, type of water supply of the farm, number of upstream and downstream fish farms, movement of fish, health status of source farms, the bio-security system applied at farm level. Preliminary results show that 59.5% of the selected farms grow fish for human consumption (95%CI 42.1-75.2), representing a minor risk factor for spreading diseases to other farms. As for the possibility of contracting diseases, 62.2% of the selected farms may be exposed to this risk due to the type of water supply (river water - 95%CI 44.7-77.5) while 33.3% of farms may be exposed as they receive fish from undetermined health status (category III, according to 2006/88 UE Directive) facilities (95%CI 14.6-56.9). To obtain a better overview of farm management characteristics and risk factors profile, this study will be completed trough the collection of the above mentioned data in all the facilities in the framework of the 2006/88 UE Directive.

*The work was undertaken in a cooperation Art 36 project "Risk categorization for Aquatic Animal Health surveillance" (CFP/EFSA/AHAW/2011/03) of the European Food Safety Authority (EFSA).*

# OCCURRENCE, SEASONALITY AND INFECTIVITY OF *VIBRIO* STRAINS ASSOCIATED TO NATURAL POPULATIONS OF MUSSELS (*MYTILUS GALLOPROVINCIALIS*)

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Numerous causes have provoked the widespread mortalities of bivalve molluscs, therefore affecting their production. Several pathogens have been reported as the primary cause of death for oysters or clams, especially bacteria of the *Vibrio* genus. The main objective of this study was to evaluate the occurrence, seasonality and infectivity of *Vibrio* strains associated with natural mussel (*Mytilus galloprovincialis*) populations. Specifically, *Vibrio splendidus* and *Vibrio aestuarianus* were analysed because they were associated with important oyster mortalities in areas where mussels are cultured without presenting mortalities. The presence of both *Vibrio* spp. was analysed bimonthly in mussels, water, sediment, plankton and other associated fauna from two sites in Galicia (NW Spain), the region with the highest mussel production in Europe. Environmental factors were also considered. The pathogenicity of different *Vibrio* isolates was analysed by performing experimental infections with strains isolated from the field. Results showed that *Vibrio* populations were influenced by environmental conditions. *V. splendidus* was dominant during the warm months and *V. aestuarianus* was predominant throughout the cold season. The sediment was the most important natural reservoir for bacteria. Experimental infections showed the extreme resistance of mussels to bacterial pathogens. *V. splendidus* and *V. aestuarianus* were only moderately pathogenic for mussel in intramuscular infections and bath infections, and mortalities only occurred when animals were infected with a high bacterial concentration in adverse environmental conditions. Although the pathogenicity of the *Vibrio* strains isolated from the wild was low for mussels, their potential risk for other bivalves cannot be forgotten.

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# *MIKROCYTOS* INFECTION IN MANILA CLAM, *RUDITAPES PHILIPPINARUM* IN THE NETHERLANDS

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In the spring of 2012 a mortality of 50% was reported in a batch of *Ruditapes philippinarum* from a land based aquaculture culture system in the Netherlands. By histology a microcell-like parasite was observed in 14 out of 35 specimen analysed. The parasite was present in the mantle tissue and, to a lesser extent, in the foot of the clam. No other pathogens or abnormalities were observed by histology. With *in situ* hybridisation using a probe for *Mikrocytos* spp. a positive labelling of the microcells in connective tissue was shown. *Mikrocytos* spp. are intracellular protistan parasites of vesicular connective tissue cells of uncertain affiliation. In the genus *Mikrocytos* only one species has been described, *Mikrocytos mackini* with *Crassostrea* and *Ostrea* oyster species as host. In a PCR assay using primers for *Mikrocytos* species PCR products were obtained. Subsequent sequencing of the PCR products revealed a sequence of a *Mikrocytos* species most closely related to the *Mikrocytos* species observed in the wedge shell clam *Donax trunculus* from France.

# FISH HEALTH IN FINLAND IN THE LAST DECADE

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The amount of food fish produced in Finland in the last decade was on average about 13 thousand tons yearly, the majority of this being rainbow trout.
The second most important species is whitefish. Fish is also produced for restocking and export of juveniles. The species farmed include Baltic salmon, landlock salmon, brown trout, sea trout, brook trout, char, pikeperch, grayling, pike, cyprinids, sturgeon and crayfish.
Finnish Food Safety Authority Evira and its predecessors have offered a voluntary based fish health service since 1969. Some fish disease diagnostics are also provided by Åbo Akademi University and one private company. In addition to these voluntary investigations, compulsory fish disease screening is done according to EU requirements since 1995.
In general, fish health in Finland is monitored extensively and is considered to be good. Viral diseases have not been problematic until year 2002 when VHS was encountered in altogether three different geographic areas on the coast. Eradication of VHS has been successful except for Åland Islands were the disease is still present. IPN is encountered yearly at the coastal area, continental Finland has been considered IPN free until 2012 when the virus was encountered at 6 inland fresh water farms. The introduction of vaccines against vibriosis and furunculosis has altered the situation for bacterial diseases so that the most important bacterial diseases encountered nowadays are infections caused by Flavobacteria. *Yersinia ruckeri* B2-infections, especially in brackish water, have been an emerging problem since 2007, but also in this case vaccination has been effective to at least some degree. BKD is encountered yearly and is subject to an eradication program in the main brood stock farming area. Official statistics concerning parasitological and fungal diseases are lacking due to the fact that these are usually diagnosed on-farm. Statistics over viral and bacteriological diseases diagnosed in 2002-2012 will be presented.

# CHARACTERIZATION OF *PERKINSUS MARINUS* FROM CULTIVATED OYSTER *CRASSOSTREA CORTEZIENSIS* IN THE PACIFIC OF MEXICO

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This study assayed polymorphisms in two regions of rDNA of *Perkinsus marinus* detected in the oyster *Crassostrea corteziensis* from wester Mexico to determine the genetic variability of the parasite, and assess the relationship between *P. marinus* from the Pacific and the Atlantic, based on phylogenetic inferences. *C. corteziensis* is a native oyster that naturally grows in mangroves. Collected oyster seeds are cultivated in river estuaries under high temperatures (30°C). The protozoan was identified by PCR amplification of the internal transcribed spacer (ITS) region of the rDNA of *Perkinsus* spp. Prevalence was 92% and 77% in oysters from two estuaries in the State of Nayarit. Using digestion with *Rsa*I and *Hinf*I to ITS PCR products, were obtained the restriction fragments size expected for *P. marinus*. The digestion pattern of *P. olseni* and *P. chesapeaki* were not found. The most frequent ITS sequence, (GenBank JQ266236) had 100% identity with the ITS locus of *P. marinus* from New Jersey, Maryland, South Carolina, and Texas, and the second most frequent observed sequence (GenBank JQ266240) was 100% identical to ITS sequences of *P. marinus* from New Jersey, South Carolina, Louisiana, and Bahía Kino, in the State of Sonora, Mexico. According to ITS analyses, *P. marinus* from Nayarit had high similarity to genotypes from Maryland to Texas, but not with genotypes from Virginia. The sequences from the non-transcribed spacer (NTS) was 98% similarity to *P. marinus* from Texas. Both the ITS and NTS sequences of *P. marinus* obtained from the oyster *C. corteziensis* were grouped into two clades, identifying two allelic variants of *P. marinus.*

# CONSEQUENCES OF INFECTION WITH THE MICROSPORIDIAN *FACILISPORA MARGOLISI* IN THE SALMON LOUSE *LEPEOPHTHEIRUS SALMONIS*

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The microsporidian *Facilispora margolisi* occurs in three species of parasitic copepods belonging to the genus *Lepeophtheirus* in the northeast Pacific Ocean. The prevalence in *L. salmonis* ranges up to 90% and copepods parasitic on wild Pacific and farmed Atlantic salmon are infected. The association of spores with developing copepod embryos suggested the microsporidian is vertically transmitted. The purpose of this study was to confirm the occurrence of vertical transmission and to assess the influence of microsporidian infection by measuring copepod infectivity to fish and gene expression. A portion of cephalothorax and one egg string from each of 20 copepods were separately screened for *F. margolisi* by PCR. The second egg string from each copepod was cultured in aerated filtered seawater. The resulting larval copepods were screened by PCR and in-situ hybridisation. In four such experiments, *F. margolisi* was detected only in copepodids from infected females, supporting a hypothesis of vertical transmission. There was no evidence that infectivity to juvenile chum salmon differed between infected and uninfected copepodids. Pools of infected and uninfected copepodids were maintained in seawater with or without 1 μg $L^{-1}$ emamectin benzoate (EB) for 24 h. Transcription among groups was compared by using a 38K oligo-array and by quantitative reverse transcriptase PCR. The number of differentially regulated genes, belonging to seven Gene Ontology (GO) categories, ranged from 456 to 1,554 among pair-wise comparisons of treatments. Principle component analysis suggested microsporidian infection accounted for 33% of variation in microarray data and cluster analysis identified a synergistic effect of EB and microsporidia on the expression of genes belonging to the GO category Protein Folding. The implications of *F. margolisi* infections on the efficacy of EB in *L. salmonis* are discussed.

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# HEMOCYTOSIS, A NEW EMERGING DISEASE IN CULTURED SHRIMP, *FENNEROPENAEUS INDICUS* AND MORTALITY AFTER EXPOSING TO WSSV

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Parasites sometimes cause diseases that could be invasive for shrimp. Among these, protozoa are very important. The objective of our study was to express the features of hemocytosis, a new emerging disease in shrimp, *Fenneropenaeus indicus* and showing prevalence, and intensity of that. Our study was designed as three groups, one treatment and 2 controls, in triplicate. Our result revealed that Hemocytosis associated with decreasing in Hyalinocytes and Large-granulocytes (less than 8%) and considerable increasing in Semi-Granulocytes and it made the shrimp, *Fenneropenaeus indicus* susceptible to WSSV disease. It is concluded that Hemocytosis causes a sub-clinical disease and result in a rapid mortality among susceptible species, *Fenneropenaeus indicus* in exposure to WSSV.

Keywords: Shrimp, *Fenneropenaeus indicus*, Hemocytosis, Intrahemocyte, Mortality

# RISK ANALYSIS ON STURGEON DISEASES APPEARANCE IN THE AQUACULTURE OF SOUTH RUSSIA

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The development of aquaculture is impossible without movement of fish and fish products. However unreasoned movement of fish could lead to introducing and spread of pathogens into fish farms and serious ecological consequences. This especially actual for south Russia where sturgeon aquaculture develops intensively. Some diseases are dangerous for definite age and species, others – appears only at definite season and under definite environmental conditions. However one is known that intensification of aquaculture cause the influence on fish health and lead to appearance of new diseases. Diseases can be prevented only when the risks are recognized and managed before the diseases occur.
The method of factorial modeling was used for risk analysis on sturgeon diseases appearance for the following steps: 1) fish transportation in closed water systems; 2) sturgeon rearing in tanks and ponds. The calculation method of the possible measure of fish mortality depended on parametrical model "exposure (present concentration of the causative agents) – protection (preventive measures and treatment) – fish susceptibility (average level of fish mortality for certain group of fish)" which include the data on registered causative agents and measured hydro chemical indexes. This method differs from earlier used methods of factorial modeling of risk analysis. This is the process and result of determination of material form of integrated risk (the share of the diseased fish among reared sturgeon or fish mortality in certain population during definite time) of preconditions of fish mortality, their connection and negative environmental factors. Thus integrated risk is determined as step by step algebraic sum of multiplication of possibility (indistinct) of fish mortality on the present quantity of sturgeons in definite population. The hazard identification and risk management depend on this calculation.
There is urgent need for sturgeon health monitoring program at the aquaculture of south Russia. It is especially important at changing environment conditions and usage of new technologies in fish rearing. Systematization of published and new data gives us the opportunity for better understanding of the disease occurrence and to prevent the epizooty at future. The developed method allows 1) to determine the probability and the consequences of entry, establishment and spread of the disease causative agents which could lead to fish mortality in complex hydro technical system, 2) make the quantities analyses of monitoring system and risk communication, 3) to determine the urgent measures for hazard decrees and at the end, to save the bio-resources.

# CRAYFISH PLAGUE IN SLOVENIAN FRESHWATERS: RESULTS OF A 2009-2012 STUDY

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The crayfish plague (*Aphanomyces astaci*) is a detrimental disease for European, Australian and Asian indigenous crayfish species (ICS). It was introduced to Europe from North America by the resistant non-indigenous carriers (NICS); *Pacifastacus leniusculus, Procambarus clarkii* and *Orconectes limosus* represent permanent plague reservoirs, endangering also the three ICS inhabiting Slovenian freshwaters (*Astacus astacus, Austropotamobius pallipes* and *Austropotamobius torrentium*) in addition to the Australian *Cherax quadricarinatus* recently discovered in Slovenia.
All five crayfish species inhabiting Slovenian freshwaters (three ICS, *C. quadricarinatus* and *P. leniusculus* introduced in 2003), in addition to retail specimens of *P. clarkii*) were inspected for the presence of plague agent *A. astaci*. Sampling of animals was performed from 2009 to 2012, mostly from wild populations in Slovenian freshwaters showing no clinical signs of disease. In total, 398 crayfish were collected, 226 of them representing *A. torrentium* as recently shown to sustain a persistent infection indicating the presence of a less virulent *A. astaci* strain (Kušar et al., 2013). Crayfish samples were subjected to molecular detection: *A. astaci*-specific real-time PCR (Vrålstad et al., 2009) and conventional 42/640 PCR assay adopted from Oidtmann et al. (2006); when applicable, confirmative sequencing was also performed.
In our study, 43/398 (10.8%) animals were found positive for the plague: 2/57 (3.5%), 2/32 (6.2%) and 32/226 (14.2%) specimens of *A. astacus*, *A. pallipes* and *A. torrentium*, respectively, representing ICS, and 5/50 (10.0%) *P. leniusculus*, 1/21 (4.8%) *C. quadricarinatus* and 1/12 (8.3%) *P. clarkii*. The prevalence of infection did not increase during the four years of sampling and the maximal level of infection was moderate (2/43), in other cases low (10/43), very low (14/43) or possible (17/43), according to agent levels adopted form Vrålstad et al. (2009). As expected, higher levels of infection could mostly be confirmed by sequencing. Our findings confirm the presence of latent infection in ICS, placing the problem of crayfish plague in Europe into a new evolutionary perspective affecting both ICS and NICS which should be further investigated.

**References**:
Kušar et al. (2013) Dis Aquat Org 103:157-169; Oidtmann et al. (2006) Dis Aquat Org 72:53-64; Vrålstad et al. (2009) Vet Microbiol 137:146-155

# REAL-TIME RT-PCR FOR DETECTION, IDENTIFICATION AND ABSOLUTE QUANTITATION OF VHSV

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Officially, diagnosis of viral hemorrhagic septicemia (VHS) must be performed by isolation of the virus (VHSV) in cell culture and identification by immunological or molecular techniques. However, nowadays, the PCR-derived techniques -mainly real time RT-PCR- are being used not only to identify isolates, but frequently for direct detection of the virus in fish tissues.
In the present study, two systems of real time RT-PCR -one based on SYBRgreen and another on TaqMan probes have been designed to detect strains from any genotype of VHSV, with a high sensitivity and repeatability/reproducibility (R&R). In addition, the method has been tested to be used for quantitative purposes (RT-qPCR).
Primers were designed to amplify a fragment of 163 bp located at the nucleoprotein (N) gene. Specificity was tested against 26 isolates from the 4 genotypes (1 from genotype I, 6 from genotype Ia, 3 from Ib, 5 from II, 7 from III, 3 from IVa and 1 belonging to genotype IVb). Sensitivity was determined using different standards: RNA from crude (limit of detection [LD]= 1 fg, equivalent to 160 genome copies) and purified virus (LD= 100 ag/ 16 copies), plasmid cDNA (2 copies), and in vitro transcribed RNA (15 copies). Both probe and SYBR Green based RT-PCR approaches showed comparable dynamic range and sensitivity. All the experiments were performed by triplicate and in three different days in order to evaluate repeatability and reproducibility (respectively), and the analysis of the Ct values yielded levels of coefficient of variation (CV) always $\leq 5$, independently from the type of standard used. This fact, together with the high efficiency ($90 \leq E \leq 110$) and correlation ($R2 \geq 0{,}995$) values yielded by the standard curves in all cases encouraged us to mathematically analyse the reliability of the method to be applied for viral quantitation. The results not only demonstrated that the procedure can be used for detection, identification and quantification of this virus, but has also demonstrated a clear correlation between the regression lines obtained with different types of standards, what will help scientists to compare results on sensitivity (in terms of LD) between different authors.

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# HEMOCYTE TRANSCRIPTOMICS OF MEDITERRANEAN MUSSEL (*MYTILUS GALLOPROVINCIALIS*) AFTER AN IMMUNE STIMULATION THROUGH AN RNA-SEQ APPROACH

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The Mediterranean mussel (*M. galloprovincialis*) is a worldwide cultured bivalve with important commercial and ecological value. It has been used as a model in bivalve and even lophotrocozoan studies. The objective of this study is to increase the knowledge of the molecular mechanisms of the *Mytilus* immune-cells from a qualitative and quantitative point of view. RNAseq is a whole-transcriptome quantification method, the next step to microarrays. We studied RNA from immune-stimulated mussel hemocytes and compared its transcriptome to other naïve tissues (mantle, muscle and gill) to identify hemocyte specific transcripts and study the expression differences between these immune specialized cells and other tissues.
cDNA libraries were sequenced with the Illumina HiSeq™ 2000 technology.
A total of 393,316 million raw reads (112,706 from hemocytes) were obtained and assembled into 151,320 non-redundant transcripts (107,045 from hemocytes), with an average length of 570 bp. The average coverage of tissues was 69.87% with 256.55 mapped reads per transcript. Hemocytes were the tissue with the lowest coverage but the highest number of mapped reads, meaning than although it was the tissue with the smaller transcriptome, the genes were highly transcribed. The annotation step identified 33.7% of the transcripts and a lot of immune-related KEGG pathways (chemokine, NF-κB, JAK/STAT or TLR signaling pathways, complement cascade…). Of the total 151,320 non-redundant transcripts 54.57% were shared by all the tissues. The most different tissues were hemocytes and gill with only 59.55% of shared transcripts. Hemocytes are the immune-related tissue per excellence and it was reflected in the top non-shared transcripts, with a very high percentage of antimicrobial peptides and other immune related proteins. In fact, the hemocytes immune-related profile showed more than 200 fold-increase of expression of AMP transcripts, pore forming molecules, lectins and other genes playing roles in apoptosis and immunity.

# OUTCOMES OF AN EXPERT CONSULTATION TO PARAMETERISE A MODEL FOR RISK CATEGORISATION OF FISH FARMS FOR AQUATIC ANIMAL HEALTH SURVEILLANCE

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Experts were consulted to quantify parameters for a model to generate scores for the likelihood of infection of aquaculture holdings with selected infectious hazards. The hazards were four fish diseases listed by Council Directive 2006/88/EC as endemic in some or several European countries: Infectious salmon anaemia (ISA), viral haemorrhagic septicaemia (VHS), infectious haematopoietic necrosis (IHN), koi herpes virus disease (KHD). The experts assessed the relative importance of 5 risk themes for each hazard to be introduced into and infect susceptible fish at the destination. The themes were: 1) live fish and egg movements; 2) exposure via water; 3) on-site processing; 4) short-distance mechanical transmission and 5) distance-independent mechanical transmission. The experts also estimated parameters for hazard transmission pathways within the themes. The consultation exercise comprised a 2-stage approach: an online survey was followed by a gathering of experts. The opinions were that live fish movements and exposure via water formed the major relevant risk themes. Experts were recruited from several European countries and thus covered a range of farming systems. The conclusions from this expert consultation therefore have relevance across Europe.

*The work was undertaken in a cooperation Art 36 project "Risk categorization for Aquatic Animal Health surveillance" (CFP/EFSA/AHAW/2011/03) of the European Food Safety Authority (EFSA).*

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# ANALYSIS OF FOUR REFERENCE GENES OF BIVALVES (*MYLILUS GALLOPROVINCIALIS* AND *RUDITAPES PHILIPPINARUM*) INFECTED WITH BACTERIA FOR THEIR USE AS INTERNAL CONTROLS IN GENE EXPRESSION STUDIES

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Quantitative real-time PCR is probably the most used method for gene expression quantification because of its high sensitivity and specificity. Nevertheless, this technology can undergo experimental errors and variations. Normalization of the results using a reference gene is therefore necessary to minimize these variations. As the study of immune genes in bivalve mollusks has increased in the last years, the establishment of adequate reference genes for bivalves is strongly required. We analyzed the behavior of four putative reference genes: ribosomal RNA 18S, actin, elongation factor 1-α and α-tubulin. The suitability of these four genes as internal control for qPCR was evaluated in *M. galloprovincialis* and *R. philippinarum* hemocytes after bacterial challenge. For these particular circumstances, the most stable expression both in control and challenged hemocytes was ribosomal 18S. The remaining three genes were not appropriate to be used as reference genes as none of them passed the BestKeeper control.

# EPIDEMIOLOGY OF AMOEBIC GILL DISEASE (AGD) IN CHILEAN SALMON INDUSTRY BETWEEN 2007 AND 2010

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The recent report of amoebic gill disease (AGD) and of *Neoparamoeba perurans* in Chile has made it necessary to develop practical tools that will be useful for carrying out epidemiological studies. AGD was confirmed with PCR specific to N. perurans. The prevalence of AGD in At- lantic salmon was 55.7% (29/52 farms) and the epidemic curve was observed between May 2007 and June 2008 closely related with low rainfall and high salinity (32 ppt). Fish weighing more than 300 g reared in Los Lagos Region during summer and autumn season showed 3.7 (p=0.0004), 4.2 (p=0.0178) and 6.2 (p=0.0031) times greater risk to be AGD positive, respectively. The reduction of Atlantic salmon biomass reared in Chile closely related with ISA crisis could considerably have increased the infection pressure of *N. perurans* to rainbow trout (63.2%, 12/19 farms) and coho salmon (90.9%, 10/11 farms).

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# FIRST MOLECULAR IDENTIFICATION OF *DIPHYLLOBOTHRIUM DENDRITICUM* PLEROCERCOIDS FROM FERAL RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) IN CHILE

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Between April and June 2009, 1,075 feral rainbow trout from 10 different lakes involved with aquaculture activities in Los Lagos Region, Chile, were inspected for *Diphyllobothrium* species. All viscera and muscles of the fish were examined using stereomicroscopy; pyloric cecae and stomachs infected with plerocercoids were checked by histology and scanning electron microscopy. Plerocercoids of *Diphyllobothrium dendriticum* were confirmed by PCR and sequencing of COI and 18S rRNA þ ITS1 þ 5.8S rRNA þ ITS2 genes for the first time in Chile. Overall prevalence of plerocercoids of *D. dendriticum* was 9.2% (99/1,075) in Los Lagos Region and 17.4% (99/570) for Chiloe Island. Plerocercoids were not detected in the continental lakes of the Los Lagos Region (Chapo, Rupanco, and Llanquihue). Tarahuı́n Lake exhibited a prevalence of 50.9% (81/159), Cucao Lake 5.1% (4/79), Natri Lake 4.7% (5/107), Huillinco Lake 3.6% (5/138), and San Antonio Lake 66.7% (4/6). Abundance was 1.1 plerocercoid larvae per fish (1,169 larvae/1,075 fish). All the plerocercoids were found encysted in the viscera of the fish. Plerocercoids were 10.9 6 3 (7–16) mm long by 0.4 6 0.2 (0.2–0.6) mm wide. The scolex was enlarged, with 2 bothria and a frontal pit. The body was covered with short capilliform filitriches, 4–6 mm long. The Chilean COI and 18SrRNA - ITS1 - 5.8SrRNA - ITS2 gene sequences indicated 96.34–96.52% and 99% similarity with *D. dendriticum* sequences, respectively. *Diphyllobothrium dendriticum* is reported for the first time in freshwater ecosystems as far as 438S on Chiloe Island. These findings and previous reports of plerocercoids of *Diphyllobothrium* spp. in farmed rainbow trout at Tarahuín Lake support the putative life cycle of this parasite in lakes of southern Chile where there are aquaculture activities.

# RISK ASSESSMENT FOR THE INTRODUCTION AND ESTABLISHMENT OF SALMON PANCREAS DISEASE VIRUS (SPDV) THROUGH THE IMPORT OF FERTILIZED EGGS INTO CHILE

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Movement of live fish and their products represent one of the practices with highest risk for the introduction of exotic pathogens. The most recent example in Chile was the emergency of ISA in 2007. The objective was to evaluate the introduction and spread risk of *Salmon Pancreas Disease Virus* (SPDV) through the import of salmon eggs into Chile. The identification of the import process of salmon eggs was carried out and a qualitative and semi-quantitative risk analysis was executed. The probability that SPDV enters Chile is extremely low as import is made from free countries and the virus has a low probability to be transmitted vertically. Salmon eggs are healthy certified and import volume has decreased with respect to the volume before to ISA. If SPDV enters Chile, the probability that susceptible species are exposed to a sufficiently high dose so as to originate infection is extremely low. Pancreas Diseases (PD) is in List 1 in Chile and is monitored actively, no vectors have been detected and wild fish analyzed have been negative. The impact of the establishment of SPDV would have moderate consequences in Chile. Import risk of SPDV is acceptable and is consistent with the requirements of the competent authority in Chile. The semi-quantitative analysis showed that the probability to select broodstock infected with SPDV (P(A)) was 0.0800; probability to spawn and fertilize infected fish eggs and that virus survives the first disinfection (P(B)) was 0.07693; probability that SPDV survives the incubation period and the second disinfection (P(C)) was 0.05586; the probability that SPDV survives transport to Chile, the third disinfection and incubation in the destination center (P(D)) was 0.00085 and the probability that PD appears in Chile (P(E)) was 0.001. Spread probability (P(A) x P(B) x P(C)) was 0.0007690 and exposure probability (P(D) x P(E)) was 0.00000166; the probability of SPDV establishment was 0.0000000002922. Consequences were 0.5; therefore, final estimation of risk was 0.0000000004218. After the ISA crisis, regulations were optimized and productive standards and good practices for the industry were developed in order to prevent introduction and spreading of exotic diseases in Chile.

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# STUDY OF THE EFFECTS OF NANO-SILVER PARTICLES ON SOME VITAL TISSUES OF ZEBRA FISH (*DANIO RERIO*) FED VIA ORAL ADMINISTRATION

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This study was initiated to enhance our insight on the health and environmental impact of silver nanoparticles (Ag-np). In this study, 300 Zebra fish with mean weight of (2± 0.5) grams were used. Tests were performed statically based on instructions of OECD under fixed water quality conditions at the temperature 28 and pH 6.8-7 in a completely random trial with nine concentrations treatments of AgNps (0,10, 50, 100, 200, 400, 600, 800 and 1000 mg/gr of food) in three replications. According to the results of acute tests, the 96hr LC50 values were 195.208, 323.696, 486.637 and 804.601mg/L AgNPs for the Zebra fish. Accordingly, investigated colloidal AgNPs are classified as “toxic” to this fish. Clinical signs such as hunched spinal column, thrilling, clot in caudal fin and skin, and irregular swimming were observed in the studied fish specimens. According to the results of chronic toxicity tests, fed via oral administration of AgNPs significantly made the histopathological effects. The most important histopathological effects of AgNPs were observed in the liver (vasculature and exposure, vacuolization and degeneration of some hepatocytes), intestine (Increase the submucosa layer, Narrowing of the intestinal lumen, fusion of parts of the mucosal layer and reduced intestinal absorption ), gills (clubbing of gill secondary lamalea, hyperplasia of gill primary and secondary lamalea, Hyperemia primary and secondary lamalea, and shorten the primary lamalea) and kidney (degeneration in tubules, high increasing in interstitial cells and dilatation of Bowman's space of glomeruli). After biological measurement, heavy metals were measured by spectrum photometry reveal, the greatest bioaccumulation of silver occurred in the liver, gills and muscle of fish respectively. With respect to the observed damages by oral administration of AgNPs, its direct application as antimicrobial agent in aquaculture and also its release into the environment should no longer be allowed.

# RISK CATEGORIZATION FOR MARINE SALMON FARMS – A CASE STUDY OF INFECTIOUS SALMON ANAEMIA (ISA)

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The present study is part of the EFSA funded project "Risk categorization for Aquatic Animal Health surveillance" (CFP/EFSA/AHAW/2011/03). The aim was to develop a methodology for categorizing (risk ranking) fish farms using farm and disease specific characteristics to develop risk based surveillance for demonstration of disease freedom (EU Directive 2006/88/EC). For Norwegian marine salmon farms, infectious salmon anaemia (ISA) was used as a case disease, focusing on 4 main pathways of pathogen introduction and spread: movements of live fish and eggs; ISA virus spread via water connection; distance-dependent exposure of fomites including contaminated avian or mammalian predators, sea lice and mussels; and distance-independent exposure of fomites including contaminated vehicles and equipment. Literature review and expert opinion were used to establish risk estimates of various steps in the pathways. The method accounts for uncertainty and variability of input parameters.
The output is given as a quantitative score that can be applied by Competent Authorities to risk rank fish farms according to their production routines and exposure to infection. Retrospective data from farms at risk of ISA infection during the period 2008-2009 were used for validation of the method. We will present and discuss the principle elements of the risk ranking methodology, as well as specific relevance of ISA in marine salmon farms, model outputs, further development of the risk ranking method including application to other aquatic animal diseases, and challenges.

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# USE OF A SIMULATION MODEL FOR EVALUATION OF CONTROL STRATEGIES OF SALMON PANCREAS DISEASE (PD) IN A HOMOGENEOUS POPULATION OF FARMED ATLANTIC SALMON

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Salmon pancreas disease virus (SPDV) is the etiological agent of pancreas disease (PD) affecting farmed Atlantic salmon. Outbreaks of PD cause substantial economic losses due to high mortality, growth reduction, and reduced flesh quality of fish, etc. Understanding disease behavior is the key of success in disease prevention, control and eradication. Development of PD in SPDV infected salmon can be described using a mathematical modeling approach.
A mathematical model simulating transmission dynamics of SPDV subtype 3 and magnitude of a PD epidemic within a homogeneously mixed population of farmed Atlantic salmon in Norway was made. We monitored time-sequence of changes in the numbers of susceptible, subclinically infectious, clinically infectious, recovered and dead fish over the whole period of the epidemic. The simulation model was calibrated using an iterative process of comparing the simulated number of deaths with observed mortalities, and adjusting the model inputs so that the simulated model represented the actual system. Model validation includes graphical visualization of model outputs and the operating characteristic curve, comparing the time series of the number of dead fish generated by simulation models with the historical series of observed mortality to determine if the simulation mode's output behavior has an acceptable range of accuracy. Subsequently, the simulated model was used for evaluation of effectiveness of various PD prevention and control strategies, including vaccination, functional feed treatment, and early harvesting. An optimal control strategy was determined by measuring the prevalence of infectious fish at the end of epidemic, and the cumulative PD-specific mortality and the concentration of shed virus during the entire epidemic. Our results showed that the simulation model yielded a reasonable capability for describing patterns of SPDV transmission, provided essential information regarding PD epidemic extent, and a useful tool for determining effectiveness of PD control strategies.

# A HYDROLYSIS PROBE BASED INTERNAL qPCR INHIBITION AND EXTRACTION CONTROL SYSTEM FOR RELIABLE DETECTION OF THE CRAYFISH PLAGUE PATHOGEN *APHANOMYCES ASTACI*

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To date, three PCR assays for specific detection of *Aphanomyces (A.) astaci* for crayfish plague diagnostics are established. It was shown, that among them, the qPCR method by Vrålstad *et al.* (2009) has the highest sensitivity. Reproducible positive qPCR results are obtained with DNA from only a single zoospore (Tuffs & Oidtmann, 2011). However, to fulfil the requirements of modern molecular diagnostic techniques, the method lacks proper controls including a positive extraction control and an internal inhibition control. Such controls were established in this study. With a single internal PCR control reaction, the PCR assay is simultaneously controlled for successful DNA extraction, as well as PCR inhibition. This leads to higher reliability of PCR results, by excluding false negative results from test reports.
The control can be used as an endogenous or exogenous system. The choice of the application system depends on the sample material. Primers Deca3F and Deca4R, as well as the probe Deca2P, were designed to amplify and detect a fragment from *Decapoda* DNA, representing the endogenous control, which is used for clinical samples derived from crayfish. The exogenous control is used for non-*Decapoda* samples, e.g. plate cultures. For this purpose a plasmid containing an artificial DNA sequence, with binding sites for Deca3F, Deca4R and Deca2P has to be added to the PCR mastermix. A PCR inhibition can be detected in case of missing fluorescence signals of fluorescence dyes from both, the *A. astaci*-specific hydrolysis probe and the Deca2P hydrolysis probe. Each additional reagent of the control qPCR was tested for its influence on sensitivity of the Vrålstad-qPCR assay. Finally, the method was optimised for use in a multiplex reaction with the *A. astaci* specific method by Vrålstad *et al.* (2009). No negative impact of the control PCR on the specific assay was observed. Therefor the assay can be recommended to use in combination with the *A. astaci* specific qPCR by Vrålstad *et al.*

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# EPIDEMIOLOGICAL TYPING OF VIBRIO PARAHAEMOLYTICUS INFECTING MARINE ANIMALS IN MARICULTURE SYSTEM

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*Vibrio parahaemolyticus*, a zoonotic pathogen, is one of the important causative agents of food poisoning in coastal areas in summer and autumn. In order to understand the epidemiological typing of *V. parahaemolyticus* in China 82 strains of *V. parahaemolyticus* were collected from clinically ill penaeid shrimp, sea crab and marine fish in main mariculture districts using Thiosulfate Citrate Bile salt Sucrose (TCBS) agar combined with biochemical identification and HSP60 gene sequencing. Multilocus sequence typing (MLST) scheme was then conducted on all of the 82 isolates by comparing a partial DNA sequence of seven housekeeping genes (recA, dnaE, gyrB, dtdS, pntA, pyrC, and tnaA) recommended by pubmlst (http://pubmlst.org/*v. parahaemolyticus*). Sequence data from both strands were assembled and entered into the *V. parahaemolyticus* MLST database for comparison to existing allele types. With 38 new sequence types (STs) and 32 novel allele types being identified, the MLST scheme defined 44 sequence types in all. However, a cluster analysis of ST demonstrated that 37 among which were unique. Though so many new sequence types were partially due to a small MLST database of *V. parahaemolyticus*, the results of this study still revealed high genetic diversity within the species of *V. parahaemolyticus* infecting marine animals in mariculture system.

# AN UNKNOWN MICROSPORIDIAN PARASITE OF PIKEPERCH (*SANDER LUCIOPERCA*) AND PERCH (*PERCA FLUVIATILIS*) IN FINNISH LAKES

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Microsporidia parasites are found in many fish species. We studied an unknown microsporidian parasite that was found from the skeletal muscles of two fish species, pikeperch (*Sander lucioperca*) and perch (*Perca fluviatilis*) that are important species for fisheries in Finland.
We found two forms of the parasite, which we believe to represent the same species. We referred to the first form as “non-xenoma form”. This non-capsulated form was rare, but it occupied a large portion of the fish muscle appearing as white spots that were easily seen with the naked eye and existed as free in the muscle tissue. The second form was much more common and we referred to it as “xenoma form”. This form was enveloped by a thick, brownish and melanised cyst. The non-xenoma form occupied a larger portion of the fish muscle and appeared as white spots that can be easily seen visually. The (small-sized) xenoma form occupied a lesser portion of the fish muscle and thus it was more difficult to observe without a microscope. Both forms were found in same fish individuals but occurred together only rarely.
The main study questions were a) How common is the parasite in perch and pikeperch populations? b) Are there genetic differences between microsporids of pikeperch and perch? c) Are there genetic differences between two different forms of the parasite?
Occurrence of the parasite was variable. In pikeperch it occurred in 5 out of 6 lakes studied (n=336 fish) with maximum prevalence of 25.5 %. The microsporidian existed in both sexes and also in young fish. In perch the parasite occurred in 4 out of 6 lakes (n=126, two of the study lakes same as for pikeperch). The maximum prevalence in perch was 20.0 %
Genetic studies are still on going. However, the rDNA results so far indicate that 1) the non-xenoma form and the xenoma form and 2) the microsporidian found in perch and pikeperch are the same species genetically. Comparison to known sequences revealed no close relatives to this species but suggested relationship with *Pseudoloma* or *Loma* genera.

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# DIGENEAN PARASITES OF FEW TELEOST FISH FROM GULF OF TUNIS

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Parasites showing the greatest biodiversity represent about 40% of the world's known species but the discrete life of these organisms contributes to their neglect for long time by ecologists. Later, parasites are considered as an effective tool to provide information on the ecosystems structure and function, the diet and the migration of the hosts. Despite the ecological importance, parasites and especially digeneans are still little known in the eastern Mediterranean and in Tunisia.
To improve our knowledge of this group of parasites, we are interested in this work to study the diversity of digeneans of a few teleost fish from the Gulf of Tunis.
The digenean fauna of 140 fish (23 *Trachurus trachurus,* 15 *Trachinotus ovatus,* 23 *Sardinella aurita,* 20 *Mullus surmuletus,* 19 *Mullus barbatus,* 25 *Scorpaena notata* et *15 Merluccius merluccius*) consists of the 6 species *(Bucephalus margaritae, Opecoeloides furcatus, Monascus filiformis, Prodistomum polonii, Ectenurus lepidus et Helicometra fasciata).*
The special study of the distribution of digenean species within the host shows that *Ectenurus lepidus* is limited to the stomach; others parasites (*B. margaritae, M. filiformis, P. polonii, O. furcatus, H. fasciata*) colonize 2 or 3 levels of intestine.
The analysis of parasite infracommunities shows that the majority of host species (61,94%) harbour only one species of parasite and the occurrence of two (28.06%) or three (10%) is much less frequent.
The most diverse digenean fauna was found in *Trachurus trachurus* (3 species). *M. filiformis* and at the lessen degree *P. polonii* seem to be the most frequent parasites. Other fish (*S. aurita M. surmuletus, M. barbatus, S. notata* and *T. ovatus*) showed a lower diversity, not exceeding 1 parasite. No species of parasites were harvested in *Merluccius merluccius.*
Our results show that parasites are generally absent in small fish (10-12 cm). Moreover, the values of parasitological indices augment generally with increasing size of the host. This is probably related to a qualitative and quantitative change of prey consumed, carrying infective stages, depending on the size classes of the host.

# NEW EVIDENCES OF *PARACARTIA GRANI* (COPEPODA, CALANOIDA) INVOLVEMENT IN *MARTEILIA REFRINGENS* (PARAMYXEA) LIFE CYCLE

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Around 10% of the national shellfish production in France are coming from Thau lagoon. Dynamics of the protozoan parasite *Marteilia refringens* was investigated monthly during a year into three suspected host species in this lagoon. The targeted species were the Mediterranean mussel *Mytilus galloprovincialis*, the grooved carpet shell *Ruditapes decussatus* and the copepod *Paracartia grani*. Samples were first screened by PCR and positive results were then confirmed in mussels by histology and in *P. grani* and clams by *in situ* hybridization (ISH). ISH performed on *R. decussatus* showed *M. refringens* necrotic cells in digestive epithelia suggesting that this species is not involved in *M. refringens* life cycle in Thau lagoon. In opposition, presence of different parasite stages in mussels in histology and ISH positive labelling observed in some copepod sections, indicate that these two species contribute to *M. refringens* cycle at our study site. The observations of *M. refringens* mature sporangia in spring and autumn in mussel suggest that (i) the parasite has two cycles per year in Thau and (ii) that mussels could release parasites from spring to autumn. PCR detection of *M. refringens* in *P. grani* copepodid stages between June and November supports the hypothesis of the transmission of the parasite from mussels to copepods but also from copepods to mussels. Indeed, a new peak of infection is observed in mussels at the end of summer, when *P. grani* abundance and PCR detection are maxima. ISH analyses performed on *P. grani* copepodites showed unusual parasite plasmodial like cells in digestive tract and gonad from the 3rd copepodid stage. In addition, mussels efficiency retention was measured on all developmental stages of *P. grani* (from eggs to adults). Results suggest that all *P. grani* stages could contribute to the transmission of the parasite to mussels, especially eggs and nauplii which were retained by mussels up to 90 %. The PCR detection of parasite DNA in *P. grani* eggs from *M. refringens* PCR positive females let think that eggs could contribute to the parasite spreading in the water and could allow *M. refringens* overwintering. Our results contribute to better understand relationships between the parasite, bivalves and copepods.

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# INVESTIGATIONS ON THE CERCARIAE OF DIGENETIC TREMATODES IN LAKE BALATON AND ITS TRIBUTARIES

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The trematode flatworms of phylum Platyhelminthes are important human and animal pathogens. They have a complicated life cycle with one ore more intermediate host. The first intermediate host is often a mollusc. The developmental stages of trematodes in molluscs are well represented in the scientific literature, but the whole developmental cycle is known only in a few cases. Among the larval stages living in molluscs, the cercariae show some morphological features of the adult worms, therefore it is possible to identify them by morphological examinations up to the genus level.
During our study, snails and bivalves were collected form the Lake Balaton and Kis-Balaton Reservoir, Hungary. The cercaria were isolated from molluscs kept individually on so called „cell well plates" using distilled water or photoperiodic induction. The cercariae were morphologically determined before further examinations.
The collected cercaria samples were examined by molecular methods, during the investigation the internal transcribed region (ITS) were amplified. Up to now, we have partial or complete sequences from 17 samples. One of the samples showed a 99,4% similarity to the species *Echinostoma revolutum*. Four of them were identified as the larval stage of *Echinoparyphium recurvatum* (97.4 -100%). Two samples belonged to the *Trichobilharzia frankii* (99,9%), which can cause human dermatitis forming itchy red spots on the human skin. *Diplostomum pseudospatheceum* (99,9%) was observed too, which metacercariae infect the eye lens of fresh water fishes. Several samples could not be identified on species level, because there are only a few available sequence data regarding those genera and species. Two of those unidentified samples belong to the genus *Plagiorchis*, one of them is an *Echinochasmus*, and one cercaria was a specimen of genus *Thylodelphis*. . Furthermore, one of our sample was morphologically identified as *Apatemon strigea*, but there are no published sequence data about this species, therefore exact identification was not possible as well. The incomplete identifications suggest that it is necessary to gain sequences from the larval stages (metacercariae) of the fishes too, because there is a possibility that cercariae can be identified by matching sequences of metacercariae. So far we have only four metacercariae sequenced, two of them from the genus *Paryphistomum* collected from rudd and roach, and the remaining two are *Echinocasmus* metacercariae from tench and Chinese sleeper.

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# PARASITES OF *MORA MORO* (RISSO, 1810) FROM THE WESTERN MEDITERRANEAN SEA: A PILOT STUDY

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The common mora, *Mora moro* (Risso, 1810), a cosmopolitan bathypelagic fish species of moderate commercial interest, inhabits at depths between 450 and 2,500 m. This species, which is one of the main contributors to biomass at depths of 1,000-1,200 m in the western Mediterranean Sea, is a large species with high energy requirements and a generalized diet. Very few studies exist on the parasite fauna of this species and there are no parasite records from the Mediterranean. The aim of this study is to reveal the parasite fauna of *M. moro* in the western Mediterranean and to assess the seasonal and geographical variability of parasite communities of this deep water marine fish species. A total of 77 individuals was collected in 2010 and 2011 at depths between 1,000 and 1,400 m, during two seasons (summer and autumn) and at three different locations of the Balearic Sea in the western Mediterranean: two on the continental margin (Barcelona and Tarragona) and one on the insular margin (Balearic Islands). Fish were dissected and analyzed according to a standardized parasitological protocol. Eighteen parasite taxa were found; five digeneans, three cestodes, nine nematodes and one acantocephalan. There was a marked dominance of larval forms. The highest prevalences and abundances were achieved by the larval nematode *Anisakis* type II (96%, 10.73, respectively), the larval tetraphyllidean cestode *Scolex pleuronectis* (95%, 21.48, respectively) and the larval nematode Anisakidae gen. sp. (77%, 31.94, respectively). Anisakidae gen. sp. and *S. pleuronectis* showed significant differences for abundances between continental and insular margins (Generalized Linear Models, $\chi^2_4 = 100.159$, $p < 0.001$; $\chi^2_4 = 39.061$, $p < 0.001$). Significant differences in the abundance of the digenean *Lepidapedon* sp. and a larval trypanorhynch cestode were observed between localities and seasons (Generalized Linear Models, $\chi^2_4 = 46.259$, $p < 0.001$; $\chi^2_4 = 14.722$, $p = 0.005$), the former being significantly less abundant in fish sampled off Barcelona in summer, and the latter significantly less abundant in fish collected at Barcelona in autumn, than in the rest of localities and seasons.

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# PROLIFERATIVE KIDNEY DISEASE MODULATES THE TRANSCRIPTION OF CC AND CXC CHEMOKINES IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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The myxozoan parasite, *Tetracapsuloides bryosalmonae,* is the aetiological agent of Proliferative Kidney Disease (PKD), an emerging disease of farmed and wild European and North American salmonid populations. Extrasporogonic proliferative stages within the trout kidney induce a considerable lymphocytic hyperplasia and development of granulomatous lesions, which leads to a gross swelling of the kidney in advanced clinical stages. Chemokines, as chemotactic agents, are important mediators of immune responses to pathogens and, thus, have the potential to be exploited as immunotherapeutic tools to counteract disease pathogenesis. Several families of chemokines have been recently characterized in fish some of which are known to be modulated by pathogens, although their modulation in response to myxozoan parasites still needs to be characterised.
CC and CXC chemokines form the two largest groups, including molecules with both inflammatory and homeostatic functions. A RT-qPCR survey was carried out on kidneys obtained from farmed rainbow trout (*Oncorhynchus mykiss*) during a natural outbreak of PKD. The transcription of CC and CXC chemokine genes was examined in relation to parasite burden by monitoring the abundance of the *T. bryosalmonae* homologue of the house-keeping gene, Ribosomal Protein L18 and clinical kidney swelling. The expression of several CC and CXC chemokines, including fish specific transcripts, correlated positively with PKD clinical progression and parasite burden with CXCL13 being the most highly induced. Interestingly, CXCL_F1c and CK8 were significantly down-regulated and/or negatively correlated with infection readouts.
The potential to exploit trout chemokines as future therapeutic tools to alleviate the chronicity of PKD pathogenesis is discussed.

# PARASITES OF BLACKSPOT SEABREAM, *PAGELLUS BOGARAVEO*, AS BIOLOGICAL TAGS FOR STOCK IDENTIFICATION IN NORTH-EAST ATLANTIC PORTUGUESE WATERS

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The blackspot seabream, *Pagellus bogaraveo* (Brünnich, 1768), is an important sparid fish targeted mostly by Portuguese and Spanish fisheries in the north-east Atlantic. In this study, 348 wild blackspot seabream from 6 different localities in Portuguese waters were subjected to a parasitological analysis. Sampling was carried out in 4 localities off the mainland Portuguese coast and also the archipelagos of Madeira and Azores.
Thirty-seven parasite taxa were detected, including twenty-four new records in this host. The parasites *Diphterostomum vividum* (Digenea: Zoogonidae), *Anisakis simplex* s.l., *A. physeteris*, *Anisakis* sp. PB-2010 (Nematoda: Anisakidae), and *Bolbosoma* sp. (Acanthocephala: Polymorphidae) were selected as biological tags for blackspot seabream stock identification in the north-east Atlantic. Results point to the existence of three *Pagellus bogaraveo* stocks in this Atlantic area: one in the Azores region (ICES Area X), one in continental Portuguese shelf/slope waters (ICES Area IXa), and a third in the waters around Madeira (sub-area 1.2 of FAO 34, central-eastern Atlantic).

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# MOLECULAR CLONING AND EXPRESSION ANALYSIS OF PEPTIDASE GENES IN THE FISH-PATHOGENIC SCUTICOCILIATE *MIAMIENSIS AVIDUS*

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Parasite peptidases have been actively studied as vaccine candidates or drug targets for prevention or treatment of parasitic diseases because of their important roles for survival and/or invasion in the host. Like other parasites, in the facultative histophagous *Miamiensis avidus*, peptidases may be responsible for important roles in the process of transforming into the infectious stage to invade the host tissues and survive. The 17 genes encoding peptidases, including seven cathepsin-like cysteine peptidases, four serine carboxypeptidases, a eukaryotic aspartyl protease family protein, an ATP-dependent metalloprotease FtsH family protein, three leishmanolysin family proteins and a peptidase family M49 protein were identified from a *Miamiensis avidus* cDNA library by BLAST X search. The deduced amino acid sequences of the isolated genes have typical features of corresponding peptidase family proteins. As results of RT-PCR, differential mRNA expression of two cysteine peptidases, three leishmanolysin family proteins, and a peptidase family M49 protein gene were shown in *M. avidus* grown under the cell-feeding culture conditions suggesting the crucial roles of peptidases in tissue invasion. In conclusion, the genetic information obtained from this study could help to design specific vaccines and inhibitors of peptidase proteins, for prevention and control of fish scuticociliatosis.

# OUTBREAK OF PLEROCERCOIDS *DIGRAMMA INTERRUPTA* IN AZOV ROACH (*RUTILUS RUTILUS HECKELI*)

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The focus of ligulosis – dirammosis has been existed in waters of the Azov sea basin for many years. The presence of a number of factors – shallow water, good warming of water, intensive vegetation of water plants, abundance of fish eating birds – definite hosts of parasites, create favorable conditions for the circulation of causative agents of the disease.
The data were collected in summer of 2012 in Don River delta and eastern part of the Azov Sea during ichthyologic survey on ship at standard points in sea and in the river. Total 3600 specimen of fish were taken for clinical, parasitological ad ichthyological investigations. Further studies were provided using routine methods in parasitology and ichthyology.
The age structure of diseased fish was presented by two generations of two-year old fish and three – year old fish. All studied fish were invaded by *Digramma interrupta.* During post-mortem autopsy the atrophy of the internal organs caused by the mechanical action of plerocercoids, developing in the body cavity, was marked. The fish were depleted, fat reserves were too small, and food masses were absent in the gastro-intestinal tract. It was found that infected fish had lower growth rate.
The size of detected parasites *Digramma interrupta* varied within the following limits: length = 3,5 - 67,5 cm, width = 0,2 - 1,5 cm. The analyses of gathered material showed that prevalence of invasion by *Digramma interrupta* was equal 100% in all studied points. The highest intensity of invasion II = 1-6 specimen and average intensity of invasion $II_a$=2,3 ± 0,16 specimen, and also index of abundance IA=2,3 ± 0,16 specimen were registered in Taganrog bay in the Azov sea. Roach in the majority had 2-3 invasive plerocercoids, but others were not invasive and had the sizes 3,5–6 – 6,5–8 cm. In the analyzed material of three – years old fish the parasites of too large sizes were registered. They caused pathological influence on fish. Three year old roach (15-15,5 cm in length) were invaded by 1 to 6 warms with the length 66-67,5 cm. There were no significant sex differences in prevalence of invasion. In some cases, the definition of fish sex was hindered by the degradation of the gonads.
The circuit simulation was used to study the formation of helminthes cenosis in aquatic systems of the Azov sea basin. The role of helminthes – indicators of food components species was taken into account as the foundation of structure of helminthes cenosis. Favorable development of representatives of family Ligulidae depends on direct influence of environmental factors whish determine the quantity of intermediate and definitive hosts, the regions of possible distribution of the parasites, development of the preventive measures in the waters of southern Russia.

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# THE ROLE OF PARASITISM IN THE EVOLUTION OF CONSISTENT INDIVIDUAL BEHAVIOURAL DIFFERENCES

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Consistent, individually characteristic, expressions of behavioural traits (i.e. personalities) and their evolutionary importance are currently a topic of intensive research interest among aquatic and terrestrial ecologists. We, together with other researchers, have suggested that parasites and pathogens may provide an ultimate explanation for the evolution and diversification of animal personalities. This proposition is based on the negatively frequency-dependent selection generated by parasites and pathogens - which are ubiquitous. By inducing and maintaining genetic variation in host immune function, parasites affect the optimal behaviour of individuals. This occurs if the fitness benefits and costs of different behavioural types are dependent on individuals' immunological capacity. In this scenario (that contradicts recent ideas based on the structure of pace-of-life syndromes), individuals that are genetically resistant or able to improve parasite resistance through high food intake rate behave more boldly than less resistant individuals. Moreover, the stronger is the risk of parasitism, the more strictly individuals are predicted to follow their optimal behavioural trajectories. Therefore, consistent individual differences in behaviour should most commonly be detected in highly parasitized populations.

# *CARDICOLA LARUEI*, A BLOOD FLUKE OF THE SCIAENID *CYNOSCION NEBULOSUS*: ELUCIDATION OF THE LIFE CYCLE INVOLVING TEREBELLID POLYCHAETES

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*Cardicola laruei* is an aporocotylid whose definitive host includes the sciaenid, *Cynoscion nebulosus*. In South Carolina (USA) *C. nebulosus* is commonly infected by this blood fluke. Adult worms live in the lumen of the heart where they release eggs, many of which become encapsulated in both the bulbus arteriosus and the myocardium. Aporocotylids are unique among Digenea with development of larval forms in polychaetes (Annelida) or bivalves. Few cercariae of the family Aporocotylidae have been described previously and the complete life cycle has been elucidated for only two species that develop in marine polychaetes. Examination of *Enoplobranchus sanguineus* and *Amphitrite ornata*, both terebellid polychaetes collected from the South Carolina coast revealed infections with sporocysts and cercariae not previously described. The cercariae observed from *E. sanguineus* and *A. ornata* most closely resemble the cercariae of the family Aporocotylidae. The morphological characteristics of the cercariae include being apharyngeate, brevifurcate, and possessing an anterior organ instead of an oral sucker. Analysis of lsrDNA and ITS-2 sequences from sporocysts and cercariae revealed close identity with the *Cardicola* clade of the Aporocotylidae, yet these were not identical to any sequence available from GenBank. Conversely, ITS-2 sequences revealed 100% identity of sporocysts dissected from the coelom of both polychaetes. Previous studies in the region identified high prevalence rates of *Cardicola laruei* in spotted sea trout and ITS-2 sequences confirmed conspecific infections in spotted seatrout and the terebellid polychaetes. This is the first report of larval aporocotylids in *E. sanguineus* and *A. ornata* and the first confirmation of intermediate hosts for *C. laruei*.

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# INTRASPECIFIC GENETIC DIVERSITY OF DIDYMOSULCUS KATSUWONICOLA PARASITISING BLUEFIN TUNA (*THUNNUS THYNNUS*) GILLS

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In total five loci of a nuclear (ITS1, ITS2) and mitochondrial (COX, COI, ND1) DNA were used to asses intraspecific genetic diversity of a didymozoid digenean species Didymosulcus katsuwonicola, parasitising gills of Atlantic bluefin tuna (Thunnus thynnus). It is the most prevalent and abundant parasite species in tuna reared in the Mediterranean, whose population numbers have been shown to decline during the rearing period of one and a half years. These hermaphroditic didymozoids are propagated to bluefin as cercaria through ingestion of infected small pelagic fish and cephalopods that further migrate from the digestive system of the final host to its highly vascularized gill tissue. In gills they encyst always in pairs, although they are hermaphrodites that do not need each other for reproduction. Generally, there is a lack of knowledge about pathways of their migration from gut to gills or mechanisms that cercaria might use to find each other in gill tissue and encyst in pair. This for, our goal was to assess if two individuals of D. katsuwonicola encysted in a single cyst share the same genotype, how much such genotype differs from the genotypes of individuals from other cysts present in the same or different host. Results showed that most of individuals encysted together have similar size, with only few exemption when juvenile individuals were found encysted alone or together with an adult. This suggests that formation of the early tissue cyst precede the “meeting” of two individuals. Intraspecific diversity showed that intricate infection and reproduction mechanisms exist in this didymozoid, presumably as an adaptation for successful fulfillment of their life cycle.

Keywords: Bluefin tuna, Thunnus thynnus, Digenea, Didymosulcus katsuwonicola

# PARASITE COMMUNITIES OF *LEPIDION LEPIDION* (TELEOSTEI: MORIDAE) AS INDICATORS OF NATURAL VARIABILITY IN THE MIDDLE SLOPE FROM THE WESTERN MEDITERRANEAN SEA

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*Lepidion lepidion* (Risso, 1810), endemic in the Mediterranean Sea, is the most abundant morid and the dominant species in the Balearic deep-sea between 1000 and 1500 m depth, and subdominant below this depth. The parasite fauna of *L. lepidion* is completely unknown. Samples of *L. lepidion* were obtained seasonally from the middle slope at two different localities (Barcelona and Vilanova) at depths between 425 and 1100 m during 2007 and 2009. A total of 101 individuals were examined for parasites. Prevalence and abundance of parasites were grouped according to season and locality of capture. Sixteen endoparasite taxa were found: two digeneans, four cestodes, two acantocephalans and eight nematodes. Total mean abundance of parasites was significantly higher in Vilanova summer than in Barcelona summer and Brillouin's diversity index was significantly higher in Barcelona spring than in Barcelona winter. The acantocephalan *Echynorhinchus gadi* was the most prevalent and abundant parasite species in all sampled fish (56.4% and 1.83, respectively), especially in Vilanova summer (prevalence of 61.5%). The nematode *Capillostrongyloides* sp. was the second parasite in prevalence and abundance (36.6% and 1.26, respectively), being the most common and abundant parasite in Barcelona summer (66.7% and 2.0, respectively). Adults and juveniles of the digeneans *Bathycreadium elongatum* and *Lepidapedon* sp. were found within the digestive tract with prevalences between 25 and 36%. *Capillostrongyloides* sp., *E. gadi* and *B. elongatum* were the species which contributed most to the dissimilarities among the locality/season categories, being considered as key discriminating species in the majority of contrast associated with the spatial/temporal assessment. Deep-water fauna is believed to be homogenous, however the differences found between these close localities point to seasonal and local differences in the intermediate host distributions.

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# ACETYLCHOLINESTERASE (ACHE) ACTIVITY IN COD (*GADUS MORHUA*) AND ITS NEMATODE PARASITES (*ANISAKIS SIMPLEX* AND *CONTRACAECUM OSCULATUM*).

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Acetylcholinesterase (AChE) is one of the most important enzymes involved in nerve impulse transmission in both vertebrates and invertebrates. The main role of this enzyme is to regulate the acetylcholine levels by the rapid hydrolysis of acetylcholine into the inactive products choline and acetic acid. The AChE activity of cod (*Gadus morhua*) and its parasites *Anisakis simplex* and *Contracaecum osculatum* has been measured spectrophotometrically. Fish has been caught in 2 regions of the southern Baltic Sea (the Polish Exclusive Economic Zone, EEZ). This is the first documentation of the presence of AChE in *Contracaecum osculatum* and first comparison of AChE activity of cod and its nematode parasites. Generalized Linear Models (GLMs) were applied to analyze AChE activity in host and parasite tissue in relation to biological parameters of the fish. The effect of the sampling region has been taken into the account. AChE activity of cod was higher in samples from the western area compared with fish caught in central area of Polish EEZ. Inverse relationship has been observed in *A. simplex* AChE activity. Similar level of average AChE activity has been observed for both nematode species.

# PRELIMINARY DATA ON THE PARASITIC FAUNA OF *ALEPOCEPHALUS ROSTRATUS* RISSO, 1820 IN THE BALEARIC SEA

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*Alepocephalus rostratus* Risso, 1820 is the only species of the family Alepocephalidae in the Mediterranean Sea and it's exclusive for the western basin. *A. rostratus* can be found at depths between 800 and 2250m reaching its maximum abundance between 1000-1400m. This species mostly feeds on macroplanktonic organisms (mainly gelatinous) and it's adapted to food scarcity. This study is the first attempt to describe the parasitic fauna associated to the fish *A. rostratus* in the Mediterranean Sea. Samples were obtained with a semi-balloon otter-trawl on the ANTROMARE project in 2010. Specimens of *A. rostratus* were collected at depths of 1000-1400m and 1400-2000m in two localities (Barcelona-Besòs and Mallorca). All material was processed by routine parasitological techniques. Seven different taxa were found: the digenean *Paraccacladium jamiesoni*, the monogenean *Paracyclocotyla cherbonnieri*, plerocercoids of Tetraphyllidean cestode, and the nematodes *Anisakis* Type II, *Capillostrongyloides* sp., Cucullanidae gen. sp. and *Hysterothylacium aduncum*. Except for *Capillostrongyloides* sp. and *Paracyclocotyla cherbonnieri*, all parasites were immature forms. Tetraphyllidean plerocercoids, reported under the collective name *Scolex pleuronectis*, were found in all host specimens (100% of prevalence). The most abundant parasite and the second in prevalence was the 2nd stage larvae of Cucullanidae gen. sp. Significant differences on the abundance of Cucullanidae gen. sp. were observed between depths (Generalized Linear Model, $\chi^2_3 = 85.87$, $p < 0.001$), being higher at 1000-1400m in both localities. *Paraccacladium jamiesoni* was found in 32% of host individuals. Larval stages of this digenean are commonly found in gelatinous plankton, the main food source of this deep-water fish. More studies are needed in order to elucidate the natural diversity of the parasite fauna of *Alepocephalus rostratus* and their potential use as biological tag.

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# CAN INVASIVE BROOK TROUT (*SALVELINUS FONTINALIS*) ACT AS A HOST FISH FOR THE FRESHWATER PEARL MUSSEL (*MARGARITIFERA MARGARITIFERA*) IN EUROPE?

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Most of the freshwater pearl mussel (*Margaritifera margaritifera*) populations live in Europe and North America. This species uses salmonid fish as its host in early life parasitic stage. In Europe, the host species are Atlantic salmon (*Salmo salar*), and brown trout (*S. trutta*), while in North America the main host fish is thought to be brook trout (*Salvelinus fontinalis*). During the last decades, brook trout has spread out its territory efficiently with for example many introductions, and it is now common species in many parts of Europe, too. It is considered as an aggressive invader, which may replace the original salmonid fish, brown trout, in brooks and streams. Thus, an interesting question is, whether the European *M. margaritifera* can successfully infect brook trout, as the American *M. margaritifera* is doing, and furthermore, can this American species be a threat for *M. margaritifera* by decreasing the number of its suitable, original host fishes. We studied these questions by infecting brook trout (and salmon and trout as comparison) in laboratory and field cage experiment, and by electrofishing in pearl mussel rivers. Both in laboratory and field cages, it was observed that only about 20 % of brook trouts got infected, which was significally lower prevalence than in the native host species (mostly 100 %). In laboratory study it was also noticed, that all glochidium larvae dropped off from brook trouts in 3 months, as undeveloped. Also the total numbers and growth rate of glochidium larvae was significally lower in brook trout in both in the two laboratory tests and in cage experiment. In electrofishing from the field, no infected brook trout was caught. Altogether, the American invader, brook trout appeared to be not suitable host for the European pearl mussel populations. Therefore introduction and spreading of this species to European pearl mussel rivers should be avoided in the future.

# PHYLOGEOGRAPHY AND POPULATION GENETICS OF THE ENDANGERED FRESHWATER PEARL MUSSEL (*MARGARITIFERA MARGARITIFERA*) STUDIED USING MITOCHONDRIAL DNA

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The freshwater pearl mussel (*Margaritifera margaritifera* L.) is one of the most endangered freshwater mussels in the world. Freshwater pearl mussel populations are declining in Europe as a result of increasing human activity and habitat loss. In Finland, at the start of the last century, there were 200 pearl mussel rivers—at the end of the century there were only 70 rivers where the species occurred. This study investigated the genetic structure and variability of 17 freshwater pearl mussel populations originating from Finland, Russia, Ireland and Spain using mitochondrial cytochrome oxidase subunit I sequences (*COI*). *COI* sequences of 11 populations were from the NCBI gene bank and *COI* sequences of the rest 6 populations were collected from those populations for this study. By their haplotype richness, number of unique haplotypes and diversity index, Finnish and Russian populations were genetically more diverse than those from Ireland and Spain. Haplotypes from geographically closer populations showed less difference in their nucleotides than haplotypes from distant populations. Results show a high degree of differentiation of Finnish pearl mussel populations and thus emphasize how important it is to maintain these populations in future. The *COI* gene also indicates the existence of two evolutionary lineages: one extending northwards from Ireland and the other extending southwards from Ireland.

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# FURTHER CHARACTERIZATION OF *WEISSELLA CETI* INFECTIONS IN BRAZILIAN RAINBOW TROUT FARMS AND DEVELOPMENT OF AN ADJUVANTED VACCINE

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*Weissella ceti* is an emerging bacterial pathogen affecting rainbow trout farms with several outbreaks recently described in China, Brazil and United States. Currently, the genetic diversity of *W. ceti* and the efficacy of vaccines against disease are poorly understood. The aims of this study were to genotype *Weissella* ceti strains isolated from distinct geographic origins and to determine the efficacy of an adjuvanted vaccine against the illness. Between 2010 and 2012, outbreaks were recorded in five Brazilian farms with no epidemiological linkage among them. A total of 34*Weissella ceti* isolates were genetically characterized by REP-PCR, ERIC-PCR and PFGE. Two different *Weissella ceti* vaccines were tested: a bacterin and an adjuvanted vaccine. Their efficacy was evaluated in rainbow trout fingerlings and adults, at 30 and 60 days post vaccination. *Weissella ceti* was shown to be a high homogeneous population in Brazil, with genotypes clonally related, being responsible for outbreaks around the country. Adjuvanted vaccine promoted the best ($p< 0,05$) protection against disease, reaching RPS values of 100%in fingerlings and adults at 30 and 60 days post vaccination. Bacterin presented RPS values of73% and 20% at day 30 in adults and fingerlings, respectively. At 60-day post immunization the bacterin was not able to protect the fish (RPS 0%). Our results suggest a clonal population structure of pathogenic *Weissella ceti* in Brazilian farms of rainbow trout. The adjuvanted vaccine provided an effectively long-term protection of fingerlings and adults against infection.

# THE UTILITY OF EXPRESSION LIBRARY IMMUNIZATION TOWARDS THE DEVELOPMENT OF A VACCINE AGAINST PROLIFERATIVE KIDNEY DISEASE IN FARMED SALMONIDS

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Proliferative kidney disease (PKD), caused by the myxozoan parasite *Tetracapsuloides bryosalmonae*, is one of the most economically important diseases facing rainbow trout aquaculture in Europe and North America. The disease is driven by the temperature-dependent release of parasite spores from the bryozoan, *Fredericella sultana*. Parasite proliferation in fish kidney tissues causes a chronic lymphoid hyperplasia giving rise to the characteristic kidney swelling response. Importantly, recovering fish acquire protective immunity to subsequent parasite encounters, thus providing the impetus for vaccine development.
We have created a pipeline for antigen discovery by developing host-specific parasite genomic resources, including cDNA libraries from bryozoan-derived parasite spore sacs and trout kidney-derived laser capture microdissected parasites. We have, to date, utilized a bryozoan-derived parasite library to aid identification of protective antigens by expression library immunization (ELI). Rainbow trout were DNA immunized at a PKD-free site and subjected to a natural exposure to the parasite, at a trout farm characterized by recurring annual epizootics, 8 weeks post-immunisation. Protection was determined by assessing the extent of kidney swelling using the kidney swelling index system devised by Clifton-Hadley and colleagues and by Q-PCR detection of viable parasites based on the parasite house-keeping gene, 60S ribosomal protein L18 (RPL18). From our ELI studies we have identified a protective gene pool (R-14) consisting of 182 contiguous sequences. Following bioinformatic analysis, several known / unknown putative surface-expressed antigens have been identified in R-14, including tetraspanins, antioxidant proteins and heat shock-related genes all with homologues known to be highly immunogenic in other host-pathogen interactions. Some of the unknown antigens possess a signal peptide and hydrophilic amino acid repeats that could be targeted by cytophilic antibodies.
Further studies are currently underway to determine the individual antigen(s) in R-14 eliciting protective immunity and to employ next generation sequencing methods to further enhance the future discovery of *T. bryosalmonae* protective antigens by identifying host-specific parasite transcripts.

# THE STIMULATION OF THE INNATE IMMUNE SYSTEM BY SJNNV PROTECTS JUVENILE EUROPEAN SEA BASS (*Dicentrarchus labrax*) AGAINST SUPERINFECTION WITH RGNNV

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European sea bass is highly susceptible to the infection by Viral Nervous Necrosis Virus (VNNV) whose genome is composed of two positive sense and single-stranded RNA segments: RNA1 (encoding the RNA dependent RNA polymerase, RdRp) and RNA2 (encoding the coat protein, CP). Betanodaviruses have been classified into four genotypes based on the sequence of the T4 region of the CP coding gene, although only the SJNNV and RGNNV genotypes have been detected in the Mediterranean area to date. Furthermore, the coexistence of these two genotypes in the same individual has been recorded by molecular techniques in a high percentage of wild and farmed fish species.
The interferon (IFN) system is the first defense against fish viral infections. Mx is the most studied IFN-induced protein; having being shown that some of them possess antiviral activity against fish viruses. The aim of the present study has been to determine the effect of the SJNNV-RGNNV coexistence on the pathogenesis of the RGNNV in European sea bass and to establish the role of the IFN-mediated immune system in the course of the RGNNV infection under coexistence.
In this study, viral replication and transcription of innate immunogenes have been determined by RT-real time-PCR (RT-qPCR) in the course of an experimental infection. Three different experimental conditions were considered: i) RGNNV-inoculated animals; ii) SJNNV-inoculated animals and iii) animals inoculated with SJNNV and superinfected with RGNNV. Superinfection was performed 24 h after the SJNNV inoculation. Control animals were mocked-injected with L-15 medium.
The RGNNV-infected group showed typical symptoms of the disease and displayed 76% cumulative mortality at the end of the experiment, whereas the mortality in the superinfected group was 4%, and no mortality was recorded in the SJNNV-inoculated group. The analysis of the Mx transcription by RT-qPCR showed a clearly differential induction of the sea bass innate immune system by RGNNV and SJNNV, since no transcription was recorded at any time tested (from 0 h to 48 h p.i.) after the RGNNV inoculation, whereas the injection of SJNNV resulted in an important increase of the Mx transcription from 24 h p.i. onwards. In the superinfected group the induction of the Mx gene transcription follows the same patterns that the ones described for the groups inoculated with SJNNV and RGNNV separately. These results suggest that the induction of the IFN mediated system by the previous infection with SJNNV could be responsible for the decrease in the mortality recorded in the superinfected group, protecting sea bass of the posterior infection with RGNNV.

# TARGETED DISEASE PROPHYLAXIS IN EUROPEAN FISH FARMING (TARGETFISH)

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TargetFish is a large collaborative project funded by the European Commission under the 7th Framework Programme for Research and Technological Development (FP7) of the European Union (Grant Agreement 311993 TARGETFISH). The project started November 2012 and will run for 5 years, bringing together leading European research groups that are experts on the fish immune system and enterprises from the Biotech and Veterinary sectors with a shared interest and experience with vaccination of fish. TargetFish will advance the development of existing (but insufficient) and new prototype vaccines against socio-economically important viral or bacterial pathogens of Atlantic salmon, rainbow trout, common carp, sea bass, seabream and turbot. TargetFish will also establish a generic knowledge- and technology-base for rational development of next generation fish vaccines. Improved vaccines will be brought closer to industrial application by addressing practical issues such as efficacy, safety and delivery route. The main objectives of the project are to: 1) generate knowledge by studying antigens and adjuvants for different routes of administration while analyzing the underpinning protective immune mechanisms; 2) validate this knowledge with response assays for monitoring vaccine efficacy and safety, including issues associated with DNA vaccines; 3) approach implementation of prototype vaccines shortening the route to exploitation and 4) optimize vaccination strategies in order to obtain maximum protection in different sizes of fish. To achieve these challenging tasks, we brought together 30 partners from 10 EU member states, 2 associated countries and 1 International Cooperation Partner Country (Chile). In this large multidisciplinary consortium an approximate equal number of RTD and SME partners will cooperate closely while keeping an intensive communication with the large vaccine and nutrition industries via an Industry Forum.

# COMPARATIVE ANALYSIS OF THE ADAPTIVE IMMUNE RESPONSE TO LIVE AND INACTIVATED *EDWARDSIELLA TARDA* IN GINBUNA CRUCIAN CARP, *CARASSIUS AURATUS LANGSDORFII*

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*Edwardsiella tarda* is an intracellular pathogen that causes edwardsiellosis in fish. Our previous study suggested that both cell-mediated immunity (CMI) and innate immunity are important for protection against *E. tarda* infection. Although many studies have been reported on effective attenuated *E. tarda* vaccines, there has only been limited report on effective formalin-killed cell (FKC) vaccines. Therefore, in this study, we compared the immune responses to *E. tarda* live cells or with those to FKC vaccines in ginbuna crucian carp (*Carassius auratus langsdorfii*). Fish were sensitized with *E. tarda* as either live cells or FKCs at $10^5$ CFU/100 g body weight (0.2 $LD_{50}$) by intraperitoneal injection, and then kidney leukocytes (KLs) and plasma were collected on various days post immunization (dpi). The expression of *IFNγ*, *T-bet*, and *GATA3* in KLs was analyzed by quantitative real-time PCR. The proportion of $CD8\alpha^+$ lymphocytes in KLs were examined using monoclonal antibodies against ginbuna CD8α. The antigen-specific cytotoxicity against *E. tarda*-infected cells was analyzed, and the *E. tarda*-specific antibody titer in plasma was determined by ELISA. In fish immunized with *E. tarda*, *IFNγ1* and *IFNγ2* expression was highest at 2 dpi, and the expression in live *E. tarda*-immunized fish was significantly higher than that in FKC-immunized fish. *T-bet* expression was significantly up-regulated from 8 to 12 dpi in live *E. tarda*-immunized fish. In contrast, *GATA3* expression was significantly up-regulated in FKC-immunized fish. The percentage and cytotoxicity of $CD8\alpha^+$ cells increased significantly from 4 to 8 dpi in live *E. tarda*-immunized fish, while those of $CD8\alpha^+$ cells in FKC-immunized fish were not significantly changed. The antibody titer in FKC-immunized fish was significantly higher than in live *E. tarda*-immunized fish. These results indicated that live *E. tarda* and FKC immunization in fish promote CMI and humoral immunity, respectively. Since CMI is only weakly induced after FKC immunization, FKC vaccine is ineffective for protecting fish against *E. tarda* infection in which CMI plays important roles.

# VIBRIO INFECTION IN FISH IN THE CZECH REPUBLIC

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The genus *Vibrio* comprises, in particular, the most significant marine fish bacterial pathogens. Species of this genus have also been isolated from freshwater environments and have been found to be responsible for fish disease outbreaks. As with aeromonads in fresh water, isolation rates increase with increased environmental temperature and where organic loads are high.
Pathogenic ***Vibrio cholerae non* O1/non O139** was isolated in the fry of the Cardinal tetra, *Paracheirodon axelrodi* (L.P.Schultz, 1956) and in adult Raphael catfish, *Platydoras costatus* (Linnaeus, 1758) and spiny catfish, *Agamyxis albomaculatus* (W.K.H. Peters, 1877) in aquarium conditions. During August, an epizootic occurred in the wild populations of common nase, *Chondrostoma nasus* Agassiz, 1832; chub, *Squalius cephalus* (Linnaeus, 1758); gudgeon, *Gobio gobio* (Linnaeus, 1758); and schneider, *Alburnoides bipunctatus* (Bloch, 1782). Mortality was not identified in Eurasian minnow, *Phoxinus phoxinus* (Linnaeus, 1758). The disease was accompanied by a long period of increased water temperature (above 20°C, unusual under the existing conditions) with a slowed-down flow of water, affected by faecal contamination. Clinical signs of vibriosis infection in common nase and chub manifested themselves as diffused and focal haemorrhages located especially in the abdominal region, within the mouth and at the base of fins. It was only in common nase infection that the whole eye was affected, causing rupture of the globe and destruction of ocular structures.
***Vibrio vulnificus*** was isolated from a purulent focus of absceding nephritis in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792). Histologically significant lesions included chronic fibroproductive inflammation with ample presence of purulent abscesses.
***Vibrio fluvialis*** was the cause of intestinal inflammation in cultured rainbow trout. The strain was isolated from the inflammatory foci in the gut wall.

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# PREVENTING DISEASE IN RAINBOW TROUT CAUSED BY *AEROMONAS SALMONICIDA*

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Furunculosis is the first bacterial disease described from Denmark in the 1950s. Currently this disease causes the greatest problems in sea reared rainbow trout production, which first takes place in freshwater and then at sea. It is in the marine phase, trout can get infected by the bacteria *Vibrio anguillarum* and *Aeromonas salmonicida*, the causative agents of vibriosis and furunculosis respectively. While applied commercial vaccines provide adequate protection against *V. anguillarum*, outbreaks of furunculosis occur repeatedly (especially during elevated temperatures) with *A. salmonicida* antigen included in the vaccines. For that reason *A. salmonicida* is responsible for economically devastating losses in aquaculture. My PhD is a part of the collaborative project "Targeted disease prophylaxis in marine fish farming (ProFish)" which will develop and implement tailored vaccines with Danish strains of *A. salmonicida* to marine trout farming. Focus will be on epidemiology and characterization of *A. salmonicida*. A "quantitative polymerase chain reaction" (qPCR) based on self-quenched, fluorogenic primers will be developed and implemented in both diagnosis and monitoring. The primers show high sensitivity and will thus be used in detection of carrier infected fish. Since there is still little known about the latent phase, *A. salmonicida* will additionally be studied by bioluminescence in order to follow the route of infection and proliferation of the bacterium in fish. Strains of *A. salmonicida* isolated from fish farmed in freshwater and saltwater for several years and from different geographical areas of Denmark will be examined in a multi-locus sequence typing (MLST) assay. A representative panel of bacteria will be selected for genome variation analysis through next generation sequencing and the results will be used for the establishment of a PCR-based genotyping analysis. Recent genome sequencing data will be used as a reference for mapping the identified variable sites. The developed assay will be used to determine proliferation and the variability of *A. salmonicida* which causes disease in marine farming.

# SCREENING OF PLANT ETHANOLIC EXTRACTS FOR ANTIBACTERIAL ACTIVITY AGAINST FISH PATHOGENS

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The present study aimed to investigate the *in vitro* antibacterial activity of fifteen commercial ethanolic extracts from medicinal plants against five relevant bacterial fish pathogens (*Listonella anguillarum* serotypes O1 and O2, *Yersinia ruckeri*, *Photobacterium damselae* subsp. *piscicida* and *Lactococcus garvieae*). Their antimicrobial potential was assessed by performing the disc diffusion assay (Alsaid *et al*., 2010, partially modified), then minimal inhibitory (MIC) and bactericidal (MBC) concentrations were established by using the broth micro-dilution method (Manfrin *et al*., 2008). Significant differences in the antibacterial activity of the investigated plant extracts were found, depending on herbal species and on bacterial strain. The extracts of *Lavandula officinalis*, *Melissa officinalis*, *Ocimum basilicum*, *Origanum vulgare*, *Rosmarinus officinalis* and *Salvia officinalis*, which belong to the *Labiatae* botanical family, were the most effective. These extracts exhibited a broad spectrum of inhibitory effects both on Gram negative and Gram positive bacteria, displaying the largest zones of growth inhibition and the lowest MIC and MBC values against the tested strains (MICs ranging from 10.5 mg/ml to 168 mg/ml, MBCs ranging from 21 mg/ml and 336 mg/ml). The extract of *Vaccinium vitis idaea* showed a moderate antibacterial activity on *L. anguillarum* serotypes, *P. damselae* subsp. *piscicida* (MICs ranging from 42 mg/ml to 84 mg/ml, MBC equal to 84 mg/ml) and *L. garvieae* (MIC equal to 42 mg/ml, MBC undetectable), while it was less active on *Y. ruckeri* (MIC equal to 336 mg/ml, MBC undetectable). The plant species *Achillea millefolium*, *Arnica montana*, *Calendula officinalis*, *Cetraria islandica*, *Equisetum arvense*, *Grindelia robusta*, *Orthosiphon stamineus* and *Thymus vulgaris* showed lower or negligible effects. Overall, *P. damselae* subsp. *piscicida* was the most susceptible bacterial species while *Y. ruckeri* was the most resistant.
To the best of our knowledge, this is the first report on the antibacterial properties of these medicinal plants on a wide range of fish bacterial pathogens. The results can be considered as a base for further investigations finalized to identify novel natural antimicrobial compounds that might be used in aquaculture for the control of bacterial infections.

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# AN OUTBREAK OF MICOBACTERIOSIS IN GOLDFISH

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A *Micobacterium*-like infection has been observed in the viscera and muscles of few goldfish, *Carassius auratus auratus*, kept in poor condition in an aquarium. The majority of infected fish were smaller then other fish in the same aquarium, because they lost the normal apetite. The signs were lethargy, inappetence, weight loss, imbalance, and sometimes lepidortosis, ascites or reddening of the lateral of the belly, followed by death in about one week. Milliary granulomas has been observed in the kidney, mesentery, liver and sometimes in the muscles of the body cavity. HEA (hematoxylin-eosin-metylene blue staining -Ciurea method- has been performed and a chronic granulomatous nefritis has been recorded. The granulomas were rounded by Langerhans cells. Few acid fast bacteria were observed in Ziehl-Nielsen smears from the kidney. It is suspected that the origin of the infection should be two Florida turtles kept in a different tank but in the same room, managed by the same person with the same facilities and the infection has been transmitted through water from turtles to fish.

# NEPHRITIS BY AN ATYPICAL *EDWARDSIELLA TARDA* STRAIN IN WHITE GROUPER *EPINEPHELUS AENEUS* CULTURED IN EILAT (ISRAEL, RED SEA)

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*Edwardsiella tarda* is a motile member of the Enterobacteriaceae family. It has a worldwide distribution and is known to be responsible for severe infections in various species of cultured fish. Although more frequently reported in freshwater fish, *E. tarda* has also been associated with other animals from various - and for the most part aquatic or humid – ecosystems. *E. tarda* displays also a non-negligible zoonotic potential, as gastrointestinal and extraintestinal infections have been frequently described in humans. *E. tarda* has been held responsible for severe infections in flounder in the Far East and turbot in Europe, and is considered as an emerging pathogen in marine aquaculture.
An atypical (non-motile, non-hemolytic, unable to grow at 42°C) *Edwardsiella tarda* strain was isolated from the kidneys of eight white groupers (*Epinephelus aeneus*) that were either moribund or that had died overnight. The affected fish were the offspring of individuals caught several years before off the Mediterranean coast of Israel and part of the brood stock cultured in Eilat (Red Sea). Confirmation of the identifications obtained on the basis of phenotypic characteristics was sought by partially sequencing the 16S ribosomal RNA gene, but due to the high similarity of our strain to both *E. tarda* and *E. ictaluri*, the identification of this bacterial pathogen could not be confirmed at the molecular level. In addition, instead of two native plasmids that are characteristic of *E. ictaluri*, our strain carried a 10-15 kb plasmid. Histopathology analyses revealed a severe suppurative nephritis with large abscesses occasionally spreading into the surrounding musculature, typical of *E. tarda*. Bacteria were conspicuously visible in masses of degenerate neutrophils in the necrotic, liquefied renal tissue. The larger abscesses were generally irregular in shape, bounded by an extensive inflammatory cell (macrophages and neutrophils) response, fibrin coating and filled with fluids and hollow areas, the latter probably occupied by gas, most likely $H_2S$ produced by *E. tarda* metabolic activity. The disease had an evident chronic character. Our study is the first record of mortalities from edwardsiellosis in a marine fish in Israel. The source of contagion remains for now elusive.

# ANALYSIS OF ANTIMICROBIAL RESISTANCE GENES IN *AEROMONAS* SPP. ISOLATED FROM AQUATIC ANIMALS IN CHINA

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The development of resistance to antimicrobials used in aquatic animals is an increasing concern for aquaculture and public health. As little is known about antimicrobial resistance and its determinants in Chinese aquaculture, this study was conducted to monitor the occurrence of antimicrobial resistance and resistance genes in *Aeromonas* isolated from different aquatic animals (freshwater fish, shrimp and turtles) in China. A total of 106 *Aeromonas* strains were identified, with *Aeromonas hydrophila* (54.7%) and *A. veronii* (19.8%) as the dominant species. Antimicrobial susceptibilities were determined by the disk diffusion method. The highest resistance percentage occurred with rifampin, streptomycin and nalidixic acid. Most strains were sensitive to fluoroquinolones, doxycycline, cefotaxime, and amikacin. The isolates from turtle samples had the highest levels of resistance to 11 of the 12 tested antimicrobials when compared to those from fish or shrimp. Forty one (38.7%) strains showed multidrug resistance phenotypes, 27 (25.5%) of which harbored at least one antimicrobial resistance gene. PCR and DNA sequence results showed that all trimethoprim/ sulfamethoxazole resistant strains contained sulfonamides resistance gene *sul1*, and 37.0% (10/27) were positive for tetracycline resistance gene *tetA*. The streptomycin resistance gene *ant(3'')-Ia* was identified in 13 (59.0%) streptomycin resistant strains. Plasmid-borne quinolone resistance genes were detected in 5 *A. hydrophila* (4.7%), two of which carried *qnrS2* while the other three strains harbored *aac(6')-Ib-cr*. Two *A. hydrophila* were positive for the extended-spectrum β-lactamase genes $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-3}}$. To our knowledge, this is the first report characterizing antimicrobial resistance in *Aeromonas* isolated from different aquatic animals in China.

# ISOLATION AND CHARACTERIZATION OF POTENTIAL PROBIOTIC BACTERIA FROM RAINBOW TROUT (*ONCORHYNCHUS MYKISS*, WALBAUM) REARING UNITS, WITH INHIBITORY ACTIVITY AGAINST BACTERIAL PATHOGENS

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Potential probiotic bacteria were searched for use probiotics against vibriosis, yersiniosis and lactococcosis in rainbow trout in this study. A total of 79 bacterial strains were isolated from rainbow trout rearing water and screened for antagonistic activity against *V. anguillarum, Y. ruckeri* and *L. garvieae* using an well diffusion agar assay. Antagonistic strains were characterized for enzymatic activity (protease, lipase), hydrophobicity. *Vibrio* spp. showed inhibitory activity aganist *V. anguilarum* and *L. garvieae*, while *Aeromonas* spp. showed antagonistic effect against *L. garvieae*. However, *Lactococcus garvieae* strains displayed inhibitory activity against all pathogen. *Vibrio sp.* A12 and *Aeromonas* sp. A5, G1 were found to have enzymatic activities and hydrophobicity. *Lactococcus garvieae* generally showed week hydrophobicity. However, as this is a preliminary study, it will be necessary to study patogenicity and than probiotic effects of these bacteria in rainbow trout.

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# BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *VIBRIO VULNIFICUS* BIOTYPE 2 ISOLATED FROM MARINE ENVIRONMENTS

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In the biotyping of the *Vibrio vulnificus* strains isolated from sea water, oyster and sediment samples in the South sea of Korea, the majority of the isolates (94.7%) were belonged to biotype 1, and remainings (5.3%) were belonged to biotype 2. In the analysis of the type of 16S rRNA of *V. vulnificus* strains isolated from marine environment using the multiplex PCR, it appeared that type A and type B were 35% and 65% respectively. RAPD-PCR was used to analyze the genomic difference of the *V. vulnificus* between biotype 2 strains isolated from marine environments and those obtained from the diseased eel. These two groups that can be included in biotype 2 *V. vulnificus* strains showed clearly different profiles of the produced amplicons in RAPD-PCR with R1 primer. Additionally, in biochemical comparison of these strains, all 4 strains isolated from marine environments were differed from strains isolated from eel by the ability of mannitol utilization. Additionally, only two isolates (NH 1 and NH 2) out of four isolates of biotype 2 from marine environments showed pathogenicity to eel in challenge test and differed from the remaining two isolates, HD 1 and HD 2, that did not induce the fish mortality in the same experiments.

# ACTIVITY OF *STREPTOCOCCUS INIAE* ON *DICENTRARCHUS LABRAX* LEUKOCYTES

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*Streptococcus iniae* is a Gram-positive bacterium that causes meningoencephalitis and death in commercial fish species and has also recently been identified as an emerging human pathogen producing fulminant soft tissue infection. In this study, we have investigated the effect of *S. iniae* in the non-specific immune response of European sea bass (*Dicentrarchus labrax*) leukocytes, production of intracellular superoxide radicals and total peroxidase content in infected cells, and the changes in the relative expression of some immune-related genes as Interleukin 1β, tumor necrosis factor-α, interleukin-6 interleukin-10, Mx and caspase-3.
Production of intracellular superoxide radicals and total peroxidase content was observed in infected cells. Our findings show that there is a direct relationship between the dissemination of bacteria and the resulting infection-associated inhibition of respiratory burst, apoptosis, and the pro- and anti-inflammatory gene expression profiles. It has also found macrophage depletion producing more robust infection, and bacterial growth ensuring its dissemination and spread into the host.

# *VIBRIO VULNIFICUS* OUTBREAKS IN DUTCH EEL CULTURE: MOLECULAR GENOTYPING, ANTIBIOTIC RESISTANCE, AND ZOONOTIC IMPACT

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*Vibrio vulnificus* is an agressive potentially zoonotic bacterial pathogen of fish. In this study, from 1996-2011, *V. vulnificus* was isolated 24 times in the Netherlands, as causative agent of serious eel disease outbreaks (23 strains) with high mortalities at 9 indoor eel farms, of which one outbreak was related to a severe zoonosis of the eel farmer (necrotic fasciitis, 1 strain).
In this study, we tested the genetic relatedness of the 24 mentioned strains of *V. vulnificus* by two genotyping techniques, MLST (using HiMLST technology) and rep-PCR (DiversiLab®). Additionally, the antibiotic resistance was tested.
The 24 strains could be separated into 8 HiMLST types and 8 rep-PCR types, which corresponded almost exactly to each other. Both methods were appropriate to distinguish a zoonotic strain form the other eel pathogenic strains of this study. Only one of the 8 HiMLST types was present in the online MLST database and the other eight each had one or more new allele variations (ST 137-143). This indicates that many, yet unknown *V. vulnificus* genotypes occur in eel farms. Most farms harboured a single genotype, and most genotypes were restricted to a single farm. However, two farms harboured two genotypes in time. The eel farmer that suffered a zoonosis from *V. vulnificus* carried the same genotype as his diseased eels, a demonstration of the zoonotic potential of this strain (HiMLST ST 112).
The antimicrobial resistance patterns were diverse amongst the genotypes of *V. vulnificus* and no clear correlation was found between genotype and antibiotic resistance profile of a strain. Most *V. vulnificus* strains were resistant to cefoxitin and showed multi-resistance to quinolones, properties that seemed to develop after prolonged use of flumequin bath and other antibiotics in the eel farms.
Although *V.vulnificus* related contact zoonosis from fish are scarce, individual cases may be severe, especially in immuno compromized patients. Risk assessment and prevention are needed to protect fish farmers and fish processors against *V.vulnificus* infections, particularly from eels. Medics should be aware about the potential zoonotic risk of *V.vulnificus* infections in our geographical area, associated with indoor eel farming.

# DIFFERENTIALLY EXPRESSED GENES IN FISH PATHOGEN *AEROMONAS SALMONICIDA* SSP. *SALMONICIDA* STRAIN JF2267 ON THE BASIS OF MICROARRAY ANALYSIS

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*Aeromonas salmonicida* is a causative agent of furunculosis in salmonids. The disease symptoms are acute septicemia, formation of ulcers and furuncles, internal hemorrhages in peritoneal cavity. The fish usually die within 4 days. A broad range of *A. salmonicida* toxins, enzymes and effector proteins contributes to infection by the interaction with fish immune cells and influence their defense mechanisms. *A. salmonicida* can respond to the environmental stimuli and optimize proliferation ability by induction or repression of specific genes during infection. It is known that *in vitro* and *in vivo* conditions like culture medium composition or interaction with host organism and its immune system induce the changes in expression of bacterial metabolic and virulent proteins.
In this study we aimed to analyze the effect of different *in vitro* treatments on the gene expression and evaluate the changes in the production of virulence factors. Our results indicate significant changes in the bacterial growth and metabolism induced by increased temperature, limited iron, calcium and oxygen conditions. The generation time of bacteria was the highest for normal condition and limited oxygen while limitation of iron and calcium slowed the bacterial growth significantly. Additionally, the iron and calcium deficient conditions reduced the maximum cell number reached during stationary phase. In further parts of presented study, three *A. salmonicida* ssp. *salmonicida* JF 2267 strains of different pathogenicity (wild type, attenuated and of increased virulence) were sequenced using 454 sequencing and obtained results showed different genome composition with set of genes present or absent just in one strain. On the basis of the more virulent strain an oligonucleotide microarray containing 5399 features representing over 4500 genes have been developed. Performed analysis indicated high dependence of the transcriptome usage on the environmental conditions. Thus, while the anaerobic conditions hardly change the expression the limitation of iron and calcium affects the transcriptome more dramatically. The expression pattern was also confirmed by qPCR. Overall, these findings contribute to a better understanding of *Aeromonas salmonicida* behavior during manipulation of environmental conditions.

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# *DE NOVO* GENOME SEQUENCING OF *PISCIRICKETTSIA SALMONIS*

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*Piscirickettsia salmonis*, the causative agent of piscirickettsiosis in salmonids (among other species), is a serious bacterial fish disease with major financial implications in Chilean aquaculture. Piscirickettsiosis has earlier also been detected in Norway. It is a Gram-negative, pleomorphic, facultative intracellular, non-motile bacteria, belonging to the *Gammaproteobacteria*. Due to fastidious growth requirements *Piscirickettsia salmonis* is relatively challenging to work with. Consequently, knowledge about many key areas such as life cycle and virulence mechanisms are still fairly limited.
Currently there are two *Piscirickettsia salmonis* genomes in GenBank. They are not annotated, and are available in a relatively high number of contigs. Accordingly, we have conducted *de novo* genome sequencing of two different strains of *Piscirickettsia salmonis*. We have chosen to take advantage of Shotgun genome sequencing (GS FLX Titanium XL+) with Paired End Library to render our genomes in as few scaffolds as possible. Automatic gene annotation has been carried out using the IGS Annotation Engine followed by manual annotation in Manatee. Preliminary results indicate an unusually high content of repetitive elements, a large number of flagellar genes and a Dot/Icm secretion system. We have also carried out a comparative analysis with other relevant genomes to better understand the evolution and genome dynamics of this bacterial species.

# PHARMACOKINETIC PROFILE AND MUSCLE RESIDUE ELIMINATION OF TILMICOSIN IN CRUCIAN CARP AFTER ORAL ADMINISTRATION

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In this study, we investigated the pharmacokinetics and muscle tissue residue elimination of tilmicosin (TLM) in crucian carp (*Carassius auratus*) at water temperature of 26±1 ℃The crucian carp were randomly divided into two groups: one group was used to explore the pharmacokinetics of TLM at a single dose of 50 mg/kg body weight by oral administration, and the other group was used for investigating of muscle tissue residue elimination of TLM at a dosage of 50 mg/kg per day by oral administration for 3 days. TLM concentrations in plasma and muscle tissue were analyzed with ultra high-performance liquid chromatography (UPLC, Waters, USA). Using the 3p97 software, the data of pharmacokinetics is conformed to a two-compartment model. The absorption rate constant (*K*a) and absorption half-life ($t_{1/2\ ka}$) of TLM were 1.99 $h^{-1}$ and 0.349 h, respectively. The distribution half-life ($t_{1/2\alpha}$) and elimination half-life ($t_{1/2\beta}$) of TLM were 2.87 h and 39.89 h, respectively. The maximum concentration ($C_{max}$) of TLM in plasma was 19.36 μg/ml and the time to peak concentration ($T_p$) was 1.27 h. The area under the plasma concentration-time curve (AUC) was 240.326 μg·h/ml. The distribution volume ($V_d/F$) of TLM was calculated as 1.968 l/kg. For determination of the muscle residue elimination of TLM, used by oral administration a dosage of 50 mg/kg body weight per day for 3 days of TLM. The results revealed that the elimination of TLM in the crucian carp muscle tissue required a long half-life time 3.12 d. Based on the above residues, we suggest the withdrawal time should be 33 days at least, Which was calculated by the equation of MRL=$C_0\ e^{-k(WDT)}$.

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# ISOLATION AND CHARACTERIZATION OF *NOCARDIA SERIOLAE, A CAUSATIVE AGENT OF* SYSTEMATIC GRANULOMA IN SPOTTED BUTTERFISH, *SCATOPHAGUS ARGUS*, LINN

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In Taiwan, a *Nocardia seriolae* was observed in diseased spotted butterfish (*Scatophagus argus*, Linn). The cumulative mortality within one month was 30% (660 out of 2200). The diseased fish were two years old with lengths from 18 to 25 cm. Most fish suffered from haemorrhages and ulcers of the skin. The most significant gross pathological changes were enlargements of the spleen, kidney and liver. White nodules, vary in size, were found in these organs. Bacteria were either coccal or filamentous in appearance, with bead-like staining. The identification of NS128 was verified by PCR assay for *Nocardia seriolae* that gave the expected specific amplicon of 432 bp, its 16S rDNA sequence had a 100% sequence identity with *N. seriolae* (GenBank accession number AF380937). A partial sequence of the 16S rRNA gene (GenBank accession number EU147501), the RNA polymerase gene (rpo B) (GenBank accession number DQ119300) and the heat shock protein gene (GenBank accession number DQ431437) of the organism, NS128 and the type strain of *N. seriolae* BCRC 13745 formed a monophyletic clade with a high sequence similarity and a bootstrap of 100%. White nodules that were induced in experimental fish, spotted butterfish and amberjack were similar to those in naturally infected fish cases and *N. seriolae* were re-isolated using brain heart infusion agar. These finding provides evidence that *N. seriolae* caused systemic granulomas in diseased spotted butterfish (*Scatophagus argus*, Linn). Based on the growth characteristics, and biochemical properties of the bacterium, its histopathological changes, PCR and the phylogenetic analysis, the pathogenic organism was identified as *N. seriolae*. This investigation is the first published on *N. seriolae* infection in spotted butterfish (*Scatophagus argus*, Linn) in aquaculture. The results reveal that the *N. seriolae* isolated in the field was pathogenic to spotted butterfish and amberjack.

# GENETIC CHARACTERIZATION OF NORWEGIAN ISOLATES OF *TENACIBACULUM* SPP.

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In Norway skin ulcers in Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Onchorunchus mykiss*) in the winter season have been diagnosed since the 1980's and *Moritella viscosa* is acknowledged as an important pathogenic bacterium in this condition. It has also subsequently been shown that 60-70 % of the ulcers are infected by *Tenacibaculum* spp. The occurrence of outbreaks of skin ulceration during the winter / late winter season with the involvement of *Tenacibaculum* seems to have increased. Also ulceration in the head region seems to be more frequent and these ulcers are often associated with *Tenacibaculum* infections. *Tenacibaculum* bacteria are also isolated from skin ulcers of cod (*Gadhus morhua*), wrasse (*Labridae*), lumpsucker (*Cyclopterus lumpus*) and halibut (*Hippoglossus hippoglossus*). A bacterium belonging to the same genus, *T. maritimum*, is well known to cause ulcers and mortalities in marine fish elsewhere, but has, to our knowledge, never been isolated in Norwegian cases. In Norway a variety of *Tenacibaculum*-bacteria have been isolated. In the EU/EMIDA project "Control of *Flavobacteriaceae* infections in European fish farms" the Norwegian Veterinary Institute is currently characterizing *Tenacibaculum* isolates retrieved from diagnostic cases between 1986 and today from different fish species. We present preliminary results from multi-locus sequence typing (MLST) analysis.

# THE DOMINANCE OF OPPORTUNISTIC MICROORGANISMS IN THE MICROBIOCENOSIS OF RAINBOW TROUT IN THE EXAMPLE OF CAGE FARMS IN KARELIA

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Each body of water in its natural state is populated by microorganisms occupying different ecological niches. Pollution of water bodies has a direct impact on the local aquatic flora and microbiocenosis of fish, changing quantitative and qualitative ratio. Negative changes of symbiotic interaction of microorganisms with fish facilitate the boom of bacteria associations increasing their pathogenic properties. Due to the high bacteria adaptability to aggressive environmental factors their enzymatic activity increases, which results into change of "host-parasite" symbiotic relationships into dominant groups of bacteria.
The aim was to study the microbiocenosis of rainbow trout and to assess impact of cage farming on natural water bodies. There were chosen five lakes of Karelia with functioning in their waters trout farms as areas for study.
Analysis of the material showed that the taxonomic composition of the microflora of fish is represented by seven families (*Pseudomonadaceae, Enterobacteriaceae, Vibrionaceae, Neisseriaceae, Micrococcaceae, Bacillaceae, Listeriaceae*), eight genera of bacteria (*Pseudomonas, Neisseria, Azotobacter, Micrococcus, Staphylococcus, Planococcus, Bacillus, Listeria, Arthrobacter*). Opportunistic bacteria *Pseudomonadaceae* family *Pseudomonas* genus dominate in the rainbow trout microbiocenosis at all studied trout farms irrespective of their work period. At adverse environment for the macroorganism they can increase their virulence and are able to infect the stressed (weakened) fish. With the help of sanitary-indicative microorganisms there was a trend of change in environmental characteristics expressed in restructuring of fish and water microbiocenosis, appearing and spreading allochthonous bacterioflora and moulds increasing the proportions of strains with hemolytic activity and parameter Coli-index (an indirect indicator of biological contamination by environment pathogens).
Analysis of the qualitative and quantitative composition of fish microbiocenosis demonstrates the high importance of the data received and the possibility of their usage as a screening test for the detection of changes of dynamic equilibrium of the bacterial flora of fish and natural aquatic ecosystems. The data of this work can be used to predict a microflora reactions of fishery lakes on the set of the processes of natural and anthropogenic impacts, to develop principles of rational natural management in conditions of increasing anthropogenic pressure on the northern fresh waters. The level of water pollution by opportunistic bacteria is an indication of ecological and epizootic situation in the fish farms of the Republic of Karelia, and the results of the monitoring will allow to assess the pathogen diversity in their seasonal dynamics.

# WINTER ULCER DISEASE AND *MORITELLA VISCOSA* IN FARMED ATLANTIC SALMON IN THE WEST OF IRELAND

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Winter ulcer disease has emerged as a challenge for autumn transferred Atlantic salmon (S0s) (*Salmo salar*) in farms in the west of Ireland. The disease presents in two forms including:
a) the classic appearance with large flank or dorsal dermal ulcers or
b) moribund fish with no external lesions but numerous internal petechiae on visceral organs and ascites.
*Moritella viscosa* has been isolated from affected fish. The disease presents over the winter months and some cases have been treated with broad spectrum antibiotic.

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# INFLUENCE OF *FLAVOBACTERIUM PSYCHROPHILUM* INFECTION ON NONSPECIFIC DEFENCE MECHANISMS IN EUROPEAN EEL (*ANGUILLA ANGUILLA*) IN INTENSIVE SYSTEM OF CULTURE

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Infections caused by *Flavobacterium psychrophilum* and *Flavobacterium columnare* in intensive eel culture has increased rapidly in recent years. The aim of this study was to examine the influence of *F.psychrophilum* on the cellular and humoral defence mechanisms of European eel (*Anguilla anguilla*) grown in intensive rearing system. In order to determine selected nonspecific immune parameters, a total of 40 individuals of approximately 20-50 g were investigated- 20 fish with pathological changes and 20 clinically healthy fish. After clinical and anatomopathological examination, the blood and pronephros were used for the immunological study. Also tissue samples (skin lesions, spleen, liver and pronephros) were taken from fish for bacteriological study. The respiratory burst activity (RBA) and potential killing activity (PKA) of the pronephric phagocytes were measured. The lymphocyte proliferation stimulated by mitogens Concanavaline A or LPS were determined by the MTT assay. The lysozyme and ceruloplasmin activities in the plasma and total immunoglobulin level in serum were determined by spectrophotometric assay.
The results showed that the cell- mediated immunity presented by RBA, PKA and proliferative response of lymphocytes were statistically significantly lower ($P<0.05$) in eel where the *F.psychrophilum* was isolated. Also the lysozyme and total Ig levels in serum were statistically significantly lower ($P<0.05$) in infected eels. Basic information regarding cellular and humoral defence mechanisms are very important in monitoring of eel health and in the early diagnosis of infectious diseases induced by *F.psychrophilum.*

# WATER TEMPERATURE AFFECTS THE PREVALENCE OF EPITHELIOCYSTIS IN WILD FISH SPECIES IN TASMANIAN WATERS

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Epitheliocystis is a *Chlamydia*-like, intracellular bacterial condition that affects the gills and skin of finfish. It is characterised by the presence of membrane-enclosed basophilic inclusions in host cells. This condition occurs in both wild and farmed fish populations and is currently known to affect over 80 different species of marine and freshwater fish. In the present study, three species, jack mackerel *Trachurus declivis*, sand flathead, *Platycephalus bassensis* and tiger flathead, *Neoplatycephalus richardsoni*, were surveyed for epitheliocystis infections on five separate occasions over a period of 11 months from Eastern Tasmanian waters.
Epitheliocystis was present in all three species is most sampling periods. Sampling period was found to have a significant effect on the prevalence of epitheliocystis infections and this was also confirmed with a significant correlation of increasing prevalence with decreasing seawater temperatures. Membrane-enclosed cysts were filled with a basophilic material that was and was not associated with a proliferative host response. This is the first report of epitheliocystis in sand flathead and tiger flathead, adding to the ever increasing number of fish species affected by this condition.

# EFFECT OF LENGTH, WEIGHT AND BODY CONDITION FACTOR ON THE SURVIVAL OF ATLANTIC SALMON (*SALMO SALAR*) EXPERIMENTALLY CHALLENGED WITH *PISCIRICKETTSIA SALMONIS*

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The effect of weight, length and body condition factor (K) on the susceptibility to piscirickettsiosis in Atlantic salmon (*Salmo salar*) was analized. Fish had experimentally being challenged in a previous work by intraperitoneal injection with *Piscirickettsia salmonis*. Inoculated salmon (n=2,461) came from 29 full-sibling families (80-87 fish each). To assess the degree of susceptibility to piscirickettsiosis, the Kaplan-Meier analysis was used and the resulting curves were compared with the Log-rank test. For each variable, fish were separated into three groups (A, B and C) using the first and the third quartile ($Q_1$ and $Q_3$) as cutoff points (A with values > to $Q_3$, B with values ≤ to $Q_3$ and > to $Q_1$ and C with values ≤ to $Q_1$). This separation was done using all the fish as a single population and also with fish of each particular family. When all the fish were used, a pattern for weight and K, characterized by a decreasing order of survival in groups A, B and C, respectively, was seen. In relation to the body length, group C also showed the highest susceptibility to piscirickettsiosis, but in this variable group B had the highest survival. The analysis within each family showed that only few families had significant differences among groups A, B and C. Except for one family, in which group C had the highest survival, which occurred with K, in the other families group C again exhibited the highest susceptibility to this disease. In conclusion, results suggest that Atlantic salmon has a general pattern in which the susceptibility to piscirickettsiosis in fish with higher weight and K would be lower than those that are included in the bottom 25% of the distribution of these variables. However, fish of some particular families would not follow this general pattern which is probably due to their specific genetic features.

# *TENACIBACULUM MARITIMUM* ISOLATED FROM SEA BASS (*DICENTRARCHUS LABRAX*) IN CROATIA

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The most severe disease outbreaks in sea bass (*Dicentrarchus labrax*) cultivated along the eastern Adriatic coast occure during the end of winter and early spring. Fish weighing between 60 and 120 grams suffer the highest prevalence and the highest mortality rates (up to 40 %). Affected fish reveal hemorrhagies on the head, opercula, fin base and the skin lesion on the body surface such as ulcers, necrosis, frayed fins and gill necrosis. Bacteriological examination of the specimen resulted the most frequently by mixed infection with *Listonella anguillarum* and *Tenacibaculum maritimum* according to determination of morphological and biochemical properties. So far as there were several species from the genus *Tenacibaculum* decribed as opportunistic bacterial pathogen in sea bass and other cultivated marine species we tried to determine affiliation of our isolates by genotyping. Partial sequence of 16S rRNA gene reveals 99 % similarity with type strain of *Tenacibaculum maritimum* isolated in Japan (difference in single nucleotide position) and with isolate from wedge sole (*Dicologoglossa cuneata*) from Spain (difference in four nucleotide positions).

# PROTOZOAL APICOMPLEXAN PARASITES INFECTING EXTERNAL AND INTERNAL ORGANS OF FARMED AND WILD SALMONIDS

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In an inland rainbow trout farm in the northern part of Sweden an unusual disease pattern was detected in 2011/2012. The first disease symtoms occured in young fingerlings, just a few weeks after hatching. Observations included blackening of the tail region and skull swelling in the upper part of the brain region, indicating increased intracranial pressure. In the following investigations lesions were also observed in the dorsal muscles, gills and in the intestinal region of the farmed fishes.
Histological observations, as well as, other investigations in combination with investigations of wild trout collected outside the farm, indicated a new type of coccidian infection in affected fish. Coccidia (phylum Apicomplexa) are a well known protozoan group of parasites causing large problems in the poultry industry as well as in the production of other domesticated animals on land. However this group of parasites has not been demonstrated to be a threat to farmed fish, except for *Goussia*-infections in juvenile common carp (*Cyprinus carpio*). *Goussia carpelli* is regarded as serious disease in European carp hatcheries where the infections can be problematic causing enteritis in the intestinal tracts (Jendrysek et al 1994). In wild salmonids, there are several coccidial parasites described where the majority of these parasites are known to infect intestinal organs, mainly the pyloric caeca. None of these parasite species are known to cause disease problems in farmed salmonid fish.
In this presented case regarding salmonids investigated in Sweden different external and internal lesions were observed in affected fish, where many of these lesions were associated with the protozoal parasites. The parasites were observed in different organs such as skin, gills, intestines, muscle and in the central nervous systems of affected fishes. The macroscopical and microscopical findings will be presented as well as observations from wild fishes in the vicinity of the farm.

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# EPIZOOTIOLOGICALSTUDIES ON MICROSPORIDIOSIS AFFECTING MARINE FISH DUSKY GROUPER (*EPINEPHELUS GUAZA*) IN EGYPT

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Microsporidia are one of common parasites of fishes, Marine fishes are subjected to several protozoan parasites of wich sporozoan take superior position. A total number of 476 of wild marine Dusky Grouper (*Epinephelus guaza*), captured from the West North shore of Mediterranean sea at Motrouh governorate (Egypt) from September (2006-2007). The average body weights of examined fish from 100-250 gm. The fish were investigated for Microsporidiosis and the water was examined for important physical and chemical parameters. The results of these investigation revealed that the fish appeared abnormally and the post mortem findings of Grouper fish was presence of variable numbers of cysts in fish gills ranging from oval to round in shape and white to yellowish in colour. The intensity of infection per gill arch was varying from 1-5 xenomas (tumer like) in gills only and no nodules in any internal organs or musculature were seen. The microsporidian protozoa was identified as *Glugea anomala* which react with Gram ans PAS stain positively. The seasonal prevalence of *Glugea anomala* from *Dusky Grouper* fish were recorded as, spring (58%), autumn (71%), the lowest infestation rate was recorded in winter (30.33%) and the highest was in summer (82.9%). The total prevalence of *Glugea anomala* among wild Dusky Grouper fish was found to be (63.2%). The prevalence of infestation also differ according to body weight, the highest infestation rate was found in 200-250 gm (88.8%), followed by 150-200 gm (50.5%) and finaly 100-150 gm (43.3%). The highest PH (7.3) was recorded in summer and lowest (5.7) in winter, the highest chloride value was 240 mg/L at autumn. The total hardness was higher in summer 290 mg/L and lower in winter 217 mg/L. The important histopathological alteration in gills was destruction of gills filaments with degenerative changes and infiltration of mononuclear leucocytes.

# TREATMENT OF *TRICHODINA* SP. INFECTIONS IN ATLANTIC COD (*GADUS MORHUA*) WITH FORMALIN

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*Trichodina* sp. (Ciliophora) is an economically important ectoparasite of cultured marine species including Atlantic cod (*Gadus morhua*) in Northern latitudes where, in Atlantic cod, *Trichodina* sp. infections mainly present as a skin disease. This study aimed at establishing the efficacy of the typically recommended therapeutic formalin treatment for Trichodinosis in Atlantic cod. *Trichodina* - infected Atlantic cod of average mass of 0.1 ± 0.0 kg (mean ± se) and length of 19.1 ± 0.2 cm (mean ± se) were exposed to a static bath treatment at either, 0 ppm (control), 250 ppm or 500 ppm formalin for 30 min. Fish were sampled prior to exposure and 1, 7, and 14 days post-exposure where standardized skin mucous smear were made, and caudal blood samples taken for hematological and biochemical analysis. Mucous biochemistry data were also obtained from the gills. The density of *Trichodina* sp. on fish exposed to either 500 ppm or 250 ppm therapeutic formalin for 30 min significantly reduced ($p < 0.05$) when compared with the control treatment. The therapeutic formalin treatment did not appear to evoke osmotic stress as there was no significant difference ($p < 0.05$) in the plasma chloride ion concentration. There was also no significant difference ($p < 0.05$) in the differential white blood cell count and total mucous cell count. Hyperglycaemia and hyperlacticemia were not evident in the *Trichodina*-infected fish exposed to therapeutic formalin treatment when compared with the untreated fish. Formalin bath at concentration of either 250 ppm or 500 ppm for 30 min was effective in treating *Trichodinosis* in infected Atlantic cod with no effects on the physiological status of the fish.

# *EUSTRONGYLIDES* SP. IN PREDATORY FISH SPECIES

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Nematodes *Eustrongylides* sp. are parasites of piscivorous birds. Their life cycle require two intermediate hosts, aquatic oligochaetes and benthophagous fish. Human is not a typical host but may be infected if eats raw or insufficiently cooked or fried fish meat. The investigations were carried out in spring 2012 on Danube–Tisa–Danube Canal in the territory of Novi Sad where were collected 21 samples of zander (*Sander lucioperca*) weighting 250-500 g 52 and 52 samples of European catfish (*Siluris glanis*) weighting 250-450 g. Postmortem examination of abdominal cavity, digestive tract and other ventral organs was conducted. Presence of nematodes in the abdominal cavity, musculature, in the lumen of the stomach and encapsulated in stomach wall was revealed in 4 individuals of zander and 6 individuals of European catfish. The number of parasites per fish ranged from a few up to the 256 . Relative parametars were measured and identification was performed using Bauer (1987), Moravec (1994) and Anderson (2000) keys. Parasites were determined as *Eustrongylides* sp. - larval form. Samples for patohistological examination were taken from muscles and nodules in stomach walls.

# ZOONOTIC FISH PARASITES IN ROMANIA - THE RISK AND ECONOMIC IMPACT

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According to the European Commission Regulation 2005/2074, all fisheries must be visually inspected for detection of fish visible parasites, with or without optical means or magnifying using good light and candling.
All fish parasites, including zoonotic parasites destroyed by frozen, confer a disagreable aspect for human consumers. Fish meat or fish viscera infected with visible parasites can be easily detected and removed. If the parasites are present in low number in fish meat and can be entirely removed, the fish can be sold. In fish placed on the market fresh on ice (uneviscerated) or live, the parasites cannot be detected and zoonotic species can infect humans.
29 species of fish parasites were detected in fisheries products in Romania and confer disagreeable aspect: *Saprolegnia sp., Henneguya sp., Kudoa sp., Azygia lucii, Postdohiplostomum cuticola, Ichtyocotylurus platycephalus, I. variegatus, I. pileatus, Apharyngostrigaea cornu, Diplostomum sp., Triaenophorus lucii, Histerothylacium aduncum, H. bidentatum, Philometroides lusiana, Acanthocephalus sp.-cystacanth, Achtheres percarum, Ergasilus anchoratus, E. sieboldi, Neoergasilus japonicus, Sinergasilus major, S. lieni, Argulus foliaceus, A. coregoni, Lernaea cyprinacea, L. ctenopharyngodonis, L. carasii, Caligus lacustris, Lepeophtheirus salmonis, Piscicola geometra,*
8 species are zoonotic and can infect humans: *Clinostomum complanatum, Euclinostomum heterostomum, Metagonimus yokogawai, Opistorchis felineus, Apophallus donicum, Diphyllobothrium latum, Anisakis sp., Eustrongylides excisus*.
Fish species susceptible to be infected with zoonotic parasites should be placed on the market only after manual evisceration and inspection in accordance to Council Regulation 2005/2074. In that case, the economic impact is increasing because the fish price will be higher.

# THE TREATMENT OF ARGULOSIS, LERNEOSIS AND DACTYLOGYROSIS IN CARP USING EXTRACTS FROM *TANACETUM* SP.

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Rotenone and pyrethrum are natural insect killers, well known as organic insecticides from centuries. Pyrethrum was extracted from *Tanacetum (Chrysanthemum) cinerariifolium*, but other plants of this genus contain pyrethrins (*T. coccineum, T. vulgare*). While rotenone is highly toxic to aquatic animals, pyrethrins are better tolerated and less toxic then synthetic pyrethrins (pyrethroids). In this study, we have tried to treat two crustaceosis - argulosis produced by *Argulus foliaceus* and lerneosis produced by *Lernaea cyprinacea*) and one monogenosis - dactylogyrosis produced by *Dactylogyrus extensus* (sin. *D. solidus*) in common carp (*Cyprinus carpio*). We established that Ectocid Herba® (used to control flea in dogs and cats), which contains 2% *T. cinerarifollium* flowers, is effective against parasites mentioned bellow, but is more toxic to fish then infusion from flowers of *T. vulgare*. The toxic effect can be managed using light exposure and a different exposure time of natural infected carp. After medicated bath, the prevalence of argulosis decreased from 15-20 *Argulus foliac*eus/carp to 2-5 *A. foliaceus*/carp, lerneosis decreased from 10-12 live *Lernaea cyprinacea*/carp to 1-2 live *L. cyprinacea*/carp and dactylogyrosis decreased from 30-40 *Dactylogyrus extensus*/carp to 5-8 *D. extensus*/carp. All carps treated were naturally infected and were caught from a pond. Oil extracts are more concentrated, but there are difficult to be applied in fish. We consider that the extracts from *Tanacetum sp.* are organic antiparasitic drugs and their use is a eco-friendly method to control some crustaceans and monogeneans in fish. Bath with extracts from *Tanacetum sp.* should be used prior the fish restocking as well.

Key words: *Tanacetum cinerariifolium, Tanacetum vulgare, Argulus foliaceus, Lernaea cyprinacea, Dactylogyrus extensus, Cyprinus carpio*

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# EFFECT OF POLLUTION WITH HEAVY METALS ON PREVALENCE OF BACTERIAL AND PARASITIC INFECTIONS WITH THEIR PATHOLOGICAL ALTERATIONS IN SOME EGYPTIAN FISH FARMS

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This study was planned to investigate the health situation and a survey on some Egyptian fish farms. Water and fish samples were collected from three fish farms: Al-Abbasa, Kafr-elsheikh and El-Fayoum during March to May, 2011. Physico-chemical, bacteriological, parasitological and histopathological examinations with heavy metal analysis were conducted for water and tissues samples. The results indicated that the highest values of heavy metals in water and tissue samples obtained from Al-Abbasa fish farm were exceeded the FAO permissible limits. On the other hand parasitic infestation reached about 60% in Al-Abbasa, 23% in Kafr-Elsheikh and 11% in El-Fayoum. Furthermore, it was concluded that the highest bacterial infections were in El-Fayoum farm with an incidence of 80% of tested strains. The identified bacteria were *Pseudomonas spp.* in most cases. Interestingly, none of tested isolates from three farms were found positive for *Aeromonas spp.*. Histopathologiacl examinations revealed alterations with different rates in the three farms, mainly attributed to the heavy metal pollution with its levels exceeded the permissible limits especially in Al-Abbasa farm. Another stressful factor that affected the pathological picture in our samples was the bacterial infestation appeared in El-fayoum first and Kafr-Elsheikh mainly. Also different parasites were shown to invade the tissues under the pathologic examination.

Key words: Heavy metals, parasitic and bacterial examination, physical and chemical analysis, water samples, fish samples, Egyptian fish farms

# THE EFFICACY AND TOXICITY OF SLICE® (0.2% EMAMECTIN BENZOATE) AGAINST INFESTATIONS OF *LERNANTHROPUS KROYERI* VAN BENEDEN, 1851 ON FARMED SEA BASS (*DICENTRARCHUS LABRAX* LINNAEUS, 1758)

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*Lernanthropus kroyeri* is a serious copepod parasite on the gills of farmed sea bass. The presence of *L. kroyeri* causes severe gill lesions, pale and necrotic discoloration of the gills with high mucous production. These infestations could result in growth restriction, mortalities in young fish, visual deterioration of the final product and consequently associated economic losses. Practice of bath treatments against this parasite demonstrates some success but this is impractical and stressful to the fish held in large cages. The development of treatments that can be administered in-feed is necessary. The purpose of this study was to assess the efficacy of Slice (0.2% Emamectin benzoate, Intervet Schering - Plough) for the treatment of *L. kroyeri* infection in cultured European sea bass. In the present study, 600 sea bass of 80 gr average weight were collected from a commercial fish farm which is located in the Aegean Sea in eastern Greece. The fish were divided into three groups of 200 each and placed in three 7 $m^3$ circular tanks. Emamectin benzoate was administered in the form of Slice to the fish of the two tanks at doses of 50 and 100 μg/Kg of biomass respectively. Slice was coated on to commercial feed pellets with fish oil and were given to the fish for seven consecutive days. To the third tank (control) was administered feed pellets mixed with fish oil without Slice. Samples were taken from all the tanks on days -1 (one day prior to the start of treatment), 7, 17, 30 and 52, and the parasites number were counted for each fish. Slice was effective on all developmental stages of the parasite. On day 17 the Slice treated populations showed a decrease in the total number of *L. kroyeri* down to 98,80% while on day 52 there were no parasites present on the treated fish. During the experiments, fish demonstrated normal behavior without any mortality. Histological findings on various organs did not reveal any toxic effect of Emamectin Benzoate in the treated fish.

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# DETERMINING THE EXISTENCE OF A VECTOR OR RESERVOIR OF *BONAMIA OSTREAE*

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At present the complete life cycle of *Bonamia ostreae* remains unknown despite numerous studies on the issue. The means by which the parasite is transmitted is not yet fully understood. Suspicion persists that the disease may be spread by more means than just direct transmission. Studies found that even after the removal of all *Ostrea edulis* and a fallowing period of several years, oysters reintroduced into an area developed bonamiosis, indicating that a vector/reservoir of the disease may exist. A screening of several possible means of transmission was carried out. Sediment, from an oyster bed infected by the parasite, was collected over a period of one year and screened using PCR analysis. No infection was detected. A tank trail was also carried out, over a period six months, whereby attempts were made to infect naïve *O. edulis* with *B. ostreae* using sediment from an area of known infection. All oysters used in the trial were screened for infection using heart imprints and PCR analysis. Sediment used in the trial was screened using PCR analysis. No infection was detected in the oysters or sediment screened. 14 different species of macroinvertebrates, from an oyster bed infected by the parasite, were collected over a period of one year and screened using PCR analysis and *In-situ* hybridisation. Several samples of *Mytilus edulis* proved positive for the presence of *B. ostreae* using both techniques. *M. edulis* samples from two other sites of known infection within Europe were collected and screened using PCR analysis but no infection was detected.

# DISTRIBUTION OF PYRETHROIDS IN SALMON CAGES AND WELL BOATS-DIRECT QUANTIFICATION AND TRACER METHODS

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There has been increased focus on distribution of therapeutic used for sea lice treatment in salmon (*S. salar)* cages in recent years. Homogeneous distribution of the therapeutic in the treatment volume during exposure ensures efficient clearance of sea lice, and reduces risk of resistance. Pyrethroids, ALPHA MAX® with deltamethrin as the active substance (PHARMAQ) and Betamax vet with cypermethrin as the active substance (Novartis Animal Health) have been used for several years for bath treatments against sea lice. The therapeutic dose is 2µg/L of deltamethrin for ALPHA MAX and 15 µg/L of cypermethrin for Betamax.
The low concentrations of pyrethroids used for sea lice treatment have, in addition to its affinity to hydrophobic materials, pose challenges in terms of determining correct concentrations in treatment water. Chromatograph methods are developed that can detect deltamethrin and cypermethrin down to 0.3 ppb and 0.2 ppb respectively. Due to the high affinity to surfaces, including plastic and glass, special care must be taken when selecting equipment for both sampling and storage. The hydrophobic character of the pyrethroids will generally result in a higher affinity towards non polar surfaces than to polar surfaces. In addition to direct quantification of pyrethroids, two tracer methods have been tested in commercial scale: quantification of synthetic DNA and use of fluorescein.
The synthetic DNA or fluorescein was added into the mixing of water together with pyrethroids before distribution into the cages or in well boats. Water samples were collected from different positions and time points for analysis of DNA (real time PCR) or pyrethroids (GC), whereas the fluorescein was continuously detected in the water column by sensors. A comparison study was also performed in order to exclude potential interactions between the analytical methods used.
The presented methods have recently played an important role in optimizing the bath treatment methods against sea lice in Norway. Detailed information on the different methods and its practical use in the field will be discussed.

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# CHARACTERIZATION OF THE INTESTINAL TISSUE OF EMACIATION DISEASE INFECTED OLIVE FLOUNDER *PARALICHTHYS OLIVACEUS* IN KOREA

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The emaciation disease has been recently found in olive flounder *Paralichthys olivaceus* cultured in Jeju-do farms of Korea. In this study, pathological and molecular analyses were conducted to identify the causative agent of olive flounder emaciation. The experimental intestinal tissues were collected from olive flounder, a typical emaciation in farms.
Total DNA were isolated from each intestinal tissue and were analyzed using the PCR method.
From the small subunit ribosomal DNA(SSU rDNA) gene of *Enteromyxum leei* which was reported to be a causative agent of the olive flounder emaciation disease in previous study of Japan, the MM18Sf·MM18Sr primers were produced and tested. As a result, the band did not show. So, we developed a new primer set (EM-1F/EM-1R) with the basis on the previously reported sequence of the *Myxidium* sp.. In 39 olive flounder collected from farms between 2010 and 2012, PCR positive samples were identified as 9(23%).
Histological changes were mainly seen on the intestine and infected fishes showed 4 to 7 μm flood spore form in their epithelium tissue. It was noticed that plasmodia located in the intestine epithelium were positive for Uvitex 2B. On the kidney tissue with positive emaciation disease, there were a deformed distal proximal convoluted segment and a part which is similar to the glomerular in the flood spore form. The present study confirmed that the emaciation disease of olive flounder is caused by parasite.

# ENCAPSULATION OF GILL PARASITES IN RELATION TO THE TOTAL PARASITE BURDEN AND CONDITION INDICES IN THE ROACH, *RUTILUS RUTILUS*

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We examined wild populations of the roach for relationships between several condition indices, the total burden of parasites and the proportion of *Rhipidocotyle campanula* (Digenea) parasites killed by the host in gills. We found a negative correlation between the total number of parasites and the proportion of encapsulated gill parasites suggesting that individuals with stronger encapsulation response have a better immunocompetence to fight other types of parasites.

# NEW MONOGENEAN IN THE PARASITE FAUNA OF SANTER BREAM *CHEMIERIUS NUFAR* VALENCIENNES, 1830 FROM OMAN WATERS

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Starting from November 2012 a profound investigation on parasite of *C. nufar* was commenced. Samples were collected along the Southeastern coasts of the Arabian Sea, Sultanate of Oman. Different parasites were found to infect the gills of *C. nufar*, among them a Monogenean parasite that has the characteristic features of the genus *Lamellodiscus* Johnston et Tiegs, 1922 (fam. Diplectanidae) was frequently recovered. The analysis of the structure of the haptor in particular, lamellodiscs and MCO states that this species belongs to "ignoratus" group of *Lamellodiscus* (Diamanka et al., 2011). It is known that only 6 *Lamellodiscus spp.* are representatives of this group, which has specificity to fishes of the family Sparidae. Based on the morphological information obtained in this study we assume that this parasite could be the seventh species in group "ignoratus". In the present a detailed description of this new species will be given based on deferential morphological and morphometric analysis with previously reported *Lamellodiscus*. Biological investigation on the infection rate of this parasite showed that the infection intensities were often high and the infection prevalence reached 100% throughout the duration of the study. Infection intensity appeared to be affected by seasonal changes and varied from 5 up to 97 parasite/fish. Studying of the biology and distribution of this monogenean has the an important practical value, as it is known that some monogenea can cause epizootics in open waters cage culture such as the infection of meager *Argyrosomus regius* (Sciaenidae) in floating cages located in north-eastern Sardinia (western Mediterranean Sea) due to the infection by the monogenean *Sciaenacotyle panceri* (Microcotylidae) (Merella et al., 2009). The data obtained in this study can help in the development of preventive measures against this parasite in future aquaculture industry in the country.

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# COMMON PARASITIC DISEASES OF AUSTRALIAN BARRAMUNDI, *LATES CLACRIFER* AQUACULTURE AND THEIR CONTROL

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The Australian barramundi industry is divided across marine and freshwater production, pond and intensive recirculation based aquaculture. Next to viral and bacterial epizootics, parasitic diseases are the most common causes of fish losses in production. These diseases include the copepods *Lernanthropus latis* and *Argulus* spp., monogenean gill flukes (*Diplectanum* sp.; *Neobenedinia* sp.) as well as *Ichthyobodo* sp., *Trichodina* sp. and *Chilodinella* sp. Monogeneans, although normally associated with juvenile marine production, can persist in freshwater grow out for several weeks or even months. However, control is possible through extended bath treatment of fingerlings with praziquantel. Gill copepods and protozoans, can be controlled with formalin or salt bath treatments although the main factors affecting outbreaks are poor water quality and stock management. Less commonly, myxosporidians can also affect the gills.

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# ECTOPARASITC CYMOTHOIDS (CRUSTACEA, ISOPODA, CYMOTHOIDAE) FROM INDIAN MARINE FISHES

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Cymothoid isopods (Crustacea) are ectoparasitic on marine, brackish and freshwater fish. They were revealed in several earliest references on natural history. Despite these early studies, cymothoids are still poorly known or completely unknown in many parts of the world. There are more than 40 genera containing more than 350 species in the world. Likewise, Indian cymothoids has a long history, going back to a first record in 1783, but cymothoid studies are still scanty. In India a total of 12 genera including 36 valid species are listed from 74 host species of 34 families. The host specificity and host–parasite relationships including the site of infection of isopods on their respective hosts are discussed. Parasitological indexes are calculated. In our studies, the maximum number of species recorded from infected body sites were: from the body surface (17), followed by the branchial chamber (12) and the buccal cavity (7). Some buccal or branchial isopods have often been reported moving out of their normal localization, particularly after the host capture. In India, the main infected host group are the Clupeiformes including the families Chirocentridae, Engraulidae, Pristigasteridae and Clupeidae. In particular, the Clupeidae have been recorded with 21 species of cymothoid parasites. Many host families need to be studied to reveal the infection and to standardize the localization of isopods on the host.

# COMPARISON OF MICROPARASITES INFECTION LEVELS BETWEEN WILD AND AQUACULTURE SEABASS

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Seabass is one of the most important fish species in European aquaculture. As the majority of fishes, it can be found infected with several microparasites that besides their small size can cause severe pathological lesions. Therefore, they are important to survey and, in some cases, it may be worth treating the associated infections. Using seabass as a model, we evaluated whether a survey in fish from wild environment is representative or not of the microparasite infections that we will found in aquaculture environment. A comparative survey of microparasites from fish caught in the two environments was performed, with 409 wild fish and 237 aquaculture fish. In total, microparasites belonging to 12 taxa were recorded, 11 in wild fish and 9 in aquaculture fish. *Ichthyobodo necator*, *Eimeria* sp., *Sphaerospora dicentrarchi*, *Myxobilatus* sp. and *Trichodina* sp. were component microparasites (prevalence >10%) in both wild and aquaculture seabass. However, 4 additional component species were detected in aquaculture fish: *Ceratomyxa diplodae* and *C. labracis*, Scuticociliata and amoebae (the last parasites were not surveyed in wild environment). From these results, it becomes clear that none of the detected microparasites was component in wild fish exclusively, and that 3 rare species were present in the wild fish exclusively. Furthermore, the performed comparison allows us to conclude that the most important microparasite species are the same in wild and aquaculture fish. In addition, some other species may be more important in aquaculture than in wild fishes.

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# THE CELLULAR IMMUNITY DEFENSE OF EUROPEAN EEL (*ANGUILLA ANGUILLA*) DURING NEMATODE *ANGUILLICOLOIDES CRASSUS* INVASIAE

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"Eel management plan in Poland" obligates to recreate the state of eel population from 60' and 70' of last century. It means a need to control the health condition of fish, which are fished i.a. on the shores of the southern Europe and then grout out to the fry up to 200g. Fished out of natural water are in about 80% infected by the *Anguillicoloides crassus (A.C.)* nematode. Fish dedicated to restocking water of Zalew Szczecinski in 2011 were examined. In order to examine hematopoietic organs (kidney and spleen) were sectioned from two groups of fish. Group A counted 64 pieces (4 infected by A.C.), group B counted 45 pieces (7 infected by A.C.). Organs of healthy fish and those infected by nematode (AN and BN) sectioned for separate summary probes and were isolated by centrifugation in Gradisol gradient (Polfa). To assess the metabolic activity of macrophages the Respiratory of Burst Activity (RBA), stimulated by PMA and Potential Killing Activity (PKA) test to asses the phagocytosis ability of macrophages, were conducted. Proliferative response of lymphocytes was defined by MTT testing, after mitogenic Con A and LPS (Sigma) stimulation. Collected results measured by the level of extinction (T-average value) proved significant differences of parameters of cellular immunity. RBA in group A amounted 0,29 (AN 0,166) and in group B 0,26 (BN 0,326). Potential Killing Activity was on the level of extinction in group A 0,138 (AN 0,119) and in group B 0,132 (BN 0,137). Significant differences of MTT level, proliferative responce of lymphocytes B and T were observed between groups A/B ($T_A$ 1,52/$T_B$ 1,85), as well as between groups A/AN (T 1,52/1,34) and B i BN (T 1,3/1,063). Collected results of examination indicated differentiated level of cellular immunity of european eel which in new environmental conditions, after restocking, can be more susceptible to potential pathogen infection for this fish species.

# AMOEBIC GILL DISEASE IN NORWAY – STATUS AND INFECTION TRIALS

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Amoebic gill disease (AGD) attributed to infection by *Neoparamoeba perurans* has, since 1986, caused great losses amongst farmed Atlantic salmon in Tasmania. Over the last three years AGD has also caused significant mortalities in Irish and Scottish aquaculture, including recent detections in the Orkney and Shetland Islands. In Norway, AGD was first observed in 2006 affecting four sites with Atlantic salmon. While one site reported 80 % mortality (factors other than AGD were also considered significant) the remaining sites reported mortality of 10-20 %. AGD then remained unobserved until November-December 2012, when the disease was suspected in five salmon sites in the South-West of the country. In three of these cases diagnosis was based on both histopathological findings and species identification by PCR. In the two remaining cases amoebas were observed in relation to typical histopathological changes, but species could not be confirmed as material for PCR analysis was not available. All farms were left untreated and clinical signs resolved after a relatively short period. *Neoparamoeba perurans* was successfully isolated from the most affected farm. The Norwegian aquaculture industry is, at the time of writing, alert to the threat and the possibility of renewed outbreaks when water temperatures rise later in the year.
While a primary pathogenic role has been demonstrated for *N. perurans* in development of AGD in Tasmania, a similar role in European AGD had, until the present study, not been verified. In order to confirm its pathogenic role *N. perurans* isolated from farmed fish in Norway were used to successfully demonstrate Koch's postulates in Atlantic salmon. Methodology and results from the infection trials, along with the current epidemiological status of AGD in Norwegian aquaculture will be presented.

# CYPERMETHRIN AS AN EFFECTIVE TREATMENT FOR EUROPEAN SEA BASS, *DICENTRARCHUS LABRAX* L., INFECTED WITH THE ISOPOD PARASITE *CERATOTHOA OESTROIDES* (RISSO, 1826).

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Isopod infections are a serious problem currently affecting cultured sea bass and sea bream in the Mediterranean aquaculture. *Ceratothoa oestroides* (Risso 1826) is the most common isopod parasite affecting cage-cultured European sea bass (*Dicentrarchus labrax* L.). It anchors itself inside the branchio-oral cavity often leading to high mortalities in juveniles. In adult sea bass resident parasites can grow, mature and reproduce within the oral cavity of the fish in compagnionship with the male partner, often resulting in excessive outward protrusion of the lower jaw. Reduced growth is often the result of hindrance in the normal feeding activity of the fish.
Cypermethrin has been successfully used to treat parasitosis in Atlantic salmon (*Salmo salar*, L.) against sea lice (*Lepeoptheirus salmonis*, Kroyer 1838) while diflubenzuron and deltamethrin have been effectively applied against *C. oestroides* in European sea bass. However there are no reports on the efficacy of cypermethrin in European sea bass against *C. oestroides*. We conducted a series of experimental trials, under controlled laboratory conditions using cypermethrin in liquid form (Excis® 10mg $L^{-1}$ Cypermethrin, Novartis) to treat parasitized European sea bass at different cypermethrin concentrations (0,005; 0,01; 0,015; 0,02 and 0,065 ppm). Therapeutic results were achieved at 0,005 ppm suggesting that cypermethrin could constitute a valid alternative to diflubenzuron and deltamethrin against *C. oestroides* parasitosis in European sea bass.

# TOXICITY OF CYPERMETHRIN ON THE EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX,* L.) JUVENILES

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Cypermethrin has been successfully utilized against parasitosis by *Lepeotheirus salmonis* Kroyer 1838 in Atlantic salmon (*Salmo salar,* L. 1758). While recent research suggests that cypermethrin could be effective against infestations of *Ceratothoa oestroides* (Risso 1826) on farmed European sea bass (*Dicentrarchus labrax* L., 1758) in doses as low as 0,005 ppm, no information is available regarding toxicity values of Cypermethrin in European sea bass. The aim of the present study was to evaluate the toxicity of cypermethrin on European sea bass juveniles. We used cypermethrin in liquid form (Excis® 10mg $L^{-1}$ Cypermethrin, Novartis) to assess toxicity both on healthy and infected fish at 0,005; 0,01; 0,015; 0,02; 0,065; 0,085 and 0,1 ppm of cypermethrin for 96h under controlled laboratory conditions. Results showed that survival in both groups was best at cypermethrin concentrations below 0,065 ppm.

# ANTIPARASITIC EFFICACY OF DIHYDROSANGUINARINE AND DIHYDROCHELERYTHRINE FROM *MACLEAYA MICROCARPA* AGAINST *ICHTHYOPHTHIRIUS MULTIFILIIS* IN *RICHADSIN* (*SQUALIOBARBUS CURRICULUS*)

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*Ichthyophthirius multifiliis* is a holotrichous protozoan that invades the gills and skin surfaces of fish and can cause morbidity and high mortality in most species of freshwater fish worldwide. The present study was undertaken to investigate the antiparasitic activity of crude extracts and pure compounds from the leaves of *Macleaya microcarpa.* The chloroform extract showed a promising antiparasitic activity against *I. multifiliis.* Based on these finding, the chloroform extract was fractionated on silica gel column chromatography in a bioactivity-guided isolation affording two compounds showing potent activity. The structures of the two compounds were elucidated as dihydrosanguinarine and dihydrochelerythrine by hydrogen and carbon-13 nuclear magnetic resonance spectrum and electron ionization mass spectrometry. The *in vivo* tests revealed that dihydrosanguinarine and dihydrochelerythrine were effective against *I. multifiliis* with median effective concentration ($EC_{50}$) values of 5.18 and 9.43 mg/l, respectively. The acute toxicities ($LC_{50}$) of dihydrosanguinarine and dihydrochelerythrine for *richadsin* were 13.299 and 18.231 mg/l, respectively. The overall results provided important information for the potential application of dihydrosanguinarine and dihydrochelerythrine in the therapy of serious infection caused by *I. multifiliis*.

# EXPANSION OF EPIDEMIC *FLAVOBACTERIUM PSYCHROPHILUM* CLONES IN CHILEAN FISH FARMS

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Chile is the second largest producer of farmed salmon in the world. Atlantic salmon, Coho salmon and rainbow trout are intensively cultivated in Chilean fish farms. All of these species have been imported in Chile for aquaculture purpose since the second half of the 19$^{th}$ century. The bacterium *Flavobacterium psychrophilum* is an important pathogen of salmonids worldwide and a very serious concern for the fish farming industry in Chile. MLST analysis of 94 bacterial isolates collected from the main production zones, revealed the expansion of a very limited number of genotypes. These isolates are genotypically identical or very closely related to the ones previously identified in Europe and North America. Our data suggest that fish farming practices have likely contributed to the expansion of selected epidemic bacterial clones.

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# IMMUNE RESPONSE IN HEAD KIDNEY OF RAINBOW TROUT FRY FOLLOWING STRESS AND INFECTION WITH *FLAVOBACTERIUM PSYCHROPHILUM*

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The bacterial fish pathogen *Flavobacterium psychrophilum*, the cause of Rainbow Trout Fry Syndrome (RTFS), results in significant mortality in farmed rainbow trout; unless it is treated with antibiotics. Presently no commercial vaccine exists. More knowledge is required to elucidate the immune response in rainbow trout against *F. psychrophilum* in order to create preventive measures against RTFS. A limited number of studies have been carried out so far and have relied on samples from either naturally infected or injection-challenged fish. The use of naturally infected fish introduces many possible sources of error. Injection is a suboptimal approach for investigations regarding the immune response, since mucosal surfaces are bypassed. *F. psychrophilum* has a limited ability to cause disease in experimental bath challenges without applying a stressor. Recently, a bath model utilized $H_2O_2$ before pathogen exposure to elevate mortality. The model was used to examine the immune response to infection in rainbow trout fry (≈1 g); both with and without preceding $H_2O_2$ treatment. Samples from the head kidney were taken before pathogen exposure and 4 hours, 48 hours, 125 hours and 192 hours after exposure. The regulation of several immune relevant genes was examined and the relative bacterial load was assessed. Although it is not determined how $H_2O_2$ increases mortality, it is assumed to be due to stress. Exposure to $H_2O_2$ prior to infection altered the regulation of several genes, and several correlations between pathogen load and gene expression were observed.

# DEVELOPMENT OF IMMERSION IMMUNIZATION METHOD AGAINST FINNISH *FLAVOBACTERIUM COLUMNARE* STRAINS

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Despite great losses *Flavobacterium columnare* bacterium causes for fish farming every summer, there is no vaccine available against columnaris disease in the EU. The only available cure is antibiotic treatment which may cause both development of antibiotic resistant strains and impairment of maturation of fish fry immunity. Thus, preventative methods against *F. columnare* infections are needed urgently. We tested heat- and formaldehyde-inactivated *F. columnare* cells, and cells broken down by freezing and thawing, as immunogens on rainbow trout (*Oncorhynchus mykiss*) fry (1.4–3.3 g) in immersion immunization experiments conducted at a fish farm and in the laboratory. *F. columnare* strain B402, isolated in 2010 from whitefish (*Coregonus lavaretus*) during a columnaris outbreak at the fish farm, was used. The fish were immunized by dipping for 1 min into 10 l of water containing immunogen corresponding to $6.0 \times 10^6$ colony forming units of bacterial cells, and boost immunized at 21–28 days post immunization (dpi). Efficacy of the immunizations was tested during the columnaris outbreak at the fish farm starting at 69 dpi and in the experimental *F. columnare* infections in the laboratory at 35–52 dpi. *F. columnare* specific antibody titres from plasma and skin mucus were determined by Enzyme Linked Immunosorbent Assay and bactericidal activities of the plasma were tested. During the columnaris outbreak at the fish farm, mortality of the immunized fish was slightly lower compared to the non-immunized fish (not statistically significant result). In the laboratory, mortalities of the immunized fish did not differ from or were significantly higher than the mortalities of the non-immunized fish. *F. columnare* specific antibody titres or the plasma bactericidal activities of the immunized fish were not elevated in any of the experiments. Inefficacy of the immunization methods may be explained by insufficient exposure time to or the amount of the immunogen, the way the immunogen was made, or the laboratory conditions. Also, in small fish, antibody production can be weak and the antibodies may not work efficiently. However, the fish farm results justify and encourage us to continue the development of immersion immunization method for rainbow trout fry against Finnish *F. columnare* strains.

# IMPACT OF PHENOTYPIC VARIATION AND GROWTH ENVIRONMENT ON PUTATIVE VIRULENCE FACTORS OF THE FISH PATHOGEN *FLAVOBACTERIUM COLUMNARE*

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*Flavobacterium columnare* causes columnaris disease at fish farms during the warmest months of the summer around the world. In Finland, infection in salmonid fish fingerlings can lead up to 100 % mortality within the rearing unit. Under laboratory conditions *F. columnare* displays three different colony morphologies: rhizoid, rough and soft. Only rhizoid type is virulent, whereas rough and soft are incapable to infect fish. Switching between colony types has led us to the suggestion that virulence of *F. columnare* is due to carefully regulated gene expression. As activity of the fish tissue degrading enzyme, chondroitinase, has previously been shown to associate with *F. columnare'*s virulence, we measured its gene expression among other putative virulence factors using RT-qPCR. Gene expressions were compared between different colony morphologies that were cultured on agar plates and liquid medium. We explored how environmental factors, such as nutrient concentration and growth phase, affect virulence factor expression. Expression level of chondroitinase was significantly associated only with the virulent rhizoid colony type in bacteria cultivated both on agar plate and in liquid medium. In both environments, the chondroitinase expression increased towards increasing nutrient level, suggesting that nutrient availability may be an environmental cue for the bacteria and trigger virulence factor production. *F. columnare*'s virulence seems to be complicated and multilevel system that may be regulated in response to abiotic signals, such as nutrient concentration in the environment. Therefore studies of virulent and non-virulent morphologies can be the key element in solving the genetic puzzle behind virulence of *F. columnare*.

# GROWTH AND VIRULENCE OF DIFFERENT COLONY MORPHOLOGIES OF *FLAVOBACTERIUM COLUMNARE* ORIGINATING FROM FISH FARMS AND ENVIRONMENT

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*Flavobacterium columnare,* the causative agent of columnaris disease outbreaks at fish farms, can be considered as an opportunistic fish pathogen, due to its ability to grow outside the fish host. The bacterium exhibits different colony morphologies of which "rhizoid" is virulent and "rough" is non-virulent. Fish farm isolates exhibiting rhizoid colony morphology have been found to be characterized by high virulence, whereas this association is not so strong in isolates from natural waters. The presence of several colony types suggests their different biological functions in transmission and survival outside the host and during the infection. As compared to natural waters, fish farms can be considered as extreme environments in terms of available host resources, but also in terms of stress caused e.g. by chemical and antibiotic treatments. Fish farms and environment could thus impose different selection pressures for coping between the within and outside host environment. We measured growth parameters of "rhizoid" and "rough" colony morphotypes of *F. columnare* isolates both from natural waters and from disease outbreaks at fish farms in different resource concentrations and temperatures, and tested their virulence with the zebrafish challenge model. We found that the "rhizoid" morphotypes had higher virulence but lower growth rate than the "rough" morphotypes, but only if the isolate was originating from the fish farms. This suggests that expression of the virulent morphotype might be costly and decrease the ability to grow outside host environment for this opportunistic bacterium. This could emerge as a consequence of selection that occurs at fish farms rather than in the natural environment.

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# HUMORAL RESPONSE TO SYNTHETIC PEPTIDE FROM OUTER MEMBRANE PROTEIN A (*OMP*A) OBTAINED FROM CHILEAN *FLAVOBACTERIUM PSYCHROPHILUM*

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High levels of resistance to florfenicol, oxytetracycline and oxolinic acid have been observed among Chilean *Flavobacterium psychrophilum* isolates and have been associated with the high amounts of antimicrobials used at the Chilean farms to control outbreaks caused by this pathogen. To date no vaccine is commercially available to prevent the appearance of this disease. Among the antigens present in *F. psychrophilum, Omp*A has been used in vaccines against different Gram-negative bacteria, as well as human pathogens. In this study, we synthetized a peptide of 13 residues from *Omp*A sequence and evaluated its ability to develop a humoral response. This peptide was selected based on the hydrophilicity, flexibility and antigenicity to B-cells and was synthesized by the solid-phase method with a multipeptide synthesizer and purified by high-pressure liquid chromatography (HPLC). The peptide was coupled with hemocyanin by the carbodiimide conjugation method with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC). Balb/c mice were immunized with this peptide by intraperitoneal injection and serum was collected. Western-blot assays demonstrated specific reaction and recognition of *Omp*A using whole *F. psychrophilum* protein obtained from different isolates. Actually, challenge studies to assess this peptide-based vaccine are being developed.

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# SOCIAL LIFE OF A BACTERIAL FISH PATHOGEN *FLAVOBACTERIUM COLUMNARE*

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Bacteria have social behaviors that have reciprocal consequences (positive or negative) for the interacting individuals. In addition to cooperation, spiteful interactions are being increasingly demonstrated among bacteria. Indeed, production and release of spiteful toxins can benefit the bacteria by specifically killing their competitors. As multiple genotype infections are common in nature, competitive ability can influence infection dynamics and evolution of bacteria. *Flavobacterium columnare* causes large economic losses for freshwater fish farming around the world. Genetically different strains co-occur at fish farms and in nature, suggesting competition both in the within-host and outside-host environments. Here, we studied strain interactions and competition of *F. columnare* strains isolated from disease outbreaks during 2003-2010. The release of toxins and phage on the culture medium was studied by co-culturing the bacterial isolates on agar plates by double layer method.
Secretion of bacteriocins was found to be associated with local competition of the strains in time, i.e. the most recent isolates inhibited most likely the growth of earlier isolates from the same location. In some *F. columnare* strains the tocin production was contact-dependent, requiring the identification of non-relative bacteria from self and activation of targeted inhibition. In addition to toxins, some *F. columnare* strains constantly released phage in low titer to kill other genotypes in proximity.
The release of toxins and phage is costly for the bacterium, but the competitive advance is evident. Our results indicate that the bacterial populations at fish farms are not stabile, and that the toxin production needed for spite may evolve due to competition in time. Our study is the first step towards understanding the importance of social networks and competition of *F. columnare* in the outside-host environment. Competition and spite may have consequences for population structure of *F. columnare* at fish farms and in nature, and also on the evolution of virulence in recurring disease outbreaks.

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# FISHPATHOGENS.NET – A RICHLY VISUAL FISH PATHOGEN DATABASE

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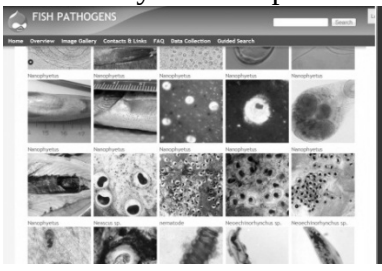
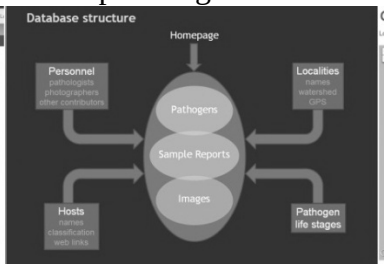
Fish are an integral part of many cultures, including those of the Pacific Northwest of North America. Widespread recreational fishing leads to the Oregon Department of Fish and Wildlife (ODFW) receiving many enquiries from the public regarding abnormal appearing fish, e.g. “What is that large white cyst in my salmon fillet?”, “What are these worms in this bluegill?”, “Can I still eat it?”. ODFW supported us (OSU) to create a public database that is an easy-to-use, richly visual, web-based source of information for the pathogens of Oregon’s fishes: **www.fishpathogens.net**.

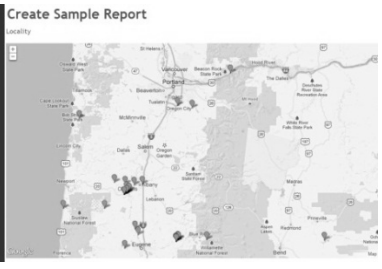
Our database cross-references three primary categories of information: >500 host and pathogen **images** from OSU and ODFW collections; >120 **pathogen information summaries** from published sources; and >600 **fish examination reports**. Data sub-categories include lists of >200 fish host species, and a Google Maps-based locality recording and searching tool. We are digitizing fish pathogen records both from OSU surveys and from ODFW’s extensive, but disconnected, health records of wild and hatchery fish. We sought to incorporate significant but poorly-circulated data (reports, personal databases) from fisheries scientists, data that is largely inaccessible to other researchers and the general public. We have also a time-sensitive opportunity to use knowledge from several key ODFW and OSU fish pathologists who are retiring.

Pathogens include viruses, bacteria, fungi, protozoans and metazoan parasites (acanthocephalans, myxozoans, crustaceans, platyhelminthes, nematodes). Non-specialists can identify common macroscopic pathogens and disease signs by stepping through a guided image search, browse through galleries of host and pathogen images, or search based on particular hosts, pathogen types or localities. Specific pathogen information can be printed out as “fact sheets”. An advanced, filter-based search is available to pathologists to synthesise pathogen records and mine patterns from the original examination reports.

The database is hosted on a dedicated MySQL database server at OSU, using CentOS Linux, with Drupal 7 for webpage creation. We have designed both the output and input to be mobile-device-friendly, to promote wide accessibility of the resource by both the public and fish pathologists.



# DEVELOPMENT OF A MEDIUM FOR ISOLATION AND PRESUMPTIVE IDENTIFICATION OF *LACTOCOCCUS GARVIEAE*

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*Lactococcus garvieae* is a facultatively anaerobic, non-motile, non-spore forming Gram-positive ovoid cocci that is recognized as one of the main threats causing lactococcosis in intensive culture fish and shellfish. The pathogen caused serious haemorrhagic septicaemia in different aquaculture species and led to substantial economic losses all over the world, such as yellowtail (*Seriola quinqueradiata*) and grey mullet (*Mugil cephalus* L.) in Japan and Taiwan, rainbow trout (*Oncorhynchus mykiss*) in Europe, Australia and South Africa, and giant freshwater prawn (*Macrobranchium rosenbergii*) in Asia. However, workers in the aquatic microbiology laboratory have not been provided a species-specific tool for detecting and isolating *L. garvieae*.
A selective and differential medium, termed LG agar, was developed for the isolation and presumptive identification of *L. garvieae* that results in colonies appearing black with red halos on this medium. In this study, only the strains of *L. garvieae* and 6 of 148 strains representing 39 species other than *L. garvieae* were able to grow on the LG medium. Those 6 strains were further differentiated from *L. garvieae* by the various colors or colony features produced on the LG agar plates. Colonies isolated from the mixing culture and the infected giant sea perch by using of LG agar plates were all positively identified to *L. garvieae* by conventional tests and 16S rDNA sequence. The testing of the specificity and differential ability of LG suggests that the agar displays considerable potential as a medium for primary isolation and presumptive identification of *L. garvieae* from pathological and environmental samples.

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# IDENTIFICATION OF CYPRINID HERPESVIRUS 3 (CYHV-3) ENCODED MICRORNAs (miRNAs) AND ANALYSIS OF THEIR EXPRESSION PROFILE

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MicroRNAs (miRNAs) are a class of small non coding RNA transcripts involved in post transcriptional regulation of messenger RNAs (mRNAs). In recent years many members of the *Herpesviridae* family in particular have been shown to produce miRNAs during infections. They are among the most prominent transcripts present during latent infection, although most of these miRNAs are common to both latent and lytic stages of infection with relative expression levels of individual miRNAs differing significantly between the two stages. The study reported here has, for the first time, identified miRNAs in Cyprinid Herpesvirus-3 (CyHV-3), a member of the *Alloherpesviridae* family.
CyHV-3, also known as koi herpesvirus (KHV), causes severe disease and mass mortalities in populations of common carp and ornamental koi (*Cyprinus carpio* ). Through deep sequencing of small RNAs from *in vitro* lytic infections followed by extensive bioinformatic analysis we identified six high probability CyHV-3 miRNA candidates. Further characterization via a combination of microarray hybridization, northern blotting and stem-loop-quantitative-PCR provided more evidence supporting the existence of these miRNAs. Also, at least one of these miRNA genes shows clear signs of conservation in other Cyprinid herpesviruses. Using computational prediction methods, we were also able to identify the most likely viral mRNA targets for these miRNAs and provide insights into their possible roles. In addition we have been able to conclusively detect three of these miRNAs *in vivo* using stem-loop-quantitative-PCR. The use of RT real-time PCR assays to detect miRNAs may provide an effective approach to diagnosing latent KHV within populations of otherwise healthy appearing fish.

# DISTRIBUTION OF *VIBRIO HARVEYI, V. ICHTHYOENTERI* AND *PHOTOBACTERIUM DAMSELAE* ISOLATED FROM OLIVE FLOUNDER (*PARALICHTHYS OLIVACEUS*) IN KOREA BY MULTIPLEX PCR DEVELOPED USING *RPOB* GENE

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The Korean peninsula is surrounded by the East, West and South seas. The marine fish culture in Korea started in the early 1980's. In 2007, the production amount of cultured marine fish was recorded at about 100 thousand tons. Olive flounder, rock fish, gray mullet and red seabream are important varieties in Korea aquaculture industry. Flounder, one of marine fish occupied more than 50% in production. In 2011, production of marine cultured fish decreased to 72 thousand tons because of several typhoon given influence in Korea. Major fish bacterial diseases in Korea are edwardsiellosis, streptococcosis and vibriosis. *Vibrio* species bacteria, such as *V. anguillarum, V. harvey*, *V. ichthyoenteri* and *P. damsela* were identified as a causative bacteria of vibriosis in flounder. During the last few years, vibriosis diseases in aquacultured fish have become a threatening reality.

In this study, we have developed multiplex-PCR method using RNA polymerase β subunit (*rpoB*) gene known a housekeeping gene for identification of *Vibrio* spp. caused vibriosis in flounder. Three pairs of PCR primers designed based on the *rpoB* sequence of three *Vibrio* spp., *V. harveyi, V. ichthyoenteri* and *P. damselae.* PCR assay using a mixture of six primers yielded amplicons of 601, 434 and 533 bp in *V. harveyi, V. ichthyoenteri* and *P. damselae,* respectively. None of the untargeted strains yielded an amplicon. The sensitivity detection for pure culture in kidney showed that the lower detection limit were 2.5 X $10^4$ cfu/g kidney for *V. harveyi* and *V. ichthyoenteri* and 2.5 X $10^5$ cfu/g kidney for *P. damselae*. From the colonies on TCBS agar plates of different samples, 632 *Vibrio* spp. isolated from aquacultured flounder between 2004 and 2010 were identified by the multiplex-PCR method. 265 strains (41.9 %) were *V. ichthyoenteri* and 115 strains (18.2 %) were *V. harveyi* and 72 strains (11.4 %) were *P. damselae.*

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# IMPROVED DIAGNOSIS OF SPRING VIREMIA OF CARP BY NESTED REVERSE-TRANSCRIPTION PCR: DEVELOPMENT OF A CHIMERIC POSITIVE CONTROL FOR PREVETION OF FALSE-POSITIVE DIAGNOSIS

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Polymerase chain reaction (PCR) is especially useful for the diagnosis of various pathogens in aquatic organisms because the rapid and sensitive detection of infectious agents facilitates the control of pathogenic diseases. However, PCR results can be confounded by DNA contamination. Specially, plasmids harboring a target gene serving as positive controls are widely used at public inspection sites and in analytical laboratories, presenting multiple opportunities for contamination. In this study, chimeric plasmids were developed to minimize the likelihood of false-positive reactions due to PCR contamination during SVCV detection by semi-nested RT-PCR. An ampicillin resistance gene was truncated by PCR amplification, and the fragments were inserted into pGEM-T Easy vectors; the resulting plasmids were named SVCV chimeric plasmid-1 and chimeric plasmid-2, respectively. The first-round PCR produced a 714-bp product with the SVCV F1 and R2 primer set that was used in a semi-nested PCR to give a 606-bp product with SVCV primers F1 and R4. The 714-bp and 606-bp PCR products were amplified using appropriate PCR primer sets (SVC F1-R2 and SVC F1-R4) with SVCV chimeric plasmid clones. The first-round PCR primers were amplified using pGEM-T Easy vector DNA as a template, using the SVCV chimeric plasmid F1 and SVCV chimeric plasmid R2 primer set in the first-round PCR, and the semi-nested PCR primers were amplified using cDNA from SVCV-infected tissue as a template and the SVCV chimeric plasmid F1 and R4 primer set. In the sequence analysis, plasmids containing the SVCV-based inserts were 100% identical with the SVCV glycoprotein gene (Gen-Bank Accession Z37505). Moreover, plasmids originating from the SVCV chimeric plasmids were 100% identical (excluding primer sequences) with the ampicillin resistance gene. The results of this study show that PCR positive controls can be created without use of viral nucleic acids or pathogen infected tissues. The technique can be applied to quarantined material and can be used to detect other pathogens.

# DEVELOPMENT OF REAL-TIME PCR FOR THE DIAGNOSIS OF FISH PATHOGENIC *STREPTOCOCCUS AGALACTIAE* AND *S. DYSGALACTIAE*

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Several *Streptococcus* species have been associated with outbreaks in fish farms worldwide. In Nile tilapia (*Oreochromis niloticus* L.) farming, *Streptococcus agalactiae* is considered an emergent pathogen, being responsible for high economic losses every year. *Streptococcus dysgalactiae* was recently described as tilapia pathogen. It was firstly recorded from a case in a Brazilian farm. The lack of rapid, accurate, and reliable tools is one of the main issues to streptococcosis control at farm level. In this regard, the real time PCR may provide a valuable alternative means, being more sensible, specific, and faster than conventional diagnostic methods based in culture and bacterial identification. The aim of this work was to develop a real-time PCR to diagnose *S. agalactiae* and *S. dysgalactiae* infections in Nile tilapia. Primers and probes were designed for the target genes *cfb* and *sodA*, respectively in *S. agalactiae* and *S. dysgalactiae*. The reactions were standardized in singleplex and duplex format. Nile tilapia fingerlings were experimentally infected with *S. agalactiae* and *S. dysgalactiae*. Samples of brain, kidney, liver, spleen, muscle and gill were collected and submitted to bacteriology, PCR and developed real-time PCR (qPCR) to evaluate the clinical sensitivity of these methods. The developed qPCR presented low detection limit, being approximately of 61 cells (genome equivalents) for *S. agalactiae* and 154 cells for *S. dysgalactiae*. The clinical sensitivity of duplex qPCR for *S. agalactiae* was 90%. It was significantly higher than bacteriology (50%) and PCR (63.3%). Better results were obtained for *S. dysgalactiae* qPCR. This method reached a clinical sensitivity of 96.67%, in comparison with 46.67% and 23.3% of bacteriology and PCR. The developed duplex real-time PCR was shown to be high sensitivity and faster than other tested diagnostic assays. This method is a promising alternative to diagnose *S. agalactiae* and *S. dysgalactiae* infections in cultured Nile tilapia.

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# DEVELOPMENT OF PCR ASSAY FOR DETECTION OF *NEOPARAMOEBA PERURANS* AND COMPARISON OF HISTOLOGICAL DIAGNOSIS

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The recent description of Amoebic Gill Disease (AGD) and *Neoparamoeba perurans* in Atlantic salmon (Salmo *salar)* farmed in Chile has necessitated the development of more reliable and sensitive diag- nostic tests. Final diagnosis of infection is normally confirmed by histology. However, the correla- tion between gross gill lesions and histological lesions is generally unclear. In the current study, moderate concordance level (k=0.5319) between gross pathology and histology was observed. The sensitivity and specificity of gross pathology was 77.91% and 71.05%, respectively. *Neoparamoeba* spp. are considered morphologically indistinguishable therefore by using histopathology limits the capacity to characterise the causative agent and it can be time consuming. We developed a PCR assay to amplify the *N. perurans* 18S rRNA gene from gill clinical samples of AGD-affected fish. High concordance level (k=0.95) between PCR and histological examination was observed. The sensitivity and specificity of PCR assay was 94.64% and 97.06%, respectively. The PCR-based assay provides a rapid tool that will be useful to the diagnostic routine for AGD in Chile.

# GOING FOR GOLD IN THE DETECTION OF CYPRINID HERPESVIRUS 3

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Cyprinid herpesvirus 3 (CyHV-3) is a highly infectious pathogen that causes fatal disease in common (*Cyprinus carpio carpio*) and koi carp (*Cyprinus carpio koi*). This virulent pathogen was first detected in 1998 in USA followed by outbreaks in Israel and the USA. The virus subsequently spread to numerous countries worldwide causing severe economic losses in farmed and ornamental carp industries. KHV detection is usually based on virus propagation using KF-1 cells or by amplification of viral DNA using the PCR technique. However, due to the limited susceptibility of KF-1 cells, it is not always possible to successfully isolate KHV even from tissues with high titres of KHV. All previously described detection methods are time consuming, laborious and require specialised equipment. To overcome these limitations, nanoparticles have been explored for direct and sensitive detection of DNA. Among the nanomaterials, gold nanoparticles (AuNPs) have been extensively used mainly due to its optical properties and easy functionalization characteristics with a variety of biomolecules. In this study, we will report about the development of a label-free colorimetric nanodiagnostic method for direct detection of unamplified CyHV-3 DNA using unmodified gold nanoparticles. Under appropriate conditions, DNA probes hybridised with their complementary target sequences in the sample DNA, which resulted in aggregation of the gold nanoparticles and a concomitant color change from red to blue due to salt induced aggregation, whereas test samples with non complementary DNA sequences remain red. The colour change of the hybridization solution is observed visually, presenting direct and rapid detection of the pathogenic DNA without prior amplification. This approach is exploited for the development and evaluation of a hybridization assay for direct and rapid detection of the highly contagious pathogen CyHV-3.

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# DETECTION OF BROWN TROUT ANTIBODIES TO GLOCHIDIA

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Relationship between fish and reproduction of mussels can induce an immune response. Enzyme-linked immunosorbent assay (ELISA) is a method which has been known for many years. This technology is based on an antibody-antigen interaction and subsequent detection of this complex by a conjugate. The purpose of the present contribution was to develop an ELISA test for the detection of specific antibodies in brown trout (*Salmo trutta m. fario*) after having encountered the ectoparasitic form of the larval stage (glochidium) of the freshwater pearl mussel (*Margaritifera margaritifera*) and to verify the method on a group of experimentally infested fish. The glochidia were taken harmlessly from a natural population of freshwater pearl mussel and used as an antigen. Purified brown trout Ig was applied to rabbits for production of specific antibodies which were after purification conjugated to peroxidase. Development and validation of the ELISA method including its diagnostic sensitivity and specificity will be presented.

# SEROLOGICAL SURVEY OF EPIZOOTIC FISH VIRAL DISEASES IN SOME REARING RAINBOW TROUT FARMS BY ENZYME LINKED IMMUNABSORBANT ASSAY (ELISA) IN IRAN

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IHN, IPN and VHS could be considered as distractive fish viral diseases in hatchery and rearing Rainbow trout farms in the country. In current study, Enzyme Linked Immunabsorbant Assay (ELISA) as a serological confirmative method was employed for detection of the causative pathogens and confirmation of virology examination. Fifty three serums of rainbow trout broodstocks (29 female and 24 male) were examined for detection of antibodies against IHNV, IPNV and VHSV that were collected from 13 hatchery and rearing farms in three provinces during March 2009 until November 2011 in Iran. Also in order to confirm results of virology examination two samples that had revealed CPE in cell lines were chosen. Finally 44 serum specimens plus 24 negative control samples (8 for each suspect virus: IHNV, IPNV and VHSV) were selected for ELISA examination. Blood samples were taken from caudal vein and serum was separated after centrifuging, nine samples were omitted for some technical problems such as hemolysis, finally serum specimens were transferred in sturdy, leak-proof plastic vials and stored at -20ºC freezers. Then was used for ELISA examination and all Optical Density (O.D) were obtained. All (O.D)s more than Cut-off Point were considered as positive samples. So regarding to ELISA findings and disease incidence percentage (Number of all positive case standing above Cut-off point divided to all examined suspected samples), IHNV had more percentage of disease in ELISA test with 23.25% in comparison with other relevant viral diseases i.e. VHSV with 14.29% and IPNV with 7.31% .These findings and disease incidence percentage show that IHN could be one of the most important viral diseases in Iran. In fact, serology findings in ELISA could be supported strongly from virology conclusion and confirmed Rhabdovirus-like particles (that was observed previously in virology examination) as a main causative agent in occurrence of IHN in hatchery and rearing Rainbow trout farms in Iran. Rapid and sensitive diagnosis methods of fish viral infectious diseases are very critical if dissemination of the viruses causing these diseases is to be controlled, because no effective vaccines or treatment currently exist for their control and prevention in the country.

Key words: Enzyme Linked Immunabsorbant Assay (ELISA), IHN, VHS, IPN, Rainbow trout, Iran

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# GROWTH OF EK-1 CELLS WITH DIFFERENT MEDIA COMPOSITION

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The availability of fish cell lines has helped to increase the knowledge about virus. The capacity to isolate and expand fish viruses in the laboratory has facilitated research on viral diseases, and has become the "gold standard" for the detection of viruses in fish cultured species. in the last decades the joint use of cell cultures together with molecular approaches has allowed our knowledge of the biology of fish viruses to grow exponentially. Even in the present molecular era virus isolation remains as an important tool for both research and diagnosis.
Eel is an important cultured fish species both economically and ecologically. Survival of this specie relays on the reproduction of adult fish in the Sargasso Sea in the middle of the Atlantic. But in the last decades the eel population has suffered a significant decline that has put them in a serious risk of disappearing. Human actions, parasites, toxics, and viruses have all been pointed to have responsibility on this decline. Some of the virus are eels specific, and a cell line (EK-1) was developed to study these viruses (Chen et al., 1982). The use of this cell line has helped to study viruses like the Eels herpesvirus, but not much has been done on the requirements to grow these cells. Our objective was to see if different composition of culture medium could influence on the growth of these cells. We used L-15 as base medium and different concentrations of Foetal calf serum (FCS) and L-glutamine. Cells were grown on these media and their number was counted at days 1, 4 and 8. Our results show different growth velocity, and therefore the number of cells at each study time differed depending on the media composition. These results seem to suggest that we can modulate the cell growth by changing media components to have a faster or slower growth depending on our needs.

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# THERE AND BACK AGAIN: A TALE OF SEA LICE RESISTANCE

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Parasitic copepods, such as *Lepeophtheirus salmonis* and *Caligus elongatus*, are a major challenge currently facing commercial salmon production. Control of the parasite using drugs has led to emergence of drug resistant strains of the organisms. Reduced efficacy of emamectin benzoate (EMB), until recently (2009) the most commonly used sea lice therapeutant in many parts of the world. Despite reports of resistance of lice to EMB in Eastern Canada, there remain populations where reduced/failed efficacy of EMB has not been reported such as in Grand Manan, NB and Newfoundland. We investigated 1) whether reports of EMB treatment success in Grand Manan, Bay of Fundy, New Brunswick, can be explained through EMB bioassay and gene expression studies, 2) if other populations of sea lice not under EMB selective pressure possess similar EMB sensitivity as Grand Manan sea lice populations, 3) the heritability of EMB sensitivity/resistance in *L. salmonis* populations, and 4) whether these populations responded similarly to other anti-sea lice therapeutics. The EMB bioassay results indicated population, species, sex-based, and temporal differences in EMB $EC_{50}$ values. *Lepeophtheirus salmonis* collected from Grand Manan and Newfoundland showed lower EMB $EC_{50}$ values compared with two reference populations in mainland NB. Sea lice reared in the laboratory maintained their EMB sensitivity status for up to three filial generations. *Caligus elongatus*, also showed lower EMB $EC_{50}$ values compared with *L. salmonis* collected from the same site, suggesting species differences in EMB sensitivity in sea lice. These results will be discussed in terms of the potential heritability of these traits and how they may interact with switching drug regimes.

# LOW-FREQUENCY ULTRASOUND FOR PATHOGEN CONTROL IN RECIRCULATING AQUACULTURE SYSTEMS

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Low-frequency ultrasound (LFUS, 25 kHz) was evaluated as a novel disinfection technique within recirculating aquaculture systems both individually and combined with UV-C. Dose-dependent inactivation rates were determined for model organisms representing different taxa of common fish pathogens: (1) heterotrophic bacteria naturally occurring in the water of recirculating aquaculture systems, determined as the number of colony forming units (CFU), (2) the ciliate *Paramecium* sp., (3) second stage larvae of the nematode *Anguillicoloides crassus*, (4) *Artemia* sp. metanauplii.
Application of LFUS up to 19 kJ $L^{-1}$ did not reduce the number of colony forming units (CFU), whilst UV-C irradiation was highly effective. Pre-treatment with LFUS reduced the particle size of suspended solids and thus increased the germicidal effect of UV-C by up to 0.6 log units. In contrast, LFUS was effective against eukaryotic organisms, and the dose-dependent inactivation could be well described by functions of an exponential decay. In clear water, the energetic efficiency of UV-C (emitted by a low pressure lamp) against *Paramecium* and *Anguillicola* larvae was higher compared to LFUS, but LFUS was more efficient against *Artemia*. However, the efficiency of LFUS against ciliates or nematode larvae would be similar or even higher than UV-C in highly turbid water or if less efficient medium pressure lamps are used.
The continuous water treatment with LFUS (2.4 kW) within a 16 m³ recirculation system reduced the number of free swimming *Trichodina* by 92 % within four days. Furthermore, it has been proved that LFUS can delay the transmission of the ciliate between tanks. The combination of LFUS and UV-C could provide an appropriate water treatment with regards to all relevant pathogens in recirculating aquaculture systems.

*The project was supported through a grant by Deutsche Bundesstiftung Umwelt (DBU).*

# BROOK TROUT (*SALVELINUS FONTINALIS*) AND HYBRID (*S. FONTINALIS* X *S. ALPINUS*) DISEASES IN RECIRCULATION SYSTEM OF DANISH TYPE

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The aim of this study was to find presence and dynamics of diseases in recirculation system in dependence with season, various origin and fish density. Samples for patho-morphological (histological), parasitological and microbiological examination were taken monthly. In brook trout we registered skin lesions mainly in caudal peduncle and tail fin. The prevalence of these changes was higher in trough with higher fish density. Two parasite species were identified (*Ichthyophthirius multifiliis* on the skin and extraordinarily on gills and *Raphidascaris acus* in the gut) by parasitological examination. The highest epidemiologic characteristics of *Ichthyophthirius multifiliis* were determined in September and October. The prevalence of *Raphidascaris acus* was 14 – 71% with maximum in September, intensity was 1-5 pieces in fish. Since October capsules with larval stages of nematods in the gut wall and in pyloric appendixes started appeared. The highest amount (100 peaces) was detected in October, the highest prevalence (100%) in January. The higher epidemiologic characteristics of this parasite were signed in *Salvelinus fontinalis* in comparison with hybrids. By microbiological examination *Yersinia ruckerii* was identified from the liver of one specimen *Salvelinus fontinalis*. From the changes on caudal peduncle mostly *F. psychrophilum* was identified. The most important problem of the monitored fish farm seems to be furunculosis of salmonid fish in all clinical forms. The most frequent incidences of *Aeromonas salmonicida* were identified in autumn in *Salvelinus fontinalis* (in October *A salmonicida* colonized gills of 86% examined fish). Sensitivity assessment showed the occurrence of sensitive and (as well) resistant *A. salmonicida* isolates against antibiotics.

*The study was supported by project NAZV (QJ 1210013).*

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# STUDY OF THE INFLUENCE OF COMBINED CHLORIDE – NITRITE CONCENTRATIONS ON HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AND NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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Nitrite is well-known toxicant for fish and its elevated concentrations can cause a great problem in waters with intensive fish husbandry. The risk of nitrite exposure for fish very closely relates to concentration of chlorides in waters. The aim of this study was focused on possibilities of increase fish tolerance against elevated nitrite concentrations and to evaluate the favourable effects of chloride before and during the nitrite poisoning of Nile tilapia and rainbow trout by using of haematological and biochemical indices.
Nitrite toxicity for rainbow trout and Nile tilapia was assessed in two toxicity tests carried out according to OECD guideline for the testing of chemicals (OECD 1992). In the first one, lethal concentrations (48hLC50) of nitrite were obtained. In the second one, the fishes were subjected for ten days to two different chloride concentrations (10 and 100 $mg.l^{-1}$) and subsequently in exposure period lasting 48h were observed responses of testing organisms to nitrite concentrations corresponding with 1/3 48hLC50. The influence of combined chloride – nitrite concentrations on haematological and biochemical parameters of fishes was assessed.
In biochemical profile, significant changes between groups were observed in PHOS, K, Na and Cl in Nile tilapia, whereas, the similar changes between groups were observed only in PHOS in rainbow trout. In haematological indices, significant differences among groups were observed in the MetHb in both species. MetHb content increased markedly ($p < 0.01$) in Group 4,6 and 7 compared to control. WBC, MCH, MCV and MCHC were changed only in rainbow trout suggested profound changes of erythrocytal structure. The PCV, RBC a Hb do not differ from control throughout the experiment in both tested species.
On the bases of the MetHb content changes, we could confirm the beneficial effect of enhanced levels of chlorides before nitrite exposition of fish.

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# OOMYCETES AS AQUATIC ANIMAL PATHOGENS

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Oomycete infections are the cause of significant diseases in aquatic animals: *Aphanomyces invadans* is the cause of Epizootic Ulcerative Syndrome, *Saprolegnia parasitica* and *S. diclina* affect farmed and wild fish populations; crayfish plague (*Aphanomyces astaci*) is one of the most devastating diseases of freshwater crustacean.

Epizootic Ulcerative Syndrome

A brief introduction to the disease. Outcomes of the risk assessment on EUS undertaken by the European Food safety Authority. Outcomes of the assessment by OIE to keep EUS listed.

Current situation and relevance of EUS in Europe. Which areas may be most at risk from introducing EUS? What surveillance would be required to detect EUS if it was there?

How good are we at diagnosing Oomycete infections? Would we recognise EUS if it arrived?

Oomycete case studies / new and emerging Oomycete diseases / Oomycete disease problems

Oomycete diseases in fish, crustacea, molluscs and amphibians. What are the most relevant current Oomycete disease problems in aquaculture / in wild aquatic animals?

What are emerging issues that we need to be aware of?

How big a problem are Oomycete infections in farmed and wild fish? How is the industry coping without malachite green? Sharing knowledge on developments regarding Oomycete treatments.

The workshop aims to answer the following questions: a summary of the most relevant developments on Oomycete diseases

- What are the most burning issues?
- What should future research focus on?

# DISEASE AND TREATMENT IN FRESHWATER RECIRCULATION AQUACULTURE

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Freshwater recirculating aquaculture systems (RAS) have long been used in the rearing of fingerlings. Today, RAS has less expensive applications than previously and is currently becoming commercially feasible even in the food fish production of middle priced fish species. It offers advantages e.g. in the regulation of water temperature and other water quality factors and in the prevention of eutrofication caused by the discharge water. Literature on the diseases and their treatment is rather limited, especially in production scale RAS of rainbow trout (and other salmonids), sturgeons, fishes of perch family and other cold water fishes.

A short review of RAS of cold freshwater fish species will be given. Some colleagues having practical experience in diagnosing and treating disease problems in recirculation aquaculture will be invited to give short talks to begin the open discussion. Information on ways to regulate the water quality and fish husbandry in order to prevent disease outbreaks and production decline is of special interest/value.

The second part of the workshop will cover disease and preventive treatments in RAS. Drug residue issues are more complicated than in flow-through aquaculture, and there is a danger of compromising the biofilters with chemical baths and other treatments.

The workshop will bring fish disease scientists and other professionals interested in diseases of the freshwater recirculation aquaculture to meet and to be able to keep contact in the future.

# AMOEBIC GILL DISEASE

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Amoebic gill disease (AGD) is a condition caused by *Neoparamoeba perurans* affecting some species of cultured marine fish worldwide with new infections reported in new species and new locations. AGD has now been reported from all continents with the exception of Antarctica. AGD is the most serious health problem of farmed Atlantic salmon in Tasmania. The only commercially available treatment is freshwater bathing. AGD has been reliably diagnosed with histological examination of gills although complementary methods such as *in situ* hybridization (ISH) and polymerase chain reaction (PCR) are required to confirm the presence of *Neoparamoeba perurans*. As molecular techniques are becoming more prevalent for pathogen identification, there is a need to adapt specimen collection and preservation so that both histology and molecular biology can be used to diagnose the same sample. Suitability of different fixatives for both histology and molecular detection was evaluated and the results suggested that sea water Davidson's fixative is the best and most cost-effective fixative. While we have much more knowledge of immune response in AGD than any other amoebic infection of fish there are still some significant gaps. This is partly due to the challenges with development of in vitro culture of the causative pathogen and a lack of an in vitro disease model. As our understanding of fish immune response increases and new tools become available our interpretation of immune response in AGD and other amoebic infections of fish will improve and lead to practical solutions for this disease.

# VIBRIOSIS IN AQUACULTURE

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Aquaculture in brackish and marine water is growing worldwide. New cultured species are introduced, and types of aquaculture vary from outdoor to indoor and from flow through to recirculated water, at various temperatures. In these types of aquaculture various *Vibrio* species play an important role, as causative agents of fish, crustacean and shellfish diseases.
In this workshop experts on vibriosis are giving short lectures. After this, there is time for a discussion, to work jointly towards a list of the current most important problems on vibriosis in aquaculture production, with definition of gaps in knowledge and recommendations on future research. It is the intention to write a joint publication for the EAFP Bulletin on the input of this workshop. This publication might then be used for future calls by national and international authorities on research related to vibriosis in aquaculture, for optimal diagnostics, prevention and control. The workshop will be opened with a lecture, in which an overview of the current vibriosis problems will be presented, followed by specific lectures on vibriosis in fish, shellfish and crustaceans.
Discussion points, among others:

1. Which are the most important vibriosis problems in fish culture, crustacean culture and shellfish culture?
2. Are diagnostic methods up to date, and are the used therapies effective?
3. Are prevention measures effective, and are specific vaccines further needed?

Which recommendations can be made for adequate prevention and control of vibriosis in aquaculture?

# THERAPY OF *FLAVOBACTERIUM COLUMNARE* AND *F. PSYCHROPHILUM* INFECTIONS

**Organizer: P. Smith**
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The workshop will discuss therapy of *Flavobacterium columnare* and *F. psychrophilum* infections and recent progress in standardization and harmonization of methods and interpretation of antibiotic sensitivity tests for these species.
Major progress has been made in these fields in the last 12 months. CLSI are about to publish standard test protocols (and QC requirements) and work on ECVs and clinical breakpoints for *F. columnare* and ECVs for *F. psychrophilum* will be available.
*The aim of the workshop will be twofold:*

1. To provide for a meeting between those who are working in this field which will allow them to coordinate future research
2. To disseminate the standard interpretive criteria. This will facilitate the harmonization of susceptibility testing in the context of planning therapeutic treatments. It will also facilitate the design of programs for the monitoring and surveillance of resistance frequencies that have been recommended in the latest Aquatic Animal Health Code of OIE.

# TARGETFISH INDUSTRY FORUM

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TargetFish is a large collaborative project funded by the European Commission 7th Framework program bringing together leading European research groups that are experts on the fish immune system and enterprises from the biotech and veterinary sectors that aim to commercialize fish vaccines for European farming. TargetFish comprises 30 partners from 10 EU member states, 2 associated countries and 1 International Cooperation Partner Country (ICPC). In this large multidisciplinary consortium an approximate equal number of RTD and SME partners will cooperate closely while keeping an intensive communication with the larger pharmaceutical, vaccine and nutrition industries via an Industry Forum. The purpose of this workshop is to hold the first Industry Forum of the project where the aims of the project, the contents of the work packages, and the results generated thus far will be presented to industry.

## ADDITIONAL ABSTRACTS

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# ULTRA-DEEP PYROSEQUENCING OF PARTIAL SURFACE PROTEIN GENES FROM NON-VIRULENT AND VIRULENT STRAINS OF INFECTIOUS SALMON ANAEMIA VIRUS (ISAV) SUGGEST NOVEL MECHANISMS INVOLVED IN TRANSITION TO VIRULENCE

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Uncultivable HPR0 strains of infectious salmon anaemia viruses (ISAVs) infecting gills are non-virulent putative precursors of virulent ISAVs (vISAVs) causing systemic disease in farmed Atlantic salmon (*Salmo salar*). The transition to virulence involves a deletion in the highly polymorphic region (HPR) of the hemagglutinin-esterase (HE) gene and a $Q_{266}\rightarrow L_{266}$ substitution or insertion next to the putative cleavage site ($R_{267}$) in the fusion protein (F). We have performed ultra-deep pyrosequencing (UDPS) of these gene regions from healthy fish positive for HPR0 virus carrying full-length HPR sampled in a screening program, and a vISAV strain from an ISA outbreak at the same farming site three weeks later, and compared the mutant spectra's. As the UDPS data shows the presence of both HE genotypes at both sampling times, and the outbreak strain was unlikely to be directly related to the HPR0 strain, this is the first report of a double infection with HPR0s and vISAVs. For F amplicon reads, mutation frequencies generating $L_{266}$ codons in screening samples and $Q_{266}$ codons in outbreak samples were not higher than at any random site. We suggest quasispecies heterogeneity as well as RNA structural properties are linked to these two molecular events involved in transition to virulence. A mechanism where selected single point mutations in the full-length HPR alter the RNA structure facilitating single- or sequential deletions in this region is proposed. The data provides stronger support for the deletion hypothesis, as opposed to recombination, as the responsible mechanism for generating the sequence deletions in HE.

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# THE IMPACT OF PARASITES AND PATHOLOGIES ON THE MARINE BIVALVES CONDITION EXEMPLIFIED BY *MACOMA BALTHICA* FROM THE BALTIC SEA

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*Macoma balthica* (Linnaeus, 1758) is one of the most widespread zoobenthic bivalve in the Baltic Sea and an important part of the food web. Baltic Sea is the only reservoir, where this clam occurs below 90 meters of depth and can survive temporal oxygen deficiency and the presence of hydrogen sulfide. Recently, decline of *M. balthica* abundance and its high seasonal mortality is observed, which results from pathologic condition of species.
The aim of study was determination and evaluation of main pathologies influencing on the Baltic clam population from the southern Baltic Sea. Research material, *Macoma balthica* (15-20 mm) was collected at 9 stations situated at 5 to 70 meters of depth. Respiration rates were determined, basing on the oxygen consumption. For cytogenetic analyses (in aim of neoplasia detection) 30 organisms were incubated in colchicine, then treated with sodium citrate solution and fixed. Slides were prepared, using an air-drying technique and stained with Giemsa stain. 30 specimens were microscopically examined for the presence of parasites or signs of disease. Gonad index (GI) of all examined clams was based on the microscopic observation of gonad stage. The condition index (CI) was calculated per individual specimen, as a ratio of soft tissue dry weight and shell dry weight. Moreover, environmental parameters (T, S, $O_2$ and organic pollution) were measured. Obtained results state that neoplasia, trematode and protozoan infection were the most severe and most common diseases. The tumor prevalence ranged from 0-60 % and depended on the investigated station as well as the season. Disease affects negative on organisms physiological activity (respiration, reproduction) and total condition. The spatial differentiation of neoplasia occurrence is also linked to sediment pollution and can be used as a marker of the contamination. Additionally, neoplasia worsens bivalve condition, therefore making them more susceptible to parasite infections, mainly for digenean trematodes and ciliates (0-30% of affected organisms). Decrease of species abundance is related generally with pathologies and seasonal worsening of environmental conditions. High prevalence of diseases in *Macoma balthica* population is a significant environmental problem for the functioning of Baltic Sea food web, due to importance of bivalves for the ecosystem.

# SALMONID ALPHAVIRUS TYPE 2 (SAV2) INFECTIONS IN RAINBOW TROUT FARMS IN POLAND

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Salmonid alphaviruses (SAV) are recognized as serious pathogens of farmed Atlantic salmon, *Salmo salar* (Linnaeus, 1758) and rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) in Europe. SAV isolates have been grouped in six distinct subtypes. In 2011, sleeping disease virus (SDV) provoked severe losses in few rainbow trout farms in Northern Poland. The typical sleeping behavior was observed on juveniles of rainbow trout. Mortalities ranged from 5 to 60 %. Severity of sleeping disease was associated to bacterial and fungal infection and decubitus ulcers occurrence. The aim of this study was the description and identification the genotype of the Polish SAV and try to trace the origin of the virus. Samples for virus isolation consisted of kidney, spleen and liver pooled from 10 fish. After inoculation on rainbow trout gonad (RTG-2) cells and chinook salmon embryo (CHSE-214) cells, a cytopathic effect induced by virus was observed. Total RNA was extracted from cell culture supernatant and submitted to RT-PCR with primers amplifying two informative regions of the genome: a conserved region in the E2 gene and a variable region in the nsP3 gene. The sequences revealed that the isolate from Poland was a strain of SAV 2, sharing a very strong genetic identity with isolates from Italy and France. We suspect that the source of the virus in Poland could be eggs imported from abroad by Polish farmers. Recently (2012-2013) sleeping disease of rainbow trout observed in Poland, does not cause mass mortality. Slight economic losses due to the lack of appetite and reduced growth rate of the fish are noted, but some farms are dealing with salmonid alphavirus infection every year.

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