Gene regulatory mechanisms in infected fish

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THE ONTOGENY OF THE IMMUNE SYSTEM OF FISH – STATUS AND FUTURE CHALLENGES

May 3 to 5, 2011

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DAFINET WORKSHOP

Danish Fish Immunology Research Centre and Network

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A THREE DAY WORKSHOP

Date:
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Venue:
Siemsens Gaard, Svaneke, Bornholm
Havnebryggen 9, DK-3740 Svaneke, Denmark
+45-56496149, hotel@siemsens.dk, www.siemsens.dk

Programme

Tuesday May 3, 2011

14.00-17.00 Arrival and check-in

18.00 Dinner

19.30 Informal get-together

Wednesday May 4, 2011

07.30 Breakfast

08.30 Meeting with welcome address by DAFINET leader Kurt Buchmann

09.00 Scott LaPatra, Clear Springs Foods, Inc., Idaho, USA
Biosecurity in rainbow trout production: Immunological strategies

09.30 Jiwan Kumar Chettri, KU-LIFE, Copenhagen, Denmark
PAMP induced expression of immune relevant genes in head kidney leukocytes of rainbow trout (Oncorhynchus mykiss)

09.45 Sidhartha Desmukh, KU-LIFE, Copenhagen, Denmark
Vaccine induced specific protection against *Yersinia ruckeri* serotype 1 biotype 2

10.00 Coffee break

10.30 Niels Lorenzen, DTU-VET, Aarhus, Denmark
Experimental fish vaccination trials: do the results reflect protective efficacy under farming conditions?

11.00 Kurt Buchmann, KU-LIFE, Copenhagen, Denmark
ICH parasites and the trout immune response

11.15 Jacob Schmidt, DTU-FOOD, Copenhagen, Denmark
Tissue regeneration and wound healing capacity in carp (*Cyprinus carpio*) during ontogeny

11.30 Jakob Skov, KU-LIFE, Copenhagen, Denmark
Gene expression profiles following β-glucan feeding and immersion vaccination in rainbow trout

12.00 Lunch buffet

13.00 Excursion to the Bornholm salmon hatchery, Nexø

15.00 Coffee break

15.15 Rzgar M. Jafaar, KU-LIFE, Copenhagen, Denmark
Effect of oral administration of the immunostimulant b-glucan on non-specific immune response parameters of juvenile rainbow trout Oncorhynchus mykiss (Walbaum, 1792)and susceptibility to the skin-parasitic ciliate Ichthyophthirius multifiliis Fouquet, 1876

15.30 Louise von Gersdorf Jørgensen, KU-LIFE, Copenhagen, Denmark
Experimental evidence for direct in situ binding of IgM and IgT to early trophonts of Ichthyophthirius multifiliis in the gills of rainbow trout

15.45 Maya M. Henriksen, DTU-VET, Copenhagen, Denmark
Challenge models for RTFS in rainbow trout fry (*Oncorhynchus mykiss*)

16.00 Martin K. Raida, KU-LIFE, Copenhagen, Denmark
Vaccination against *Yersinia ruckeri* serotype 1, biotype 2

16.30 Walk along the rocky coast

18.00 Dinner

Thursday May 5, 2011

07.30 Breakfast

08.30 Excursion to Gudhjem and Østerlars

11.00 Coffee break

11.15 Brian Dall Schyth, DTU-VET, Aarhus, Denmark
Gene regulatory mechanisms in infected fish

11.30 Kasper Rømer Villumsen, KU-LIFE, Copenhagen, Denmark
Fish immune responses to *Aeromonas salmonicida*

11.45 Karina Juhl Rasmussen, SDU-Health, Odense, Denmark
Characterization and comparison of immunological functions in the primary and secondary circulatory system in Oncorhynchus mykiss

12.30 Lunch buffet

13.30 Xueqin Jiang, East China Normal University, Shanghai, China
Comparison of four feed codes: Effects on susceptibility towards White Spot Disease, serum lysozyme and mucous cell density in rainbow trout

13.45 Discussion of present DAFINET status and future challenges

15.00 Good bye coffee

15.30 Departure

Abstracts

BIOSECURITY IN RAINBOW TROUT PRODUCTION: IMMUNOLOGICAL STRATEGIES

Scott E. LaPatra

Clear Springs Foods, Inc., Research Division,PO Box 712, Buhl, Idaho, 83316 USA

Biosecurity is a relatively new and evolving concept. For the Food and Agricultural Organization of the United Nations (FAO), biosecurity broadly describes the process and objective of managing biological risks associated with food and agriculture in a holistic manner (FAO 2003). To the farmer, concepts are not useful or practical until they result in something real, the overall objective being to ensure economic viability and profitability of the enterprise.

The application of biosecurity varies considerably at the farm level. So too does the understanding of what actions are required to achieve adequate "biosecurity." To some, biosecurity is simply about having barriers in place that are designed to ensure pathogen exclusion from a farm site. To others biosecurity encompasses not only the prevention of pathogen introduction and dissemination but disease prevention. Pathogen exclusion strategies consist of the use of a fish pathogen-free water supply, complete enclosure of facilities with "bird cages," allowing only certified lots of fish onto an aquaculture facility and in extreme instances, complete disinfection of vehicles and personnel before they are allowed to enter. Prevention of pathogen dissemination can be aided by detailed signage, separate equipment for each discreet rearing unit of fish, disinfection of eggs, equipment including transport trucks, personnel and raceways, designated mortality collection points on each farm and rapid and accurate diagnosis and treatment strategies if a disease does occur. Disease prevention strategies include good nutrition, a clean and consistent rearing environment, the use of vaccines and selectively bred fish. The effectiveness of a vaccinology program may also be enhanced by utilizing alternative mass immunization strategies. Additionally, immunological parameters can be monitored such as antibody titers in juvenile and adult rainbow trout that assist in assessing vaccine efficacy and the risk of juvenile populations of fish manifesting clinical disease. This presentation will give an overview of biosecurity strategies currently used with farmed rainbow trout and identify immunological aspects that exist for positively impacting the health of these cultured animals.

PAMP INDUCED EXPRESSION OF IMMUNE RELEVANT GENES IN HEAD KIDNEY LEUKOCYTES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Jiwan K Chettri, Martin K Raida, Lars Holten-Andersen, Per W Kania and Kurt Buchmann

Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, University of Copenhagen, Denmark

Host immune responses elicited by invading pathogens depend on recognition of the pathogen by specific receptors present on phagocytic cells. However, the response to viral, bacterial, parasitic and fungal pathogens vary according to the pathogen-associated molecular patterns (PAMPs) on the surface of the invader. Phagocytic cells are known to initiate a respiratory burst following an exposure to the pathogen, but the underlying and associated specific elements are poorly elucidated in fish. The present study describes the differential response of head kidney leukocytes from rainbow trout (*Oncorhynchus mykiss*) to different PAMPs mimicking bacterial (flagellin and LPS), viral (poly I:C) and fungal infections (zymosan and β-glucan). Transcript of cytokines related to inflammation (IL-1β, IL-6, IL-10 and TNF-α) were highly up-regulated following LPS exposure whereas flagellin or poly I:C induced merely moderate reactions. In contrast, IFN-γ expression was significantly higher in the poly I:C stimulated group compared to LPS group. When head kidney cells were exposed to zymosan or β-glucan, genes encoding IL-1β, TNF-α, IL-6 and IL-10 became up-regulated. Their level of up-regulation was comparable to LPS but the kinetics differed. In particular, TNF-α induction was considerably slower when stimulated with zymosan or β-glucan. The gene encoding COX-2 enzyme, which is a central element in initiation of inflammatory reactions, was significantly higher in stimulated cells but a depressing effect of high concentrations of LPS and zymosan became evident after 4 h exposure. This study suggests that rainbow trout leukocytes respond differently to viral, bacterial and fungal PAMPs, which may reflect activation of specific signaling cascades eventually leading to activation of different immune effector molecules.

VACCINE INDUCED SPECIFIC PROTECTION AGAINST ENTERIC RED MOUTH DISEASE (ERM) CAUSED BY *YERSINIA RUCKERI* SEROTYPE 1 BIOTYPE 2

Sidharta Deshmukh1[a], Martin K Raida[1], Inger Dalsgaard[2], Jiwan K Chettri[1] and K. Buchmann[1]

[1]Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, University of Copenhagen, Denmark. [2]National Veterinary Institute, Technical University of Denmark.

In European fish farms there is evidence of enteric red mouth disease (ERM) outbreaks in previously vaccinated farmed fish. It has been suggested that the occurrence of a *Yersinia ruckeri* variant (biotype 2) may explain this situation. Recent development of commercial vaccines has included both biotype 1 and 2. In this study, the specificity of immune protection extended by three commercial vaccines viz; AQUAVAC ERM® Intervet Schering Plough (based on biotype 1 only), ERMOGEN VET® Novartis (based on biotype 1 only) and AQUAVAC RELERA® Intervet Schering Plough (based on both biotype 1and 2) developed against ERM was investigated following intraperitoneal (IP) challenge with *Yersinia ruckeri* serotype1 biotype 2. Fish were immersion vaccinated for 30 s and challenged 2, 4 and 6 months post vaccination. The onset and severity of various pathological lesions along with their disappearance during the course of disease was also carried out to evaluate the protective index conferred by three different vaccines. After IP challenge, the overall best relative percentage survival was observed in AQUAVAC RELERA® followed by ERMOGEN VET® with least survival rates in AQUAVAC ERM® among all vaccinated groups. Interestingly a marginal better immune protection was observed between AQUAVAC RELERA® and ERMOGEN VET® vaccinated group during the last two challenge trial. The onset and severity of pathological lesions observed during challenge 2 (i.e. 4 month post vaccination) suggested a beneficial efficacy shown by AQUAVAC RELERA® in terms of milder and lesser degree of certain pathological lesions like haemorrhages in or around the buccal cavity, base of fins and intestines, when compared to ERMOGEN VET®, AQUAVAC ERM® vaccinated group and *Yersinia ruckeri* (BT2) infected group.

EXPERIMENTAL FISH VACCINATION TRIALS: DO THE RESULTS REFLECT PROTECTIVE EFFICACY UNDER FARMING CONDITIONS?

Niels Lorenzen

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Vaccine development represents one of the main applied challenges in fish immunology research. For some bacterial diseases like vibriosis and enteric red mouth, traditional vaccines based on killed pathogens have proved very efficient, but for many viral and parasitic infections, only experimental vaccines are available. One central challenge in evaluating a vaccine under experimental conditions is to design and perform vaccination and infection trials that can provide results on the base of which the usefulness of a vaccine under farming conditions can be estimated. Parameters like vaccine delivery route, route of pathogen administration, size and status of the fish, pathogen dose and variability, can all affect the outcome of the trials and it is often difficult to mimic the farming conditions. Recent experimental vaccination trials with viral and bacterial vaccines will be presented and discussed in relation to how to optimize vaccine testing under experimental conditions.

ICH PARASITES AND THE TROUT IMMUNE RESPONSE

Kurt Buchmann

Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, University of Copenhagen, Denmark

The ciliate *Ichthyophthirius multifiliis* (ICH) which is able to infect skin, fins and gills of freshwater fishes worldwide is a notorious pathogen in fish cultures. Already Fouquet noticed in 1876 its extreme capability of multiplication and named it "the fish louse with many kids". It has not only been responsible for major disease outbreaks in wild and cultured fish populations. As the pathogen in a series of host/parasite models it has made it possible to dissect the intricate immune responses elicited by ciliate parasites in fish. Although the parasite previously was referred to as an ectoparasite, due to its invasion of skins and fins, it must be considered an endoparasite due to the fact that it gets totally embedded in the fish epidermis following penetration. The protective response in fish against this organism was already described by Buschkiel in 1910. The effects of infection dosage on immunity were determined by Bauer in 1953 and since the 1970es research on the immunological mechanisms involved in protection has increased markedly. In a review in 2001 it was noted that both innate and adaptive responses were taking part in immunity of fish against ICH. Since then new molecular techniques and immunological methods, including histochemistry, have made it possible to point at the action of MHCII, CD8 positive cells, B-lymphocytes, complement factors, lysozyme, acute phase reactants such as serum amyloid protein A, IgM and IgT during development of immunity in rainbow trout. It has been shown by Olsen in 2011 and by Jørgensen in 2011 that fish can be fully immunized by intra-peritoneal injections of theronts and that interactions between the systemic and mucosal adaptive immune system exist in trout. However, also innate responses do limit susceptibility of fish to infection. This observation may prove to be of practical importance for aquaculture enterprises. It was indicated by early work by Lauridsen in 2008 that immunostimulants such as beta-glucans may confer some protection to trout against this parasitosis and Jaafar has recently shown that high beta-glucan dosages lead to a significant decrease of infection levels after prolonged feeding. This information has been used by feed-producers manufacturing feed with immunostimulants. Some of these commercial preparations were recently shown by Xueqin to confer some protection to trout against ICH. Based on these observations it can be suggested that both classical antigen presentation, B- and T-cell activation are playing roles in this antiparasitic immunity. However, the role of Toll-like receptors (TLRs) must be expected to take part in the innate responses. The exact nature of this process should be one of the tasks to explore in the future. Further, the possible deviation of T-cell responses towards either TH1 or TH2 like responses following pathogen exposure should be further analysed.

TISSUE REGENERATION AND WOUND HEALING CAPACITY IN CARP (*CYPRINUS CARPIO*) DURING ONTOGENY

Jacob G. Schmidt, Hans-Christian Ingerslev and Michael E. Nielsen

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Tissue regeneration leads to restoration of damaged tissue to the original state. Healing or repair of wounds does not. In the early ontogeny of vertebrates, the regenerative capacity is greater than in the adult. This is partly related to the development of the immune system. The immune and wound healing and/or regenerative responses are intertwined, and developmental stage is thus important for the wound healing >< tissue regeneration-axis. Different parts of the immune system form at different time points during ontogeny, and non-specific innate elements are in place before adaptive memory-based responses. Common carp (*Cyprinus carpio*) is an excellent fish species to study tissue regeneration since its genome is well-described and wound healing is readily followed visually. In this study, carps were physically damaged in the musculature using sterile needles at days 10, 16, 24, 47 and 94 post-hatch. Muscle tissue samples were subsequently taken at day 1, 3 and 7 post-damage for further analysis by quantitative real-time PCR of genes related to the inflammatory response and wound healing/tissue regeneration. The analyses are still on-going, and will be presented at the work-shop.

CHARACTERIZATION OF TROUT FACTOR B ISOFORMS

Yaseelan Palarasah[1], Mikkel-Ole Skjoedt[2], Karina J. Rasmussen[1], Karsten Skjodt [1]

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The complement system includes a number of plasma proteins and cellular factors that play a crucial role in the clearance of pathogens, self-antigens and immune complexes. The complement system can be activated through three different pathways: the classical, the alternative, and the lectin pathway. Compared to mammalians, teleost fish seem to rely on a different and more complex strategy in relation to the complement system. This is evident by the presence of multiple factors and isoforms of a variety of essential components from the complement system. The rainbow trout (*Oncorhynchus mykiss*) has developed four different C2/factor B isoforms termed TfB a1, Tfb a2, TfB b and TfB c, from which the function and phylogenetic relationship are only partially understood. These serine proteases are known to generate the essential C3 convertases from the classical/lectin pathway (C2) and the alternative pathway (factor B) in mammals. The major objectives of our study were to generate specific monoclonal antibodies against the C2/factor B isoforms to establish functional and biochemical properties of the different isoforms. Furthermore, in our recently developed assays for functional capacity evaluation of the complement pathways we wanted to investigate, which of the isoforms are functionally active in the pathways and whether some of them play a dual role in the activation of complement.

GENE EXPRESSION PROFILES FOLLOWING B-GLUCAN FEEDING AND IMMERSION VACCINATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Jakob Skov, Per Walther Kania and Kurt Buchmann

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Potential immunostimulatory effects of orally administered β-glucan were investigated in combination with immersion vaccination against enteric redmouth disease (ERM) caused by *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*). Four groups in duplicate were studied, *i.e.* Group(G)1: -vaccination, -β-glucan.; G2: -vacc., +β-gluc.; G3: +vacc., -β-gluc.; G4: +vacc., +β-gluc. A linear, pure (purity ≥ 98%) β-glucan from the algae *Euglena gracilis* was applied. β-glucan supplemented diet (containing 1% β-glucan) was administered to the fish continuously for 84 days at a level of 1% biomass day^{-1}. Fish were vaccinated after two weeks of feeding, bath challenged with live *Y. ruckeri* six weeks post vaccination, and mortality was subsequently assessed for 28 days. Expression of immune relevant genes (IL-1β, IL-6, IL-10, TNF-α, INF-γ, TCR-β, CD4, CD8, MHC I, MHC II, IgM, IgT, precerebellin, SAA, hepcidin, C3 and lysozyme) in head kidney were examined at day 0, 13 (1 day pre-vacc.), 15, 55, 59 (day 3 post challenge (p.c.)), 70 and 84. Gene expression profiles will be presented. In addition, *Y. ruckeri*-specific antibodies and lysozyme activity in plasma were assessed at the sampling time points listed above, and a qualitative detection of the *Y. ruckeri* 16S gene in head kidney samples p.c. was performed. Results on mortality showed that the vaccine was efficient (RPS = 85%) ($P = 0.0002$), whereas the β-glucan had no effect on survival ($P > 0.95$). The level of antibody in plasma against *Y. ruckeri* increased similarly in all four groups p.c. and became significantly different from pre-challenge levels at day 28 p.c. ($P < 0.05$) except for unvaccinated fish receiving β-glucan ($P > 0.05$). In all four groups, plasma lysozyme activity increased significantly on day 3 p.c. ($P < 0.01$) followed by a significant decrease to pre-challenge levels on day 14 p.c ($P < 0.05$). On day 28 p.c., plasma lysozyme activity increased significantly in the unvaccinated groups (G1 and G2) ($P < 0.01$), whereas it remained at pre-challenge levels in the vaccinated groups (G3 and G4). An insignificant trend towards a positive effect of the β-glucan was observed as a slightly greater increase in lysozyme activity in β-glucan fed groups (G2 and G4) compared to control fed groups (G1 and G3) on day 3 p.c. Likewise, a slight decrease in lysozyme activity was observed in β-glucan fed groups (G2 and G4) compared to control fed groups (G1 and G3) on day 28 p.c. This could be interpreted as an enhanced ability of the fish receiving β-glucan to instantly fight the pathogen by means of lysozyme and thereby making this immune parameter less important at a later time point post infection. This interpretation is supported by the detection of the *Y. ruckeri* 16S gene in merely 20% of the unvaccinated, β-glucan fed fish (G2) on day 28 p.c. compared to 40% of those not receiving β-glucan (G1).

EFFECT OF ORAL ADMINISTRATION OF THE IMMUNOSTIMULANT Β-GLUCAN ON NON-SPECIFIC IMMUNE RESPONSE PARAMETERS OF JUVENILE RAINBOW TROUT *ONCORHYNCHUS MYKISS* (WALBAUM, 1792)AND SUSCEPTIBILITY TO THE SKIN-PARASITIC CILIATE *ICHTHYOPHTHIRIUS MULTIFILIIS* FOUQUET, 1876

Rzgar M. Jaafar, Per W Kania, Kurt Buchmann

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A wide range of β-1,3-glucans are known to modulate immune responses in terrestrial and aquatic animals. These glucans can be isolated from a variety of sources such as fungi, yeast, algae, bacteria, and lichens. They all have a (1→3)-β-D-glucopyranosyl linked backbone, but their branching frequency, degree of polymerization, molecular weight and solubility differ which play a role in glucan-associated biological activity. β-1,3-glucans are often used as dietary immunostimulants in commercial feeds for aquacultured fish species.

The present study was conducted to investigate the effects of dietary β-1,3-glucan on some innate immune parameters of juvenile rainbow trout *Oncorhynchus mykiss* and on susceptibility to the skin-parasitic ciliate *Ichthyophthirius multifiliis*. A basal diet (dry pelleted feed, Biomar) was supplemented with 0% (control), 0.2% (low), 2.0% (medium), and 5.0% (high) of the β-1,3-glucan particulate insoluble algae glucan, Paramylon® from *Euglena gracilis* with a molecular weight ~500,000 Da and purity ≥ 98% (Sigma-Aldrich). Fish were divided into 4 groups (I, II, III, and IV) each with 55 fish and each experimental diet was fed to 2 replicate groups at a daily feeding rate of 1.5% of body weight for 56 consecutive days. Liver and plasma sampling was performed at day 0 and after feeding with β-1,3-glucans for 14, 28, 42, and 56 days. Gene expression in trout liver was investigated by real-time PCR using Elongation factor 1 α as reference gene. Genes encoding several immune molecules were investigated. These included acute phase proteins (SAA, hepcidin, and precerebellin), immunoglobulins (IgM and IgT), cytokine (IL-1β), lysozyme, and mannan-binding lectin (MBL-H1, MBL-H2, and MBL-H3). The evaluation of the lysozyme activity in plasma was performed by a turbidimetric lysozyme assay. Challenge infection was conducted after feeding with β-glucan for 14, 23, and 45 days, where 10 fish (5 fish from each replicate tank) were exposed to parasites.

Challenge infection after 14 days feeding showed that high (5.0%) glucan supplementation fish obtained significantly more parasites (trophonts) ($P<0.05$) compared to control fish (0.0%). Fish fed the diet with low glucan (0.2%) for 23 days had significantly fewer trophonts relative to the control fish. In addition, fish fed the diet with medium (2.0%) and high (5.0%) glucan for 45 days had significantly fewer trophonts compared to the control group (0.0%). Fish were under regular observation every day post-challenge and mortality rate was recorded. No significant differences were seen among groups. The serum lysozyme activity showed some variation in all groups. Plasma lysozyme activity of fish fed the low (0.2%) and medium (2.0%) glucan supplementation fluctuated, while high (5.0%) glucan supplementation initiated a consistent trend for an lysozyme increase and in some groups and at some sample point glucan-treated fish showed a significantly increased level

(*P<0.01*). This study also demonstrated the stimulatory properties of β-1,3-glucans on gene expression involved in some innate immune parameters. Groups II (low 0.2%) and III (medium 2.0%) showed a down-regulation of immune relevant genes but a significant down-regulation (*P<0.01*) was merely seen in gene MBL.H2 (mannan-binding lectin) and hepcidin at last sample point (day 56 of feeding with β-glucan) relative to control group. Group IV (high 5.0%) showed a consistent trend for up-regulation of immune relevant genes but a significant up-regulation (*P<0.001*) was merely seen with regard to the gene SAA at day 28 of feeding with β-glucan.

These results suggested that low and medium concentrations of β-1,3-glucan from algae (*Euglena gracilis*) in feed merely influence the measured innate immune parameters and the antiparasitic response. However, a high concentration (5.0%) has been found associated with a significant reduced Ich-infection and a non-significant but a consistent trend for elevated expression of various immune genes. Lysozyme activity in plasma was found significantly increased in some groups and at some sample point following feeding with β-1,3-glucan. This indicates that the concentration of the immunostimulant is very important.

EXPERIMENTAL EVIDENCE FOR DIRECT *IN SITU* BINDING OF IGM AND IGT TO EARLY TROPHONTS OF *ICHTHYOPHTHIRIUS MULTIFILIIS* IN THE GILLS OF RAINBOW TROUT

Louise v G Jørgensen[1], Rasmus D Heinecke[1], Karsten Skjødt[2], Karina J Rasmussen[2] and Kurt Buchmann[1]

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The ciliate *Ichthyophthirius multifiliis* is a protozoan parasite that infects freshwater fish worldwide causing considerable economic losses in aquaculture enterprises. It has a direct lifecycle comprising the infective theront, the feeding trophont and the external tomocyst stage producing numerous free-swimming infective theronts. Following a non-lethal infection with Ich fish can acquire immunity and several studies have indicated that both cellular and humoral factors are involved in the protective response. Specific antibodies in serum and mucus from immune fish bind *in vitro* to specific parasite antigens called immobilization antigens (i-ags). It has been shown that theronts penetrate immune and naïve fish to the same extent but they leave immune hosts within two hours. It has been suggested that cross-binding by antibodies of i-ags is the stimulus triggering pre-mature exit in immune hosts. However binding of host antibodies to the parasite *in situ* has not yet been demonstrated. The development of immunohistochemical techniques has enabled us to visualize this link in the anti-parasitic host reaction of fish. The present study demonstrates binding of specific IgM and IgT to the surface of early trophonts *in situ* two hours post-infection of immune rainbow trout. No binding at all of IgT and no or only a weak binding of IgM was observed on the parasites in the gills of similarly exposed but naïve rainbow trout. IgT was recently demonstrated not only to take part in intestinal but also gill immunity. In this study IgT also appears to have a prominent role in the epithelial response of immune fish against Ich in the gills. IgM was found mainly on the surface of the parasites however in a few cases they were also found inside organelles. This could indicate that the early trophonts start ingesting antibody within two hours, possibly as a way of overcoming low levels of antibody. Furthermore, it is shown that IgM from the systemic compartment is likely to be connected to surface immunity. The observations from this study support the hypothesis suggesting that specific antibodies in immune hosts in the epithelia bind to early trophonts and contribute to their premature escape although additional humoral and cellular factors may contribute to this reaction.

CHALLENGE MODELS FOR RTFS IN RAINBOW TROUT FRY (*ONCORHYNCHUS MYKISS*)

Maya M. M. Henriksen, Lone Madsen and Inger Dalsgaard

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The fish pathogen *Flavobacterium psychrophilum* is one of the main causes of mortality in fry of farmed rainbow trout (*Oncorhynchus mykiss*) and other salmonid fish. The disease following infection is often called bacterial coldwater disease (BCWD) in USA and rainbow trout fry syndrome (RTFS) in Europe. Presently no commercial vaccine exists, although several are under development.

Various models for experimental infection have been carried out with varying success, including challenge through injection, bath and cohabitation. Intraperitoneal challenge and bath challenges combined with various forms of stress have shown to be reproducible. Bath challenge is more appropriate for vaccine testing, since natural transmission of infection is imitated and is also more suitable due to the small size of the fry.

A bath-model using H_2O_2 as a stressor is currently being tested on 1.4g rainbow trout fry in four experimental groups: 1) no H_2O_2/no bath infection, 2) H_2O_2/no bath infection, 3) no H_2O_2/ bath infection and 4) H_2O_2/ bath infection. Mortality will be evaluated over approximately 25 days.

The project is currently in its preliminary phase and presently focused on development of a model of infection. The overall goal is also to examine gene expression and location of transcription products in rainbow trout fry, in order to optimize vaccination or immune-stimulation. The presentation will focus on previous experimental models and the experimental design of the current model as well as the future plans for the project.

VACCINATION AGAINST *Y. RUCKERI* SEROTYPE 1, BIOTYPE 1 AND 2

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During the FTP-project "Host-pathogen interactions during vaccination and infection of rainbow trout with fluorescent *Yersinia ruckeri"*, several experimental immersion vaccines against enteric redmouth disease (ERM) have been developed and tested by the use bath challenge. This is a summary of some of the results obtained during the project.

The immune mechanisms responsible for the immersion vaccine-induced protection against *Y. ruckeri* may comprise both cellular and humoral elements but the role of specific immunoglobulins in this system has been questioned and not previously described. One of the present studies demonstrates a significant increase in *Y. ruckeri* specific antibodies in plasma following immersion vaccination using a biotype 1 bacterin, and significantly reduced mortality during *Y. ruckeri* challenge. The vaccinated groups also showed a reduced bacteremia *in vivo*, and *in vitro* plasma studies showed a significantly increased bactericidal effect of fresh plasma from vaccinated fish. Passive transfer of plasma from rainbow trout surviving a *Y. ruckeri* infection to naïve rainbow trout significantly increased the survival during *Y. ruckeri* challenge which indicates a protective effect of *Y. ruckeri* specific antibodies.

A new biotype 2 of *Y. ruckeri,* which lacks motility has shown to be highly virulent for rainbow trout and is causing disease in cultured trout even in fish vaccinated with commercial ERM biotype 1 vaccines. Not much is known about immunity against biotype 2 and we have therefore produced a *Y. ruckeri* biotype 2 immersion vaccine, and tested the protection against both *Y. ruckeri* biotype 1 and 2 infections. Seven months post vaccination, both vaccinated and mock-vaccinated groups of rainbow trout were bath challenged with *Y. ruckeri* serotype O1, biotype 1 or 2. Challenge with biotype 2 resulted in very low mortalities with no significant difference in mortality between vaccinated and mock-vaccinated fish. Challenge with biotype 1 resulted in a significantly lower mortality ($P=0.0001$) in the vaccinated group. Three and seven days post challenge five fish from each group were sampled for RT-qPCR as well as immunohistochemical analysis in order to find increases in molecular markers which correlate with the increased protection in the vaccinated fish. In general the mock-vaccinated group had a higher bacterial load in all examined organs (head kidney, spleen, liver, brain, muscle, heart, intestine, skin and gill). Seven days post infection 40% of mock-vaccinated fish were still heavy infected, which corresponds well with overall mortality in this group (35%). In general pro-inflammatory cytokine expression was higher at both mRNA and protein level in the mock-vaccinated group and correlated to the higher load of *Y. ruckeri* in the tissues. Some CD markers such as CD4 and CD86 were significantly increased at the mRNA level in the vaccinated fish during challenge, probably reflecting fast induction of adaptive immunity. IgT transcripts were also significantly increased in some organs in the vaccinated trout.

The results indicate that the survival of the vaccinated fish after bacterial challenge seems to be correlated with an ability to clear bacterial infection over time. Additionally, the results indicate that immersion vaccines based on *Y. ruckeri* serotype O1, biotype 2 confers significant cross protection against biotype 1.

GENE REGULATORY MECHANISMS IN INFECTED FISH

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This talk will highlight the regulatory mechanisms of gene expression especially the programmed form of mRNA decay which is known as RNA interference (RNAi) and how this and other mechanisms contribute to the regulation of genes involved in immunity. In the RNAi mechanism small double stranded RNA molecules produced by the eukaryotic cell is used to program the RNA Induced Silencing Complex (RISC) for cleavage of specific mRNA transcripts and/or translational repression in the cytoplasm or even chromatin methylation in the nucleus. All processes leading to silencing of the target gene. MicroRNAs (or miRNAs) are one class of such small RNAs which are expressed from the genome. The RISC system allows for non-perfect base pairing of miRNAs to their target genes why one small RNA can in theory silence large groups of genes at the same time. It is therefore anticipated that they are able to depress whole pathways for the fine-tuning of physiological states like immunological reaction. But miRNAs are themselves under control of regulatory sequences for their timed expression. We will give an example of the finding of two rainbow trout microRNAs, which are up-regulated in the liver during infection with viral hemorrhagic septicemia virus (VHSV), and a genomic upstream sequence which we believe contains their promoter. Particular transcription factor binding motifs inside this potential promoter area point to its use in dsRNA induced antiviral defence. Other sites point to a role in leukocyte differentiation. Thus the expression of these miRNAs might be steered by different mechanisms in different cell types and have different roles in terms of the genes they target in different cell types. Thus gene regulation and function is better looked upon as a web of interactions. Data from zebrafish studies seem to show that these microRNAs are only expressed above a certain stage in the development of the fish.

FISH IMMUNE RESPONSES TO *AEROMONAS SALMONICIDA*

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For more than 100 years, furunculosis caused by the gram negative bacterium *Aeromonas salmonicida* subspecies *salmonicida* (A.s.s), has been recognized as a major threat to aquacultures. A broad host-range as well as a wide geographical distribution has caused large economical losses, especially within the salmonid fishes.

Development of efficient vaccines against A.s.s. infections has been an active area of research for decades, resulting in the introduction of commercially available, oil-adjuvanted vaccines for use in aquaculture. However, while providing more efficient protection, these vaccines have also been reported to cause unfortunate adverse effects including pigmentation and adhesion of internal organs as well as induction of autoimmunity in some cases. Therefore, further research into the effects of existing vaccines is needed to provide a base for the development of new efficient and safer vaccines.

The aim of the present project is to investigate the difference in immune response towards A.s.s. between naive and vaccinated rainbow trout (*Oncorhynchus mykiss*) during a water-borne infection with A.s.s. The immunological responses to infection can be described, by comparing naïve fish and fish vaccinated with either a commercially available vaccine or an experimental bacterin to adjuvant-only controls and non-infected controls. Twelve weeks post vaccination an A.s.s. challenge will be performed, mortalities in each group will be recorded and compared, and samples will be taken at 1, 3 and 7 days post challenge for examination of gene-expression using real-time quantitative PCR as well as humoral responses using ELISA and serum inactivation of bacteria. The interactions between host and bacterium will also be examined using immunohistochemistry. Histological sections will be prepared for assesment of pathological conditions caused by the infection.

The results from this project should provide a detailed insight into both the host-defence mechanisms in naïve trout as well as potential mechanisms of protection conferred by vaccination. This should provide knowledge of the host-pathogen interactions during infection with *A. salmonicida* that could also prove useful in development of future vaccines.

CHARACTERIZATION AND COMPARISON OF IMMUNOLOGICAL FUNCTIONS IN THE PRIMARY AND SECONDARY CIRCULATORY SYSTEM IN *ONCORHYNCHUS MYKISS*

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The secondary circulatory system (SCS) has mainly been described in ray-finned fishes (Actinopterygii) by Vogel (1985), Steffensen (1986) and Steffensen & Lomholt (1992). These authors suggested that the lymphatic system described so far lacked the characteristic anatomy normally seen in other vertebrates, especially mammals. Supported by dye-injected specimens, plastic casts and live images of the glass catfish (*Kryptoterus bicirrhis*), they showed that the SCS was in open communication with the systemic arteries via a large number of anastomoses of capillary dimensions. The SCS forms capillary beds situated in the outer surfaces of the fish such as the skin, gills, mouth and pharynx (Vogel (1985)). On the other hand, the SCS appears to be absent in regions of the mesenteric and renal tissues, where the lymphatic system is normally found in mammals. Together with the presence of red blood cells (albeit in low numbers), these findings led to a reassessment of whether the SCC has true lymphatic function or not (Vogel (1985), Steffensen & Lomholt (1992), Skov and Bennett (2003)).

An actual understanding of the SCS is still under debate, as the physiological function has not yet been completely elucidated. In November 2009, we initiated a project aiming to characterize and compare the immunological functions in the primary and secondary circulatory systems of the rainbow trout (*Oncorhynchus mykiss*). To this end, cell count was performed from fluid withdrawn from the primary blood circulation (PCS) and the SCS. Although the results are not conclusive, the measurements indicated that the frequency of leucocytes in the PCS (3.8*107 cells per ml) were 10 fold higher compared with the SCS (3.1*106 cells/ml). On the other hand, the share of leucocytes in the secondary fluid (66%) was substantially higher than in the PCS (4-5%).

Monoclonal antibodies against *Oncorhynchus mykiss* MHC class I, II, IgM, IgD, IgT, CD4 and CD8, were used to identify the distribution of the different cell types in the two circulatory systems. Immunocytochemistry of fixated cells from the PCS and the SCS indicated that the distribution of the cell types is decidedly different. It was especially the CD4+ and CD8+ T-cells, in combination with the CD86 positive APC, which seem to appear with a much higher frequency in the SCS.

COMPARISON OF FOUR FEED CODES: EFFECTS ON SUSCEPTIBILITY TOWARDS WHITE SPOT DISEASE, SERUM LYSOZYME AND MUCOUS CELL DENSITY IN RAINBOW TROUT

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White Spot Disease caused by *Ichthyophthirius multifiliis*, is one of the major diseases in freshwater fish and causes great economical losses in trout farming enterprises. Various feed additives have been developed for use as immunostimulants in trout feed and some have been suggested to modify susceptibility to White Spot Disease. Therefore, the present experiment was designed to evaluate the immuno-modulating effects of different types of feed containing various additives on rainbow trout susceptibility towards White Spot Disease. A total of 800 rainbow trout (10-12g) were kept in eight duplicate fish tanks in the Bornholm Salmon Hatchery. The trial was performed as a double-blind study with no involved scientists or assistants knowing the content of feed used for different groups. Only after ending the investigation the code was broken. The four feed types were named by code name Kolding (control, no additives), code name Billund (product Biofocus), code name Præstø (product Parmix) and code name Brande (product BioSyn), respectively. Upon feeding 30 days and 50 days, samples of the four feed groups were taken for testing the susceptibility to infection. In addition, samples for serum lysozyme activity, mucous cell density in fins and immune gene expression were taken. At day 30 and 50, five fish from each tank (10 from each feed code) were transferred from the hatchery to the laboratory and exposed to Ich for 48 h. One week later parasites (white spots) were counted on fish to evaluate susceptibility towards White Spot Disease of fish fed different feeds. Plasma lysozyme activities were found slightly increased in fish fed both Brande and Billund, and significantly increased in fish fed Brande and Billund at day 50, but slightly depressed in fish fed Præstø at day 30 and 50. All fish receiving feed with additives showed higher mucous cells density compared to control fish at day 30 and 50. A significantly higher mucous cell density was present in fish fed Præstø at day 30. All fish obtained infection with Ich but showed a lower infection at day 50 than at day 30 (due to a lower infection pressure imposed in the laboratory exposure facility). Fish fed Billund (Biofocus) significantly increased the disease resistance compared to control fish at both challenge experiments. Gene expression analyses are still in progress.

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