DAFINET WORKSHOP

THE ONTOGENY OF THE IMMUNE SYSTEM IN FISH

April 24th & 25th, 2012

Venue:

April 24th: Lecture Theatre 1-01
April 25th: Meeting Room Ø34
Bülowsvej 17
1870 Frederiksberg
Denmark

Organised by:

Danish Fish Immunology Research Centre and Network
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Book of abstracts

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Programme
Tuesday April 24th, 2012

13.00	Welcome address by DAFINET leader Kurt Buchmann
13.10	Professor Kurt Buchmann *Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark* Improved ERM vaccination efficacy using combined vaccine administration methods
13.30	Professor Barbara Nowak *National Centre for Marine Conservation and Resource Sustainability, AMC, University of Tasmania, Australia* Amoebic Gill Disease in the marine environment – an overview
14.20	Research director Scott LaPatra *Clear Springs Foods, Inc., Research Division, Buhl, Idaho, USA* Icthyophonus: a model pathogen for investigating infection, disease and immunity
15.00	**Coffee break**
15.30	Researcher Sonal Patel *Institute of Marine Research (IMR), Bergen, Norway* Ontogeny of adaptive immune cell markers in Atlantic halibut
16.00	Post doc Brian Dall Schyth *Department of Poultry, Fish, and Fur Animals,* *Technical University of Denmark, Aarhus, Denmark* Chemical modification of RNA-based medicine can be used to reduce its induction of the innate immune response
16.20	Post doc Hans-Christian Ingerslev *National Veterinary Institute,* *Technical University of Denmark, Frederiksberg, Denmark* Characterisation of the gut microflora in rainbow trout fry (*Oncorhynchus mykiss*) using deep-sequencing
16.40	M. Sc. student Simon Haarder *Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark* Establishment success and microhabitat preference of anisakids in salmonid fish
16.55	Wrap up by Kurt Buchmann
18.30	**Dinner at Falconer Center, Falkoner Alle 9, 1870 Frederiksberg**

Programme
Wednesday 25th, 2012

10.00 Professor Barbara Nowak
National Centre for Marine Conservation and Resource Sustainability, AMC, University of Tasmania, Australia
Chlamydia-related epitheliocystis in Australian fish

10.20 Professor Barbara Nowak
National Centre for Marine Conservation and Resource Sustainability, AMC, University of Tasmania, Australia
Host response post-vaccination and during disease challenge in yersiniosis

10.40 Ph.D. student Maya M. M. Henriksen
National Veterinary Institute,
Technical University of Denmark, Frederiksberg, Denmark
Immersion challenge with *Flavobacterium psychrophilum* in rainbow trout fry (*Oncorhynchus mykiss*)

11.00 Ph.D. student Rasmus D. Heinecke
Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
Expression and antibody staining of immune relevant molecules of early rainbow trout larvae infected with *Ichthyophthirius multifiliis*

11.20 Ph.D. student Kasper Rømer Villumsen
Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
Potential role of specific antibodies in vaccine-induced protection against *Aeromonas salmonicida* subsp. *salmonicida* in rainbow trout (*Oncorhynchus mykiss*)

11.40 Ph.D. student Jacob G. Schmidt
Biological Quality Group, National Food Institute,
Technical University of Denmark, Lyngby, Denmark
Ontogeny of the carp (*Cyprinus carpio* L.) innate immune system:
Gene expression and experimental limitations

12.00 Lunch at Gimle

Programme
Wednesday 25th, 2012

13.00 Senior researcher Jesper Skou Rasmussen
Department of Poultry, Fish, and Fur Animals,
Technical University of Denmark, Aarhus, Denmark
DNA vaccination of small rainbow trout fry against VHSV

13.20 Research assistant Diana Sindberg
Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology,
Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
Salmon lice (*Lepeophtheirus salmonis*; Krøyer, 1837) in Atlantic salmon (*Salmo salar*) from Denmark: Implications for wild and cultured stocks

14.10 Ph.D. student Dennis B. Bela-ong
Department of Poultry, Fish, and Fur Animals,
Technical University of Denmark, Aarhus, Denmark
Expression of micro-RNAs and immune-relevant genes in rainbow trout (O*ncorhynchus mykiss* Walbaum) upon vaccination with a *Viral Haemorrhagic Septicemia Virus*

14.30 Coffee break

15.00 Research assistant Jesper Kuhn
Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology,
Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
Parasites of wild cod postlarvae (*Gadus morhua* L.) in the North Sea

15.20 Research assistant Foojan Mehrdana
Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology,
Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
Association between nematods *Hysterothylacium aduncum* invasion of cod larvae and growth

15.40 M. Sc. student Lukas Neumann
Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology,
Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
Does Oral Vaccination Protect Rainbow Trout (*Oncorhynchus mykiss*) against Enteric Red Mouth Disease?

16.00 Final discussion and conclusions of the workshop

Abstracts

IMPROVED ERM VACCINATION EFFICACY USING COMBINED VACCINE ADMINISTRATION METHODS

Kurt Buchmann[1], Sidhartha Desmukh[1],
Jiwan Kumar Chettri[1], Rzgar M. Jafaar[1] and Inger Dalsgaard[2]

[1] *Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark*

[2] *National Veterinary Laboratory, Danish Technical University, Frederiksberg, Denmark*

We have previously shown that immersion vaccination (30 sec) using the Aquavac Relera vaccine (containing formalin killed *Yersinia ruckeri* serotype O1 of both biotypes 1 and 2) provides the best protection (when compared to other commercial ERM vaccines on the Danish market) against infection following i. p. challenge using *Y. ruckeri* O1, biotype 2, which at present is the main bacterial pathogen in fingerling trout production in Denmark. Despite a significant protection conferred by this vaccine (immersion) some mortality could be observed following challenge.

We have therefore performed a study in order to elucidate if different vaccine administration methods (using Aquavac Relera) can improve protection and reduce mortality of exposed trout following challenge with this particular pathogen. Rainbow trout (mean weight 7.8 g) reared at the Bornholm Salmon Hatchery under pathogen free conditions were vaccinated by 1) a single immersion (30 sec) and no booster, 2) a single immersion (30 sec) and a second immersion booster (30 sec) 410 degree-days later, 3) a single injection i.p. (0.1 ml) and no booster and finally 4) a single immersion (30 sec) and a booster injection i.p. (0.1 ml) 410 degree-days later. Fish were compared to naïve and non-vaccinated control fish. All experiments were performed in duplicate. Fish were brought to our university facilities, acclimated for 14 days and then challenged by i.p. injection of *Y. ruckeri* O1, biotype 2 (8.5×10^6 cfu/fish).

Control fish exhibited a mortality of 65 %, fish immersed only once showed a mortality of 36 %, fish immersed twice had 4 % mortality, fish injected once reached 2 % mortality. Rainbow trout immersed once and subsequently boosted by injection showed no mortality at all. This superior protection offered by a combination of primary immersion vaccination and a booster vaccination by injection may lead to improved health and reduced drug application in Danish trout farms.

Presenting author: Kurt Buchmann, kub@life.ku.dk

AMOEBIC GILL DISEASE IN THE MARINE ENVIRONMENT – AN OVERVIEW

Barbara Nowak

National Centre for Marine Conservation and Resource Sustainability,
AMC, University of Tasmania, Launceston, Tasmania, Australia

Amoebic gill disease (AGD) is a condition affecting some species of farm-reared marine fish caused by *Neoparamoeba perurans*. This disease has been reported from Australia, USA, Ireland, Spain, Scotland and New Zealand and in all these countries it has been associated with the presence of *Neoparamoeba perurans*. Amoebic gill disease (AGD) is the most serious health problem of farmed Atlantic salmon in Tasmania. However, the disease is also present in other hosts, one of the most recent records includes farmed ayu in Japan. Reservoir populations of the amoeba and the mechanism of transmission to farmed fish have not been elucidated.

Preliminary investigation showed negative results for sediments and biofouling organisms in AGD affected area. However *N. perurans* DNA was detected in alcoholic washings of salmon lice *Lepeophtheirus salmonis* collected from salmon from an affected farm in the USA. Furthermore cross-infection with another species of sea lice *Caligus rogercresseyi* was reported during an AGD outbreak in Chile. This suggests that epidemiology of this disease may depend on the geographical locations.

Amoebic Gill Disease is an interesting disease model to investigate host-parasite interactions. The effect of the disease on host at gene and protein level as well as AGD pathology will be discussed.

Presenting author: Barabara Novak, bnowak@amc.edu.au

ICTHYOPHONUS: A MODEL PATHOGEN FOR INVESTIGATING INFECTION, DISEASE AND IMMUNITY

Scott E. LaPatra

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Ichthyophonus hoferi, originally described in brown trout by Hofer (1893), is a systemic parasite that has been reported from more than 100 species of marine, brackish, and freshwater fishes. The taxonomic position of *I. hoferi* has been uncertain, however, recent molecular phylogenetic studies have grouped the organism in a novel clade of protists near the animal-fungal divergence. Because there are most likely several different species of this organism given the host range, we simply refer to it as *Ichthyophonus*. Due to the chronic nature of the disease, *Ichthyophonus*-infected hosts may survive for extended periods with little or no apparent deleterious effects.

Rainbow trout *(Oncorhynchus mykiss)* were infected or mock infected by ingestion of visceral tissue from naturally infected or uninfected adult trout, respectively. The groups were monitored by heart explant culture and when 100% infection prevalence was detected in the infected group studies were undertaken to investigate the impact this pathogen may have on a host's ability to resist disease and mount a protective immune response. Groups of fish from each treatment were challenged with *Flavobacterium pyschrophilum* or infectious hematopoietic necrosis virus (IHNV) and evaluated for their susceptibility and their capability to mount a humoral immune response.

Surviving fish in each challenge from each treatment showed no difference in their capacity to mount a specific antibody response to either pathogen. There was also no difference in susceptibility to *F. psychrophilum*, however, *Ichthyphonus* infected fish were reproducibly significantly less susceptible to IHNV. This model provides evidence that pathogen infection may be beneficial to the host in some cases.

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ONTOGENY OF ADAPTIVE IMMUNE CELL MARKERS IN ATLANTIC HALIBUT

Sonal Patel, Aina-Cathrine Øvergård and Audun Helge Nerland

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Marine larvae hatch in an environment where they can be exposed to a variety of pathogens, and often high mortality has been observed during the early stages when the immune system is not fully developed. This emphasizes the need to establish adequate preventive countermeasures such as vaccination and the use of probiotics and immunostimulants. It is important that vaccines activate the specific immune system, as it is this part of the immune system that is expected to develop memory cells. However, immunization before the fish is able to mount an effective immune response might induce tolerance. High mortality during the first feeding and the transition to dry feed stages has been observed during halibut farming, and thus we were interested in studying the development of lymphoid organs and the expression of B- and T-cell markers along with some aspects of innate immunity during developmental stages. This would enable us to estimate earliest possible time point to activate the specific immune system and vaccinate halibut fry.

Samples of fertilized eggs, larvae and juveniles up to 159 days post hatching (dph) were collected regularly. All three lymphoid organs, spleen, anterior kidney and thymus, appeared to be morphologically well developed at the end of metamorphosis. Molecular biological analysis (real-time RT-PCR and *in situ* hybridization) showed that IgM transcripts (B- cell marker) could be detected from 66 dph. Using immunohistochemistry, the presence of IgM protein was found in both kidney and spleen by 94 dph, while in thymus at 108 dph. The appearance of halibut T-cell markers during development was found to generally follow the same sequence as observed in mammals. *In situ* hybridization analysis detected genes that are essential for early T- cell development at 42 dph within the thymus anlage, while positive cells likely to be mature T- cells were seen at 87 dph. Interestingly, despite a general trend where all the investigated immune markers showed a clear increase during the early metamorphosis stage, they were down-modulated around the period of transition to dry feed.

We can thus conclude that the halibut specific immune system is probably not fully developed until the latter part of metamorphosis. Vaccination of Atlantic halibut larvae before 94 dph could possibly lead to tolerance instead of protection. An optimal timing for vaccination is thus likely to be later than 94 dph, since the strength of the immune response will be decisive for adequate protection.

Presenting author: Sonal Patel, Sonal.patel@imr.no

CHEMICAL MODIFICATION OF RNA-BASED MEDICINE CAN BE USED TO REDUCE ITS INDUCTION OF THE INNATE IMMUNE RESPONSE

Brian Dall Schyth[1], Jesper Bertram Bramsen[2],
Jørgen Kjems[2], Jesper Wengel[3] and Niels Lorenzen[1]

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[2] *Department of Molecular Biology, University of Aarhus, Denmark*

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Small interfering RNAs (siRNAs) are regarded as promising new active compounds in gene medicine. They are small 21-22bp long double stranded RNAs which act by targeting and inhibiting expression of specific mRNAs through base complementarity to one of their strands. But one serious problem with siRNA based treatment is the non-specific activities of double stranded RNAs when formulated in some effective delivery reagents. Cellular reactions upon double stranded RNAs include those of the 2´-5´ oligoadenylate synthetase system, the protein kinase R, RIG-I and Toll-like receptor activated pathways all resulting in innate antiviral defence mechanisms. Following injection of formulated siRNAs we have shown that we are able to detect the effect of such defence mechanisms as lowered mortality of rainbow trout infected with the fish pathogenic virus *Viral Haemorrhagic Septicaemia Virus* (VHSV).

We used the trout and VHSV to screen siRNAs containing various chemical modifications of the RNA backbone and found that was possible to modify the backbone so as to reduce the antiviral effect of the RNA. Antiviral protection was also dependent upon localisation of the modified nucleotide residues in the RNA strands and we found some evidence of an effect of both base composition and thermal stability of the double strands.

We conclude that our model is a potent tool for gaining insight into the triggering of antiviral cellular reactions towards RNAs in living fish. The overall perspective is to learn how to avoid non-specific antiviral responses of RNA-based gene medicine, but the knowledge gained also has a potential for use in the design of adjuvants (although adjuvance effect has not been tested for any of our siRNAs yet). The model can also be used for screening various commercial and non-commercial delivery reagents with the same perspective.

Presenting author: Brian Dall Schyth, bdsc@vet.dtu.dk

CHARACTERISATION OF THE GUT MICROFLORA IN RAINBOW TROUT FRY (*ONCORHYNCHUS MYKISS*) USING DEEP-SEQUENCING

Hans-Christian Ingerslev, Inger Dalsgaard, Mette Boye and Lone Madsen

National Veterinary Institute, Technical University of Denmark, Frederiksberg C, Denmark

For many years it has been known that the bacterial microflora in the gut of warm-blooded animals exists in harmony with the host and exert various beneficial effects on the health by their metabolic activities. Hence, the gut microbiota has a high importance for the animal. In many studies from *e.g.* humans and the pig mapping of the bacterial flora from the gut have shown dominance by some specific bacterial groups, and this bacterial profile is termed as a 'core microbiota'. For lower vertebrates like fish mapping of the bacterial flora in the gastrointestinal system is to date a relatively new research field and previous studies have mainly been done on bacterial species that can be cultured or by classical molecular techniques like T-RFLP and DGGE. In the last recent years deep-sequencing techniques have enabled sequencing and mapping of entire microbial communities from for instance an environmental sample or a tissue / fecal sample from an animal. These metagenomic studies have provided new and deeper insight into the microbial ecology and the influence of the microbiota in warm-blooded animals.

In this study next-generation sequencing of the 16S rDNA gene on the Illumina HiSeq platform was used to examine the composition of the microbial flora in the gut of rainbow trout (*Oncorhynchus mykiss*) fry. The fish were examined before and after first-feeding and after administration by commercial probiotic lactobacilli to the feed.

Presenting author: Hans-Christian Ingerslev, hain@vet.dtu.dk

ESTABLISHMENT SUCCESS AND MICROHABITAT PREFERENCE OF ANISAKIDS IN SALMONID FISH

Simon Haarder, Per W. Kania and Kurt Buchmann

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We have investigated the establishment potential of three well known closely related anisakids - *Hysterothylacium aduncum, Anisakis simplex* and *Contracaecum osculatum* – in three different salmonid fish. Brown trout (*Salmo trutta*) from river Esrum, rainbow trout (*Oncorhynchus mykiss*) from Fousing trout farm and Atlantic salmon (*Salmo salar*) from river Skjern were orally challenged with specimens of *H. aduncum*, *A. simplex* and *C. osculatum* and samples were taken 2, 7 and 14 days p.i., noting the intensity of infection and microhabitat of recovered larvae.

Brown trout exhibited the highest susceptibility towards *H. aduncum* after 2 days (59.2% recovery) whereas a low number of nematodes successfully established in salmon (17.9% recovered) and rainbow trout (14.3% recovered). Only a few larvae were recovered from brown trout 7 days p.i. and none were found in salmon or rainbow trout. The nematodes were observed to differ with regards to microhabitat preference. In brown trout viable *H. aduncum* were predominantly located in the pyloric intestine. The majority of live nematodes were, on the other hand, recorded from the post pyloric intestine in salmon and rainbow trout.

A large proportion (78.2%) of the administered *C. osculatum* larvae were recovered from rainbow trout 2 days p.i.; sampling of rainbow trout after 7 and 14 days revealed a lower recovery (28.8% and 2.5%, respectively). Brown trout and salmon showed a higher natural resistance towards *C. osculatum* when compared to data from the rainbow trout challenge experiment (35.0% and 23.1% recovery 2 days p.i., respectively). No larvae were found in brown trout and salmon after 7 and 14 days. The stomach lumen contained the majority of the experimentally inoculated larvae in rainbow trout, however, a few individuals had penetrated the pyloric caeca. *C. osculatum* were primarily found in the rectum of brown trout. In salmon the nematodes were found in all parts of the gastrointestinal system with no clear preferential site.

Finally, the recovery of *A. simplex* was slightly higher in rainbow trout than in salmon after 2 days (86.3% and 85.5%, respectively) and 7 days (65.0% and 55.4%, respectively), but not after 14 days (48.7% and 61.8, respectively). The total number of recovered *Anisakis* larvae were, however, higher in salmon (67.7%) when compared to rainbow trout (66.8%). Almost all of the nematodes from salmon were alive (92.2%), whereas this only applied to 73.1% of the experimentally introduced larvae from rainbow trout. Brown trout exhibited the strongest resistance towards the infectious *A. simplex* larvae; a third (33.2%) of the administered nematodes were recovered (57.1% were alive). Most of the nematodes were located between the pyloric caeca in rainbow trout and salmon. *Anisakis simplex* larvae in brown trout were predominantly observed in the process of penetrating the anterior intestine and embedded in the epithelia. Muscle-penetrating larvae were recorded from all three salmonids, most in salmon. Larvae were also found in the body cavity, swim-bladder and the liver.

In conclusion, the data obtained in this study shows that salmonid fish exhibit a variation in susceptibility towards experimentally introduced anisakid larvae. The implications of these findings will be discussed.

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CHLAMYDIA-RELATED EPITHELIOCYSTIS IN AUSTRALIAN FISH

Barbara Nowak

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Epitheliocystis is a bacterial intracellular infection of epithelial cells of gills and skin of fish. Many marine and freshwater species have been reported to be infected. The condition has been reported in both wild and farmed fish. Season, pollution (including eutrophication) and fish age have been reported to affect prevalence and intensity. There appears to be some difference in the infections of wild and farmed fish of the same species, for example farmed yellowtail kingfish, *Seriola lalandi* showed greater prevalence than kingfish and host response was present in the farmed fish but not in the wild individuals. While overall these infections are benign they can cause mortalities particularly in farmed fish and may require treatment with antibiotics. There was a significant increase in lysozyme activity and blood plasma osmolality in striped trumpeter (*Latris lineata)* affected by epitheliocystis.

Recent results show that epitheliocystis agents are host-specific and belong to order Chlamydiales in a lineage separate from bacteria belonging to the family Chlamydiaceae. There is a wide taxonomic variation between the pathogens causing epitheliocystis in different fish species. In some cases more than one agent has been reported from the same host species.

Presenting author: Barabara Novak, bnowak@amc.edu.au

DNA VACCINATION OF SMALL RAINBOW TROUT FRY AGAINST VHSV

Jesper Skou Rasmussen, Ellen Lorenzen,
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Technical University of Denmark, Aarhus, Denmark

Small rainbow trout fry were DNA vaccinated by intramuscular injection at 0.25g and other fish later at 0.5g. Vaccine groups included pcDNA3-vhsG, heterologous vaccine (pcDNA3-ihnG), empty vector (pcDNA3) and unhandled fish. Fry vaccinated at 0.25g were challenged with VHSV by immersion at 3wpv, 11wpv and 21wpv. The challenge at 3wpv was started 1wpv, however as no mortality was observed, the fish were re-challenged 3wpv using a modified setup. Fry vaccinated at 0.5g were challenged with VHSV by immersion at11wpv.

By early challenge (3wpv) of fish vaccinated at 0.25g both homologous and heterologous vaccines induced unspecific protection (10 % mortality for both). Challenge 11wpv showed waning unspecific protection (60 % mortality) but also a poor specific protection (30 % mortality). By challenge 21wpv, hardly no specific (75 % mortality) or unspecific (81 % mortality) protection was observed. In contrast, fish vaccinated at 0.5 g and challenged at 11wpv showed good specific protection.

The results indicate that DNA vaccination of very small fry (0.25g) can induce an early innate response. However a late adaptive immune response is apparently not established. Vaccination of fry at 0.5g induces an adaptive response like in larger fish.

The experiment was repeated with same vaccination groups. Rainbow trout fry were vaccinated at 0.25g followed by challenge with homologous or heterologous virus at 13 dpv, 11 wpv and 21 wpv. At 13 dpv unspecific protection was induced with both homologous and heterologous challenge (5% mortality). At 11 wpv an unspecific protection with 30 % mortality was observed. At 21 wpv protection against VHSV had dropped further (50 % mortality). Protection against IHNV was better (10 % mortality) but equal for both homologous and heterologous vaccines confirming previous results, that vaccination of fry at 0.25g induces unspecific protection but no adaptive response.

Presenting author: Jesper Skou Rasmussen, jsra@vet.dtu.dk

IMMERSION CHALLENGE WITH *FLAVOBACTERIUM PSYCHROPHILUM* IN RAINBOW TROUT FRY (*ONCORHYNCHUS MYKISS*)

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

Technical University of Denmark, National Veterinary Institute, Frederiksberg, Denmark

The fish pathogen *Flavobacterium psychrophilum* is one of the main causes of mortality in farmed rainbow trout (*Oncorhynchus mykiss*) and other salmonid fish. 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. Various chemicals are used against pathogens in aquacultures, including copper sulphate, chloramine-T, sodium carbonates, sodium chloride, formalin and hydrogen peroxide (H_2O_2). One of the more successful immersion models made use of formalin as a stressor, but a less harmful chemical was desirable, since the use of formalin is to be phased out in the Danish farms by 2014 due to health considerations.

The aim of this study was to establish a reproducible method for immersion challenge of rainbow trout fry to be used in investigations concerning the immune response and vaccine testing. Various concentrations of H_2O_2 were tested, before being combined with immersion exposure to the well-known, virulent strain 950106-1/1, which was used for all infections. The mortality of *F. psychrophilum* infection in fry was increased by pre-treating with H_2O_2, although variation was very high.

Presenting author: Maya M. M. Henriksen, mmah@vet.dtu.dk

EXPRESSION AND ANTIBODY STAINING OF IMMUNE RELEVANT MOLECULES OF EARLY RAINBOW TROUT LARVAE INFECTED WITH *ICHTHYOPHTHIRIUS MULTIFILIIS*

Rasmus D. Heinecke and Kurt Buchmann

Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

The protozoan skin parasite *Ichthyophthirius multifiliis* is a serious worldwide problem in freshwater aquaculture causing severe morbidity and mortality. Fish surviving an infection with *I. multifiliis* are able to obtain immunity against the parasite and thereby protection against future infections. The age of the trout larva at which full maturity of the immune response can be achieved should be determined in order to design suitable vaccination strategies. The aim of the present study was to investigate the capacity of rainbow trout larvae 10 days post hatch (84 degree days) to mount an immune response against *I. multifiliis*.

Further, the expression of a range of cytokines and acute phase proteins in the response has been described. The localization of functional immune molecules in the trout tissue compartments was examined by immunohistochemistry using specially designed specific monoclonal antibodies. This study confirmed that 10 days post hatch larvae (84 degree days) are able to regulate the expression of different cytokines and APPs in response to infection with *I. multifiliis*. The immune response is an exceedingly intricate network of cellular signalling and biochemical processes. Further studies should be conducted to reveal if the initial response detected in the larvae can develop into a mature response leading to immunity.

Presenting author: Rasmus D. Heinecke, rdh@life.ku.dk

POTENTIAL ROLE OF SPECIFIC ANTIBODIES IN VACCINE-INDUCED PROTECTION AGAINST *AEROMONAS SALMONICIDA* SUBSP. *SALMONICIDA* IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Kasper Rømer Villumsen[1], Inger Dalsgaard[2] and Martin Kristian Raida[1]

[1] *Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark*

[2] *National Veterinary Institute, Technical University of Denmark*

Furunculosis caused by infection with *Aeromonas salmonicida* subsp. *salmonicida* has now been a known threat to aquaculture for more than a century. Efficient prophylactic precautions against this disease are essential for continued growth of salmonid fish aquaculture. Ever since the introduction of successful oil-adjuvanted vaccines in the 1990's, a number of studies have been published on the protective effects of these vaccines. While most of these studies mainly focus on vaccination of salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) are also highly susceptible to infection and are therefore vaccinated as well.

In this study we have examined the levels of protection against infection with a Danish strain of *A. salmonicida* subsp. *salmonicida* in both non-vaccinated, as well as vaccinated rainbow trout. Both a commercial vaccine (AlphaJect 3000, PHARMAQ AS) as well as an experimental auto-vaccine was tested. For comparison, the isolated adjuvant used in the commercial vaccine, as well as the one used in the experimental vaccine was included in the experimental setup. The protective effects of the vaccines were tested by bacterial challenge 18 weeks post vaccination, and during the 18 weeks, the development of specific antibodies was monitored using ELISA assays.

Both vaccines resulted in significantly increased survival during a 28 day challenge period. None of the two adjuvant systems provided increased protection. A significant increase in specific antibody levels was seen in both vaccinated groups during the 18 weeks between vaccination and challenge. Additionally, further analysis showed a significant correlation between the mean level of specific antibodies measured for each group and the final survival percentages of these groups.

The positive correlation between specific antibodies and survival seems to indicate a prominent role of antibodies as a vaccine mediated protective mechanism.

Presenting author: Kasper Rømer Villumsen, krv@life.ku.dk

ONTOGENY OF THE CARP (*CYPRINUS CARPIO* L.) INNATE IMMUNE SYSTEM: GENE EXPRESSION AND EXPERIMENTAL LIMITATIONS

Jacob G. Schmidt, Dominika A. Pryzybylska and Michael E. Nielsen

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Technical University of Denmark, Lyngby, Denmark

The objective of this study was to investigate the ontogeny of the immune system in common carp (*Cyprinus carpio*, L.). The work has been focused on innate immune responses during the wound healing processes and how the innate immune response develops with age and size of the fish.

Newly hatched carp were brought to the facilities at DTU and kept in aquaria at 25°C. They were initially fed *Artemia nauplii*, and later switched to commercial dry granulate feed. Carp were anaesthetised and then experimentally wounded at days 10, 16, 24, 47 and 94 days post-hatch. Sampling was carried out at day 1, 3 and 7 post-wounding and samples were stored in RNA later for isolation of RNA. The physical tissue damage was performed using a sterile needle, which penetrated the skin and the underlying musculature in an area above the lateral line of the left side of fish. Carps at the age of 10, 16 and 24 days post-hatch were stored and processed whole, whereas just the muscle (the left (wound area) and right filet (internal control)) was sampled for the two latter time-points. mRNA was extracted from the samples, cDNA was synthesised and gene expression was quantified using real-time RT-PCR. The investigated genes were IL-1β, IL-6, TNF-α, SAA, Hsp70, TGF-β and the mucins Muc2c and Muc5bc.

It can be generally concluded that the response of the investigated genes appeared to be faster and more pronounced at earlier life stages. However, care should be taken when comparing the results for the fish wounded on days 10, 16 and 24 post-hatching with the fish wounded on days 47 and 94 post-hatching since the way of sampling differed. However, it is inherently difficult to standardise experimental procedures for fish that increase 1000-fold during the course of the experiment. The results and experimental pitfalls will be further discussed.

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HOST RESPONSE POST-VACCINATION AND DURING DISEASE CHALLENGE IN YERSINIOSIS

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This study aimed to improve vaccine performance through understanding molecular host-pathogen interactions in yersiniosis. We investigated effects of different methods of vaccine application. Survival after double dip, single dip, bath and ip vaccination was significantly better than control unvaccinated fish in a challenge 3 weeks post-vaccination. These results confirm that dip is better than bath although all vaccinated treatments have RPS values of >60%. 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. We used cDNA microarray to characterise the differential response of host genes in the gills of naive unvaccinated and vaccinated Atlantic salmon challenged with *Y. ruckeri*. Differentially expressed genes were identified using two-way ANOVA and restricted to those with >2.5-fold change at $P<0.05$. We identified 7 genes in response to infection and 4 genes specifically associated with the protective host response to yersiniosis. Furthermore, we investigated the role of antibody in protection against yersiniosis.

The conditions required for an effective yersiniosis vaccine as well as the future direction needed to investigate yersiniosis pathogenesis, host immunity and the potential identification of genetic markers to predict vaccine efficacy will be discussed.

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SALMON LICE (*LEPEOPHTHEIRUS SALMONIS*; KRØYER, 1837) IN ATLANTIC SALMON (*SALMO SALAR*) FROM DENMARK: IMPLICATIONS FOR WILD AND CULTURED STOCKS

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Sea lice (family Caligidae) infections are considered to represent one of the most serious problems in mariculture worldwide, especially in countries with intensive salmonid production, such as Norway, Scotland, Ireland, Chile and Canada. In the Northern hemisphere, *Lepeophtheirus salmonis* and *Caligus elongatus* are the most prevalent and problematic species, infecting salmonids of the genera *Salmo, Salvelinus,* and *Oncorhynchus*. Sea lice are ectoparasitic crustaceans adapted to high salinity waters and they feed on fish mucus, skin and blood, which affect the growth, fecundity and survival of the fish. Moreover, sea lice might be carrier of other pathogens potentially affecting fish host health. Management and control of the sea lice, including medical, chemical and biological approaches, have received much attention. Moreover, the impact of sea lice infestations on wild salmonid stocks is highly debated but remains to be elucidated in further depth.

Both *L. salmonis* and *C. elongatus* have previously been recorded in Denmark (maricultured rainbow trout and in wild sea trout). With the extensive governmental management plans for wild Atlantic salmon in the western part of Denmark (e.g. River Skjern and River Storå) including restocking with juvenile fish more focus should be placed on potential health threats including infestations with sea lice of smolts. Further, in association with the continuously growing salmonid production and current plans for expansion in high salinity waters, e.g. off-shore production in the North Sea, problems with sea lice are to be expected. We have performed a preliminary study on wild Atlantic salmon from the River Skjern. We examined 16 specimens of spawning Atlantic salmon (*Salmo salar*), and found all infected with between 1-10 specimens of adult *L. salmonis*. Fertilised female lice were dominating. In addition, pathogens (including fungi and monogenean parasites) were found associated with the lice and microsporidean hyperparasites are to be diagnosed. These findings suggest, that sea lice might be more prevalent in Danish waters than first assumed, and further studies framing potential lice threats to wild and cultured salmonid stocks in Denmark should be implemented.

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EXPRESSION OF MICRORNAS AND IMMUNE-RELEVANT GENES IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM) UPON VACCINATION WITH A *VIRAL HEMORRHAGIC SEPTICEMIA VIRUS*

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Development of strategies to alleviate potential disease outbreaks in sea-farmed rainbow trout (*Oncorhynchus mykiss* Walbaum) due to wildlife marine reservoir of *Viral hemorrhagic septicemia virus* (VHSV) remains imperative. A DNA vaccine expressing VHSV glycoprotein (*G*) gene has been developed and shown to protect fish in VHSV challenge experiments. Identifying key factors as biomarkers during infection and vaccination will allow understanding of the complex web of interactions involved in the underlying host immune response. These molecular signatures of immune responses may also provide suitable selection criteria for identifying disease-resistant fish under production conditions during resistance breeding as fish do not show visible signs of resistance. Micro ribonucleic acids (miRNAs) are a diverse class of small (18-22 nucleotides) endogenous RNAs that potently mediate post-transcriptional silencing of a wide range of genes and are emerging as critical regulators of cellular processes, including immune responses. In a previous microarray experiment, we observed upregulation of miR-155, miR-462, and miR-731 in fish liver following VHSV infection. Therefore, we analysed the expression of these miRNAs and those of immune-related genes in rainbow trout in response to DNA vaccination.

Quantitative RT-PCR analysis revealed the increased levels of miR-155, miR-462, and miR-731 in the skeletal muscle tissue at the site of injection and in the liver of vaccinated fish relative to saline- and empty plasmid-injected controls. The increased expression of these miRNAs in the skeletal muscle correlated with the increased levels of the type I interferon (IFN)-inducible *Mx* gene, the vaccine gene (*G*), type I IFN and IFN-γ genes, and immune cell marker genes (CD4, CD8, sec-IgM, TCR, MHCI, and MHCII) at the vaccination site. The increased expression of immune cell markers indicates infiltration of the vaccination site with activated immune cells. Since the expression of these miRNAs correlated with increased levels of the type 1 IFN gene, IFN-γ gene, and the type I IFN-inducible *Mx* gene, we then determined whether this induction depends on interferons. Injecting fish with IFN 1-13 (a type I IFN) and IFN-γ constructs resulted in the increased expression of miR-155, miR-462, and miR-731 in the skeletal muscle tissue relative to controls. The same response was obtained from injection with the general IFN stimulator and Toll-like receptor (TLR) 3 agonist, polyinosinic: polycytidylic acid (poly I:C). These suggest that the induction of these miRNAs is elicited by interferons, which are major mediators of immune responses.

These regulated microRNAs could potentially be used as biomarkers of immune responses and as suitable selection markers to identify VHSV-resistant fish. Future work will involve identifying the specific cells that express these microRNAs, as well the genes that they regulate.

Keywords: microRNA, *Viral hemorrhagic septicemia virus* (VHSV), DNA vaccination, interferons, rainbow trout

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PARASITES OF WILD COD POSTLARVAE (*GADUS MORHUA* L.) IN THE NORTH SEA

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The Atlantic cod, *Gadus morhua* L. is a common and commercially important fish species in the North Sea, but estimated stock size and recruitment has been on an overall declining path since 1980. Atlantic cod is in this sense believed in the future to become one of the most intensively cultivated gadoid species in the North Atlantic and has been the subject of several parasitological studies. Past research is however primarily concentrated around adult or juvenile fish and our knowledge concerning the larval stage is very sparse. This is in spite of the general belief that at this stage, fish larvae encounter highest levels of mortally and predation. In this sense effects of parasitization could be speculated to have a higher impact.

In this study we investigated the occurrence of metazoan ecto- and endoparasites in a school of 209 cod post larvae (9mm – 39mm) caught in the North Sea of Denmark. Skin, fins, body cavity, gut and intestinal tract were checked and parasites were identified either through morphology or with the use of PCR.

A total of 58 parasites were found counting 38 individuals of the ectoparasitic copepod *Caligus elongatus*, 7 individuals of the endoparasitic nematode *Hysterothylacium aduncum*, 5 trematodes (1 *Lecithaster levinseni*, 4 *Hemiurus*) and 5 individuals of a tetraphyllidean plerocercoid cestode larva. Intensity, abundance and prevalence will be presented as well as area of infection. Brief introduction to the parasites and known literature on the parasitic effect will be given as well as a discussion on the possibilities of increased mortality due to infection.

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ASSOCIATION BETWEEN NEMATODE *HYSTEROTHYLACIUM ADUNCUM* INVASION OF COD LARVAE AND GROWTH

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Parasitic nematodes of the superfamily *Ascaridoidea* are distributed worldwide also with numerous representatives in fish. They have an important role to play in the aquatic environment and may affect survivability of fish. The life cycle of many of these fish infecting roundworm species includes invertebrates and fish species and for some species also higher vertebrate hosts. We have recently demonstrated that fry of North Sea cod has a high prevalence of infection with regard to the nematode *Hysterothylacium aduncum* and it was indicated that these infections could affect survival of cod and thereby affect the cod stock in the North Sea. The objective of the present study was to elucidate if infections are associated with a decrease or an increase of fish size when examining fish of the same age.

We investigated effects of *H. aduncum* infections on the growth rate of cod larvae by using the otolith reading method. In our study, the prevalence of infection with *H. aduncum* in North Sea cod *Gadus morhua* larvae was studied during the years 1992-2001. A subsample of 65 cod was selected based on the body length (range 20 to 45 mm) with 32 infected and 33 uninfected fishes. For ageing the cod larvae, lapillus otoliths were removed, polished and the number of growth zones in each otolith counted by light microscopy. Each growth zone indicates one day of the fish life span. Covariance analysis demonstrated highly significant differences ($p \leq 0.001$) between the growth rate of infected and uninfected cod larvae; the infected larvae showed a higher growth rate compared to uninfected ones of similar ages. The higher growth rate in infected fish could be a result of higher ingestion of copepods which are serving as intermediate hosts of these parasites. The increased growth could reflect that fish larvae with a higher ingestion rate of copepods will have a higher probability of infection. Further studies should elucidate if the higher infection (due to an increased feeding rate) may lead to a decreased survivability at a later stage of life.

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DOES ORAL VACCINATION PROTECT RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AGAINST ENTERIC RED MOUTH DISEASE?

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The effect of oral vaccines against bacterial fish diseases has been a topic for debate in many years. Recently both M-cells and dendritic cells have been found in fish and it is therefore likely that antigens can be taken up from the intestine and induce immunity in orally vaccinated fish.

The objective for this project is to investigate whether oral vaccination of rainbow trout against *Yersinia ruckeri* O1 (biotype 1) causing Enteric Red Mouth disease (ERM) can protect rainbow trout against a subsequent experimental bath challenge with *Y. ruckeri*.

The rainbow trout were given oral vaccinations with AquaVac^{TM} ERM Oral vet. (MSD animal health) or an experimental vaccine based on killed *Yersinia ruckeri* O1, (biotype 1) bacteria. Seven groups were studied: 1) Control group (no vaccination, no infection), 2) infected control, 3) experimental vaccine, 4) experimental vaccine with booster (4 months post primary vaccination), 5) AquaVac ERM (as a primary vaccine), 6) AquaVac with booster, and 7) one group with 10 fold increase (with booster) of the experimental vaccine in the feed.

The rainbow trout were bath challenged with 6.3 x10^8 CFU/ml *Y. ruckeri* 6 month post the primary oral vaccination. The challenge induced significant mortality in all infected groups except for the group which received the experimental vaccine in a ten times higher concentration. These results show that rainbow trout can induce specific immunity against *Y. ruckeri* after oral vaccination, and that high concentrations of dead *Y. ruckeri* bacteria are need in order to obtain significantly increased immunity against the disease. These results suggest that a high amount of the vaccine is digested in the stomach of the rainbow trout and therefore did not reach the intestine as immunogenic antigens.

The project is still ongoing, and samples have been taken for several immunological assays.

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