# 2nd FRDC Australasian Aquatic Animal Health Scientific Conference

aquatic animal health subprogram

## Pullman Reef Hotel
## Wharf Street, Cairns, Qld
## Australia

## 8-12 July 2013

Australian Government
Department of Agriculture, Fisheries and Forestry

CSIRO

FRDC
FISHERIES RESEARCH & DEVELOPMENT CORPORATION

# Second FRDC Australasian Scientific Conference on Aquatic Animal Health

**The Pullman Reef Hotel**
**Cairns**

**8-12 July 2013**

## Conference Programme

# Second FRDC Australasian Scientific Conference on Aquatic Animal Health

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## CONFERENCE PROGRAMME

### DAY 1 Monday 8 July

**18.00-20.00 Registration and Welcome Happy Hour**

### DAY 2 Tuesday 9 July

8.00 Registration

8.25 Welcome and Introduction. Mark Crane, FRDC AAH Subprogram Leader

**8.35 Keynote Presentation I. Emerging Problems in Fish Pathology. Ferguson H.**

**9.35 Session 1: Finfish Disease and Pathology I (Chairs: Ken McColl and Tracey Bradley)**

9.40 Gladstone: Clearing the mud from the waters - why the animals died. Landos MA, Dennis M.

10.00 Effects of chronic heavy metals exposure on thyroid hormone pathway related genes of sand flathead (*Platycephalus bassensis*) from the Derwent River, a historically polluted estuary. Dingkun F, Bridle AR, Nowak BF, Leef M.

10.20 Dinoflagellate dilemma. Bannister J.

**10.40 Coffee Break**

**11.05 Session 2: Finfish viruses (Chairs: Agus Sunarto and Nick Moody)**

11.10 Koi herpes virus: Dreaded pathogen or white knight? McColl KA, Sunarto A, Clarke B, Prabandono A, Slater J, Hoad J, Williams LM, Ward A, Doran TJ, Crane MStJ.

11.30 Genotype analysis of koi herpesviruses detected in quarantine in Singapore from 2005 to 2011. Jing C, Chee D, Yahui W, Yee LG, Min CS, Taoqi H, Nuo LY.

11.50 Preliminary characterization of Tasmanian *Aquareovirus* (TSRV) isolates. Zainathan S, Carson J, Crane MStJ, Moody NJG, Williams LM, Hoad J, Gudkovs N, Hyatt AD, Crameri S, Nowak BF.

12.10 Experimental transmission of megalocytivirus between freshwater and marine fish populations through the use of a model euryhaline species Australian bass, *Macquaria novemaculeata*. Go J, Whittington RJ.

12.30 Sero-prevalence of Nervous Necrosis Virus across barramundi and Australian bass adult populations. Jaramillo D, Hick P, Dyrting K, Anderson IG, Whittington RJ.

**12.50 Lunch Break**

**13.35 Session 3: Pacific oyster mortality syndrome I (Chairs: Peter Kirkland and Ika Paul-Pont)**

13.40 Pacific oyster mortality syndrome in NSW (2010-2013). Doolan DA, O'Connor WA, Dove MC, Walker ML, Frances J, Lyall I, Kirkland PD, Hick P, Gabor M, Spiers ZB, Jenkins C.

14.00 OsHV-1 in Pacific oysters in NSW – molecular detection and partial characterisation. Moody NJG, Mohr P, Boyle D, Hick P, Kirkland PD, Crane MStJ.

14.20 Ostreid herpesvirus-1 (OsHV-1) in commercially farmed Pacific oysters (*Crassostrea gigas*) in the Hawkesbury River in 2013: outbreak investigation. Paul-Pont I, Evans O, Dhand N, Rubio A, Coad P, Whittington RJ.

14.40 Investigating the transmission factors of Ostreid herpesvirus-1 (OsHV-1) within the environment: Wild mollusc species as a possible source of infection? Evans O, Paul-Pont I, Whittington RJ.

15.00 Effects of husbandry practices to reduce OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas*, and first steps towards integrated management within an infected estuary. Paul-Pont I, Evans O, Dhand N, Rubio A, Whittington RJ.

**15.20 Tea Break**

**15.45 Session 4: Pacific oyster mortality syndrome II (Chairs: Richard Whittington and Serge Corbeil)**

15.50 Experimental infection of Pacific oyster *Crassostrea gigas* using the Australian OsHV-1 strain: mortality, dose-response relationship and inoculum storage conditions. Paul-Pont I, Evans O, Whittington RJ.

16.10 Design of a detection survey for Ostreid Herpesvirus using hydrodynamic dispersion models to determine epidemiological units. Pande A, Brangenberg N, Acosta H, Keeling S.

16.30 Poly I:C induces a protective antiviral immune response in the Pacific oyster (*Crassostrea gigas*) against subsequent challenge with Ostreid herpesvirus (OsHV-1 µvar). Green TJ, Montagnani C.

16.50 Susceptibility of the black-lip pearl oyster, *Pinctada margaritifera*, to Ostreid herpesvirus (OsHV-1). Dang C, Tan T, Paul-Pont I, Evans O, Barnes AC, Whittington RJ, Fougerouse A, Bichet H.

**17.10 Close Day 2**

**18.00-19.00 Happy Hour**

## DAY 3 Wednesday 10 July

8:30 Introduction. Mark Crane

**8.35 Session 5: Crustacean Health (Chairs: Jeff Cowley and Mark Crane)**

8.40 Gill-associated virus epizootics in Queensland prawn farms in 2012. Anderson IG, Bowater RO, Condon K.

9.00 Detection of a new genotype of the yellow head complex of viruses in *Penaeus monodon*. Mohr PG, Moody NJG, Hoad J, Williams LM, Anderson IG, Crane MStJ.

9.20 Detection of a new rickettsia-like organism in wild-caught sand crabs (*Portunus pelagicus*) from Darwin and Bynoe Harbours. Diggles BK, MacBeth WG, Barnes L, Dyrting K.

9.40 Teaching driving research: A case study using a novel microsporidium found in western king prawns off the Townsville coast. Elliman J, Owens L.

10.00 Endogenous virus-like elements in redclaw crayfish *Cherax quadricarinatus*. Rusaini, La Fauce KA, Elliman J, Bowater RO, Owens L.

**10.20 Coffee Break**

**10.45 Session 6: Finfish Vaccines (Chairs: Richard Morrison and Matt Cook)**

10.50 From research to development: Pioneering the use of aquaculture vaccines in Australia. Carson J, Morrison R, Cornish M.

11.10 Evaluation of an experimental and commercial state-of-the-art vaccine against enteric redmouth disease (ERM) in rainbow trout by waterborne challenge with *Y. ruckeri* O1 biotype 2. Strøm HK, Aalbæk B, Otani M, Villumsen KR, Raida MK.

11.30 Effect of inactivation method of *Yersinia ruckeri* on the efficacy of single dip vaccines. Nguyen DT, Bridle AR, Nowak BF.

11.50 Oral and anal vaccination against enteric red mouth disease protection against yersiniosis. Neumann L, Villumsen K, Strøm H, Raida M.

12.10 Use of a recombinant protein from the amoebae *Neoparamoeba perurans* as a vaccine candidate against AGD in Atlantic salmon. Valdenegro-Vega VA, Crosbie PBB, Cook M, Nowak BF.

**12.30 Lunch Break**

**13.15 Session 7: Amoebic Gill Disease (Chairs: Barbara Nowak and Phil Crosbie)**

13.20 The comparative susceptibility of four Tasmanian endemic fishes and Atlantic salmon to experimentally induced amoebic gill disease (AGD). Adams MB, Bridle AR, Nowak BF.

13.40 A cross-species approach to functional feed development for Amoebic Gill Disease. Maynard B, Appleyard S, Colgrave M, Taylor R, Cook M, Brock M, Glencross B, Brown M.

14.00 Immune gene expression in Atlantic salmon (*Salmo salar* L.) affected by AGD. Pennacchi Y, Bridle AR, Leef MJ, Nowak BF.

14.20 *In vitro* assessment of gill function in AGD affected Atlantic salmon *Salmo salar* gills. Leef M, Nowak BF.

14.40 Culture and trialling of cryopreservation techniques for *Neoparamoeba perurans*, the causative agent of amoebic gill disease in marine-farmed Atlantic salmon, *Salmo salar*. Crosbie PBB, Bridle AR, Nowak BF.

15.00 Towards the application of RNA interference (RNAi) against Amoebic Gill Disease (AGD) of farmed Atlantic salmon (*Salmon salar*). Lima PC, Botwright N, Harris JO, Cook M.

**15.20 Tea Break**

**15.45 Session 8: Aquatic Animal Health Education and Training (Chairs: Ingo Ernst and Mark Crane)**

15.50 DAFF-FRDC Aquatic Animal Health Training Scheme. Humphrey K, Ruscoe J.

16.10 The Neptune Project - a comprehensive database of Australian aquatic animal pathogens and diseases. McNamara M, Ernst I, Adlard R.

16.30 Aquatic Animal Health Technical Forum (AAHTF) - Current Status. Williams LM, Jones BK, Cornish MC.

16.50 Towards the development of a national aquatic animal health curriculum for tertiary institutions. Whittington RJ, Pyecroft S.

**17.20 Close Day 3**

**18.00- Happy Hour**
**19.00**

## DAY 4 Thursday 11 July

9:00 Introduction. Mark Crane.

**9.05 Keynote Presentation II. Gill diseases of fish. H. Ferguson.**

**10.05 Session 9: Finfish Disease and Pathology II (Chairs: Stephen Pyecroft and Rachel Bowater)**

10.10 Gill lesions in Murray cod (*Maccullochella peelii*) raised in farm dams. McCowan C, Cohen S, Bradley T, Ingram B, Mansell P.

**10.30 Coffee Break**

**10.55 Session 10: Finfish Disease and Pathology III (Chairs: Stephen Pyecroft and Rachel Bowater)**

11.00 South Australian marine mortalities, summer 2013: An overview. Roberts SD, Bastianello SB, Pyecroft S, Wilkinson C, Van Ruth P, Neverauskas V.

11.20 Common problems with fish blood samples, haematology, biochemistry and feed analysis. Stephens F.

11.40 Yellowtail kingfish (*Seriolae lalandi*) taurine deficiency - A diagnostic case study. Huynh C, Landos M.

12.00 An unusual keratitis and uveitis in farmed barramundi; history, histopathology and cause. Anderson IG.

**12.20 Lunch Break**

**13.05 Session 11: Diagnostic test development and validation (Chairs: John Hoad and Charles Caraguel)**

13.10 Design and reporting of validation studies for diagnostic assays used for detection of aquatic animal pathogens: Are improvements necessary? Gardner IA, Burnley T, Caraguel CGB.

13.30 Development and validation of a specific immunohistochemistry method for the detection and localization of *Streptococcus agalactiae* infections in fish tissues. Delamare-Debouteville J, Bowater R, Stanford J, Barnes AC.

13.50 SYBR, TaqMan, or both: Highly sensitive, non-invasive detection of *Cardicola* blood fluke species in Southern Bluefin Tuna (*Thunnus maccoyii*). Bridle AR, Polinski M, Belworthy Hamilton D, Nowak BF.

14.10 Use of real-time PCR assay for detection of *Yersinia ruckeri* and asymptomatic carriers in Atlantic salmon. Pradenas J, Bridle AR, Nowak BF.

14.30 Use of next-generation sequencing during the biosecurity response to the detection of *Flavobacterium psychrophilum* in New Zealand. Draper J, Brosnahan C, Haydon T, Spence R, Williams RE, Jones B, McDonald, WL.

14.50 MALDI-TOF for bacterial identification and application to strain typing of *Vibrio harveyi*. Buller NB, Tong J, Hair S.

**15.10 Tea Break**

**15.35 Session 12: Aquatic Animal Health Regulation (Chair: Tim Lucas and Jeffrey Go)**

15.40 A risk analysis of Australia's marine ornamental value chain focusing on biosecurity (diseases and pathogens) concerns. Erickson KP, Campbell ML, Hewitt CL, Flint N.

16.00 Risk analysis of the aquarium trade as a pathway for release of dwarf gourami iridovirus (DGIV) in Australia and the risks of exposure of wild native fish. Rimmer AE, Becker JA, Toribio JA, Whittington RJ.

16.20 Incursions of cyprinid herpes virus 2 in goldfish populations in Australia despite quarantine practices. Becker JA, Tweedie A, Rimmer A, Landos M, Lintermans M, Whittington RJ.

16:40 Welfare and Aquaculture: Where we are? Where we go? Caraguel CGB.

**17.00 Close Day 4**

**18.00- Happy Hour**
**19.00**

## DAY 5 Friday 12 July

8.30 Introduction. Crane MStJ.

**8.35 Session 13: Finfish Parasites I (Chairs: Ingo Ernst and Shane Roberts)**

8.40 Parasites of introduced freshwater fish species in Murrumbidgee Basin and their impact on aquaculture development in the region. Turner A, van Keulen M, Eulink G, Wassens S, Shamsi S.

9.00 Integrated parasite and disease management strategies for finfish aquaculture in tropical north Queensland: a JCU and QLD Department of Agriculture, Fisheries and Forestry initiative. Miller TL, Knuckey R, Reynolds A, Hutson KS.

9.20 Praziquantel as a control for fluke infections in fish. Deveney MR.

9.40 Review of the fish-parasitic cymothoid crustacean 'tongue biter' genus *Ceratothoa* (Fabricius, 1775) in Australian waters. Martin MB, Bruce NL, Nowak BF.

10.00 Development of an Australian strain of *Ichthyophthirius multifiliis* infecting rainbow trout under different temperatures. Forwood JM, Harris JO, Deveney MR, Landos M.

**10.20 Tea Break**

**10.45 Session 14: Bacteriology (Chairs: Nicky Buller and Jeremy Carson)**

10.50 Research findings from the investigation of *Streptococcus agalactiae* in Queensland grouper *Epinephelus lanceolatus*, and wild fish and crustaceans of north Queensland. Bowater RO, Delamare-Debouteville J, Barnes A, Fisk A, Condon K, Dyer K.

11.10 Novel Chlamydia-like epitheliocystis agents in Australian farmed yellowtail kingfish *Seriola lalandi*, striped trumpeter *Latris lineata* and barramundi *Lates calcarifer*. Stride MC, Polkinghorne A, Miller TL, Groff JM, LaPatra SE, Powell M, Nowak BF.

11.30 First report of *Flavobacterium psychrophilum*, the bacterial agent associated with peduncle disease, from Chinook salmon (*Oncorhynchus tshawytscha*) in New Zealand. Williams RE, Jones B, Draper J, Brunton J, Fischer J, Brosnahan C, McDonald WL.

11.50 *In vitro* use of synthetic antimicrobial peptides against aquaculturally relevant pathogens. Blumhardt M, Bridle AR, Nowak BF.

**12.10 Lunch Break**

**12.55 Session 15: Mollusc Diseases (Chairs: Rob Adlard and Tim Green)**

13.00 Detection of bacteriophage-related chimeric marine virus in cultured abalone in Taiwan. Chang PH, Chen MS, Chen YW, Kuo ST.

13.20 CSIRO AAHL-IFREMER: Collaborative studies on mollusc pathogens. Corbeil S, Arzul I, Renault T, Faury N, Berthe F, Crane MStJ.

13.40 A longitudinal study of winter mortality disease in *Saccostrea glomerata* (Sydney rock oysters). Jenkins C, Spiers ZB, Gabor M, Fell SA, Carnegie RB, Dove M, O'Connor W, Frances J, Go J, Marsh IB.

**14.05 Session 16: Quality Assurance (Chairs: Nette Williams and Peter Mohr)**

14.10 New Zealand's salmon export testing scheme. Lane H, Brosnahan C, Orr D, Spence R, McDonald WL.

14.30 A regional aquatic animal health laboratory proficiency testing program in Asia. Herbert BW, Leaño E, Crane MStJ, Gudkovs N, Hoad J, Moody NJG, Warner S.

14.50 International proficiency testing for viral pathogens of finfish. Hoad J, Moody NJG, Williams LM, Crane MStJ.

**15.10 Tea Break**

**15.35 Session 17: Finfish Parasites II (Chairs: Kate Hutson and Dave Ellis)**

15.40 Infestation of Isopod parasite *Catoessa boscii* (Cymothoidae) on Malabar trevally *Carangoides malabaricus* (Carangidae), Southwest Coast of India. Ravichandran S, Rameshkumr G, Trilles J-P.

16.00 Survival strategies of an insidious fish ectoparasite, *Neobenedenia* sp.. Hutson KS, Truong DH.

16.20 Effects of temperature and salinity on the life cycle of *Neobenedenia* sp. (Monogenea: Capsalidae) infecting farmed barramundi (*Lates calcarifer*). Brazenor AK, Hutson KS.

16.40 Differences in epithelial pathology of fish microhabitats infected with the ectoparasitic monogenean *Neobenedenia* sp.. Trujillo AG, Constantinoiu CC, Johnson LK, Hutson KS.

17.00 Efficacy of garlic (*Allium sativum*) extract in managing the fish ectoparasite *Neobenedenia* sp. (Capsalidae: Monogenea). Militz TM, Hutson KS.

**17.20 Close Day 5**

**19.00- Conference Dinner**
**22.00**

# ABSTRACTS

# Emerging Problems in Fish Pathology

Hugh W. Ferguson BVM&S, PhD, DipACVP, MRCVS, FRCPath,
Professor, Diagnostic Pathology & Microbiology,
St George's University, School of Veterinary Medicine,
Grenada, West Indies

At a time when we are being constantly encouraged to eat more fish for the health of our own hearts, it is indeed ironic that the animals themselves are suffering from increasing levels of severe cardiovascular disease, particularly in farmed salmonids. Aside from involvement in some of the alphaviral diseases, examples include cardiomyopathy syndrome of salmon, a disease that targets the largest and fastest-growing fish, and for which a viral aetiology has recently been proposed. Animals frequently die due to a ruptured atrium, a consequence of severe necrosis of the spongy myocardium. If the fish survive, the heart tissue will completely regenerate.

Bacterial diseases continue to dominate the daily diagnostic caseload. Examples include emerging chronic granulomatous diseases of warmwater fish, caused by Francisella sp. and *Edwardiella ictaluri* in tilapia in Central America and in *Pangasius* catfish in Vietnam, respectively. A range of syndromes associated with the yellow pigmented *Flavobacterium psychrophilum* can be found in trout in the UK; these include "coldwater disease" with tailrot, "necrotic myositis", "dissolving head disease" with generalized necrosis of cartilages in the head and spinal column, and most recently "strawberry disease", which causes severe lichenoid-type dermatitis with lysis of scales thereby rendering the skin fragile and easily damaged by even relatively minor management procedures such as grading. Consequent financial losses due to downgrading of the fish at slaughter are a major concern. *Candidatus Arthromitus* is a long filamentous spore-forming bacterium that is associated with an enteric disease of European trout, "summer enteritis syndrome"; mortality can be high, but we know very little about the pathogenesis of this disease, nor the relationship, if any, of these very distinctive organisms to the ensuing enteritis.

Probably due to global changes in climate, blooms of jellyfish are becoming increasingly important. These animals can cause problems to farmed fish due to nematocyst damage, but they may also be capable of acting as vectors for other significant pathogens such as *Tenacibaculum maritimum*.

*Streptococcus iniae* causes disease in farmed warmwater fish, but can also cause encephalitis in humans. This bacterium was originally described from Dolphin iniae the freshwater dolphin of the Amazon basin. It was shown to be responsible for a major mortality of reef fish in the Caribbean basin in 1999, almost completely wiping out the entire fish population of reefs round several of the Windward Islands. The source of the infection was never established, although an especially heavy outfall from the Orinoco River was suspected. At the time of the "Millenium" celebrations, there were almost no affected fish left on the beaches, and a potential source of human infection was thereby greatly reduced.

A very recent outbreak of acute disease in farmed tilapia was associated with distinctive liver lesions in which the formation of hepatocyte syncytia was characteristic. Mortality in fry exceeded 80%.

Neoplasia is seen on the skin of oil sardines from the Indian Ocean, as well as in the mouth of one of their major predators, the barracuda. The possibility of an infectious aetiology for both needs to be considered.

## Gladstone: clearing the mud from the waters - why the animals died

Landos MA[1] and Dennis, M[2]

[1]Future Fisheries Veterinary Service Pty Ltd, East Ballina, NSW, Australia
[2]Michelle Dennis, Veterinary Pathologist, Brisbane, QLD, Australia

In late 2010, the largest harbour development project in the Great Barrier Reef World Heritage Area commenced, after State and Commonwealth approval of three Liquified Natural Gas (LNG) developments on Curtis Island, and a new coal export terminal. The project involved construction of a 15km rock bund wall over a seagrass meadow in the western basin of the harbour, dredging over 26 million cubic metres of sediment for shipping access and wharf development, and disposal of sediments at sea and into the bund wall. From April 2011 large numbers of diseased animals were reported in the vicinity of the Gladstone project. Mortalities were observed well above all historical baselines in dugongs, inshore dolphins, green and hawksbill turtles, catfish, barramundi and stingrays until late 2011. Numerous other marine fish species developed red skin lesions across around 40km of coast line adjacent to the development and ocean disposal site. Commercial fishers reported mud crabs developed novel "rust spot" shell lesions at an increased prevalence.

In January 2012, FFVS was commissioned with ~$55,000 by a public charity, to undertake a veterinary investigation of the causes of sickness and mortality in Gladstone Harbour. Full report published at www.gladstonefishingresearchfund.org.au. Sampling took place where diseased fish were reported from Turkey Beach in the south to the Narrows in the north and on the oceanic side of Facing Island. Gill nets and trawl equipment was used to sample live grossly affected fish, prawns and scallops which were examined for lesions and parasitism, prior to necropsy and preservation in 10% neutral buffered formalin for histopathology. A prevalence survey of shell lesions on mudcrabs was undertaken using commercial crab pots.

A reference site at Thirsty Sound 250km to the north was sampled using similar equipment targeting similar fish species and mud crabs. Where no fish with lesions could be captured, convenience sampled fish were processed for pathology.

Increased intensities of parasites which have been associated with immunosuppressed fish, in particular monogeneans, coccidians and copepods were detected in the majority of fish in Gladstone in association with damage to host tissues. The same parasite fauna were observed at the reference site, however intensities were lower, and no accompanying damage to tissues was observed on histopathology.

Prevalence of rust spot shell lesions on mud crabs was substantially elevated in Gladstone Harbour compared to historical data and to the reference site. The lesions in Gladstone crabs were endocuticle defects which, in previous studies, are morphologically consistent with lesions described from elevated exposures to metals such as copper and zinc. Subsequent testing revealed substantially elevated copper and zinc levels in affected mud crabs in Gladstone compared to the reference site.

The temporo-spatial epidemiology of the affected aquatic animals is only able to be explained by exposure to a cocktail of resuspended metals and metalloids in addition to a toxic algal bloom generated from nutrient resuspension from the harbour development.

**Effects of chronic heavy metals exposure on thyroid hormone pathway related genes of sand flathead (*Platycephalus bassensis*) from the Derwent River, a historically polluted estuary**

Dingkun F, Bridle A, Nowak BF and Leef M

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At present there is scant information regarding aspects of health in recreationally important fish such as sand flathead *Platycephalus bassensi* that not only have significant recreational, commercial and arguably cultural value to the Tasmanian community. In teleost fish, thyroid hormones are crucial in regulating many biological processes including development, growth, and reproduction. Laboratory studies have implicated heavy metals as thyroid disruptive agents in fish; however, the linkage between mixed heavy metals exposure in field and effects on the thyroid hormone pathway of fish is not well established. The objective of this study was to clone thyroid pathway related genes and assess the use of these genes as potential molecular bio-markers for monitoring the health status of wild Derwent River sand flathead. Fish were sampled in the Derwent River at Ralphs Bay and at Cornelian Bay (reference site). The thyroid hormones related genes, including deiodinases (*D1* and *D2*), transthyretin (*TTR*), and the thyroid hormone receptors (Thrα and Thrβ), responsible for metabolism, transport and recognition of thyroid hormones, were cloned as target genes to compare expression levels in fish from the different sites. Moreover, metallothionein (*MT*) and cytochrome P4501A (*CYP1A*), which are involved in metal detoxification and oxidative metabolism, were cloned and used as additional molecular markers. The association between heavy metals and thyroid hormone gene expression in this important species will be discussed. The use of these genes as molecular biomarkers for monitoring the health of flathead and potentially other fish species will also be discussed.

## Dinoflagellate dilemma

Bannister J

Fish Health Unit, Department of Fisheries, Western Australia

*Karlodinium* is a dinoflagellate protist micro alga with a characteristic apical groove. This alga has been implicated in fish kills worldwide with numerous cases reported in the United States, south-western Africa and in Perth, Western Australia. The species that is commonly identified in Western Australia, is *Karlodinium veneficium*. This toxic alga prospers in saline water and high levels of nutrients and organic matter can precipitate algal levels to bloom out of control. The alga is known to produce toxic compounds that destroy gill architecture and has harmful effects on all aquatic animals. These toxins have been shown to generate pores in membranes which increase ionic permeability resulting in membrane depolarization, disruption of motor functions, osmotic cell swelling and lysis.

In late May 2012, fish began dying in a river in Perth, Western Australia. The Western Australian Department of Fisheries Fish health unit received three separate fish kill specimen submissions up until July that year. Across all three submissions histopathology revealed significant gill lesions, and dinoflagellate cells with their characteristic apical groove, were identified on histological examination from fish in the third submission. There were no significant lesions in other organs.

The most likely cause of this extended algal bloom in 2012 is the lack of winter rainfall in Perth that year. Research has also shown that dinoflagellate cells need to lyse for fish kills to occur. Lysing may occur at the end of blooms or as a result of dinoflagellate mortality from a sudden change in environmental conditions such as temperature or salinity. Currently, the only management option to control an algal bloom like this is to manipulate the operation of oxygen plants in a river system. *Karlodinium veneficium* does not present a direct risk to public health, however the community should be advised not to swim or fish in affected waterways with dead or decomposing fish.

## Koi herpes virus: dreaded pathogen or white knight?

McColl KA[1], Sunarto A[1], Clarke B[1], Prabandono A[1], Slater J[1], Hoad J[1], Williams LM[1], A Ward[2], Doran TJ[1] and Crane MStJ[1]

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[2]School of Medicine, Deakin University, Geelong Waurn Ponds Campus, VIC 3216, Australia

On a global scale, there is a definite ambivalence about the value of the common carp (*Cyprinus carpio*). On the one hand, it is the fourth most-farmed fish in the world, being particularly important as a food source in China, south-east Asia, eastern Europe and the Middle East. However, by contrast, in Australia, New Zealand and North America, carp are regarded as a significant introduced pest species that is a major environmental problem with a negative impact on many native species of fish.

Disease associated with koi herpes virus (KHV), formally known as cyprinid herpesvirus-3, was first described in common carp in Israel in 1998, although retrospective studies have since shown that the virus was responsible for mortality in common and koi carp from about 1996. Since then, the virus has spread throughout much of Europe and Asia, and to South Africa and the United States. As with its host species, there is also a certain ambivalence about KHV. While it represents a serious threat to important carp industries throughout much of the world, in Australia it is seen as one of a number of potential weapons that might be used in a multi-pronged attempt at controlling carp in this country. So, depending on your country of residence, KHV may be seen as a dreaded pathogen (in carp aquaculture), or as a white knight (for biological control). In the AAHL Fish Diseases Lab, we are addressing both possible roles of KHV.

In Australia, the Invasive Animals CRC has developed an integrated pest management plan for the control of carp that includes an examination of both classical and innovative control strategies, including the use of KHV as a potential biological control agent. So far, work at AAHL with KHV has demonstrated that: (1) Australian carp are susceptible to the virus; (2) KHV kills carp quickly (thereby precluding any major animal welfare issues); (3) 10 native, and one introduced, non-target species of fish are not susceptible to infection; (4) carp-goldfish hybrids are less susceptible than common carp; and (5) cross-reactive viruses of carp are unlikely to confer much, if any, protection against KHV. Current work aims to examine the susceptibility of further non-target species including not only fish, but also a mammal, a bird, a reptile, an amphibian, and a crustacean. We are also developing an agent-based epidemiological model that will assist in devising the best means of distributing KHV throughout the Murray-Darling Basin in the event that public and political support are obtained for release of the virus.

In many areas of the world, significant resources are being directed toward developing control strategies for KHV in order to protect farmed carp. Many groups have attempted to develop vaccines for use against the virus, but, to this point, no vaccine has been uniformly adopted throughout the world. For this reason, we decided to investigate the possibility of producing a genetically modified carp that would be resistant to KHV. To develop the technology, we used zebrafish (*Danio rerio*) challenged with viral haemorrhagic septicaemia (VHS) virus as a model. The basis for protection in our model is that a transgenic zebrafish will have RNA hairpins, targeting complementary VHS virus gene sequences, integrated into the genome of every cell in the fish, and, when these viral sequences are expressed as non-coding RNA in an infected host cell, an RNAinterference (RNAi) response will knock down virus production, and protect the fish. At this stage, we have successfully produced germ-line transgenic zebrafish, and we are about to challenge these fish with VHS virus. On another front, we have already identified a number of genes from KHV that, when targeted by an RNAi approach, result in knock-down of KHV in cell culture. These results augur well for the future production of a KHV-resistant carp.

**Genotype analysis of koi herpesviruses detected in quarantine in Singapore from 2005 to 2011**

Jing C[1], Chee D[2], Yahui W[1], Yee LG[1], Min CS[2], Taoqi H[1], Nuo LY[1]

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Koi herpesvirus (KHV) is the causative agent of koi herpesvirus disease and it is an OIE listed notifiable disease. The Agri-food & Veterinary Authority of Singapore (AVA) requires all imported consignments of koi to be subjected to compulsory 4-week quarantine and sampling at 10% prevalence and 95% Confidence Interval to ascertain freedom from KHV. Under this programme, fifteen independent KHV positive cases were identified by PCR testing from koi fish imported from the East Asia and South-east Asia regions from 2005 to 2011. Cell culture isolation of KHV in koi fin (KF-1) cells was successful in one case. Initial analysis of the thymidine kinase gene sequences of these fifteen cases indicated that the cases detected were more closely related to the KHV Asian 1 lineage than the European lineages. It has been shown by others that there are three major KHV lineages circulating worldwide, the Japan lineage (J), the Israel lineage (I) and the United States lineage (U). Therefore, further genotype analysis was carried out by duplex PCR and sequencing for marker Ⅰ and marker Ⅱ to determine the lineages of KHV in the positive cases. It was found that 9 of the 15 KHV positive cases were related to KHV lineage J ( $^{+}$Ⅰ$^{+}$Ⅱ$^{+}$), while another 4 cases were related to KHV lineages I/U ($^{-}$Ⅰ$^{-}$Ⅱ). Another 2 cases were associated with variants of an intermediate lineage ( $^{++}$Ⅰ$^{-}$Ⅱ). Our results shed some lights on the geographical prevalence of KHV genotypes in this region and help to understand the origin and potential diversification of KHV.

**Preliminary characterization of Tasmanian Aquareovirus (TSRV) isolates**

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Fourteen isolates of TSRV originating from various locations in Tasmania, covering a 20-year period obtained from various host species, host tissues and isolated on different cell lines, were selected in an attempt to increase the probability of detecting virus variants. Typical and atypical variants of TSRV were identified based on genotypic and phenotypic characterization of the different isolates. Electron microscopic examination demonstrated the existence of at least three variants based on viral particle size. This study revealed preliminary evidence of vertical transmission of TSRV from brood-stocks to eggs and horizontal transmission from farmed salmon to wild fish. Finally, this characterization study demonstrated the existence of at least one variant TSRV isolate other than the more commonly isolated, typical TSRV in farmed Tasmanian Atlantic salmon.

**Experimental transmission of megalocytivirus between freshwater and marine fish populations through the use of a model euryhaline species Australian bass, *Macquaria novemaculeata***

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Megalocytiviruses similar to infectious spleen and kidney necrosis virus (ISKNV) have previously been detected in gourami species entering Australia through the live trade in ornamental fish (Go et al., 2006 *Mol Cell Probes* **20**: 212-222). Although Murray cod (*Maccullochella peelii*) have been shown to be highly susceptible to megalocytivirus derived from imported dwarf gouramis (*Trichogaster lalius*) (Go and Whittington 2006. Aquaculture **258**: 140-149), the susceptibility of other native Australian species is not well characterised. More recently an ISKNV-like iridovirus obtained from pearl gourami (*Trichopodus leerii*) has been shown to be pathogenic to rock bream (*Oplegnathus fasciatus*) a marine species widely cultured in Korea (Jeong et al., 2008 DAO **82:** 27-36). However, due to the vastly different ecosystems in which these two species occur, opportunities for direct contact between them are likely to be limited.

Previously, the euryhaline Australian native species Australian bass (*Macquaria novemaculeata*) has been shown to be experimentally susceptible to infection with an ISKNV-like megalocytivirus. Further, Australian bass were capable of acting as a model vector for transmission between this species and Murray cod (Go and Whittington 2011. 1st FRDC Australasian Aquatic Animal Health Scientific Conference).

In this study, the experimental susceptibility of an Australian native marine species, silver sweep (*Scorpis lineolata*) was tested both by intraperitoneal (IP) injection and cohabitation. Australian bass were then used as a model euryhaline vector in a series of experimental transmission trials in an attempt to transmit an ISKNV-like megalocytivirus from a freshwater species, Murray cod, to a marine species, silver sweep and vice versa.

Silver sweep were found to be highly susceptible, both by IP injection and cohabitation, to an ISKNV-like megalocytivirus derived from imported dwarf gourami (DGIV-10). Further, when naive Australian bass juveniles were exposed to infected silver sweep, transitioned to a freshwater environment, and then exposed to naïve Murray cod fingerlings, megalocytivirus was transferred to Murray cod juveniles resulting in severe mortality in that species. Conversely, when naïve freshwater acclimated Australian bass were exposed to Murray cod fingerlings that had been IP injected with DGIV-10, then acclimated to marine conditions and exposed to naïve silver sweep, megalocytivirus was transmitted to this species again inducing severe mortalities. In all cases, clinical signs consistent with megalocytivirus infection were observed pre-mortem, and further confirmation of megalocytivirus infection in these species was achieved through quantitative polymerase chain reaction, representative histology and in situ hybridization.

These findings suggest that the potential host range of ISKNV-like megalocytiviruses in Australian native fish species may be relatively broad, and that spread of this virus group between freshwater and marine species might be possible. This group of viruses could pose a greater biosecurity risk than previously thought.

**Sero-prevalence of Nervous Necrosis Virus across Barramundi and Australian bass adult populations**

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Viral Nervous Necrosis (VNN), caused by infection with a Betanodavirus, is a disease causing significant losses in marine aquaculture worldwide. Affected fish usually display abnormal swimming behavior and outbreaks commonly result in 100% mortality in larval fish. Understanding transmission is crucial for developing prevention policies and broodstock screening is considered to be an important component of disease control. ELISA to detect serum antibodies against NNV is a plausible non-lethal assay for this purpose. A longitudinal serological survey was carried out with barramundi (*Lates calcarifer*), Australian bass (*Macquaria novemaculeata*) from different locations in Australia. Results were analyzed by exposure status and correlated with the occurrence of outbreaks and subclinical infection in populations of young fish.

A total of 649 serum samples collected between 2003 and 2012 were used in this study. They were classified as naturally exposed or of unknown exposure status according to clinical histories and results from molecular tests for NNV. Specific antibodies against NNV were measured using an in-house indirect sandwich ELISA developed and optimized using sera from immunized barramundi and Australian bass.

We also found that sero-prevalence was significantly higher in fish populations known to be naturally exposed to NNV compared to populations of unknown exposure status ($p<0.001$) in endemic regions. The apparent prevalence of anti-NNV antibodies in barramundi populations known to be exposed to infection was 70% (n=96) and 10% for a population with unknown exposure status (n=323). For Australian bass, where the same infected population was followed over time, antibodies were detectable in 45% and 35% of the individuals at 3 and 5 years post-exposure, respectively. Hence, we consider that detection of anti-NNV antibodies is a useful method of determining the exposure status of barramundi and Australian bass populations.

## Pacific Oyster Mortality Syndrome in NSW (2010–2013)

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Pacific Oyster Mortality Syndrome, or POMS, is the name given to the disease affecting Pacific oysters that is caused by Ostreid herpesvirus-1 microvariant (OsHV-1 µvar). POMS was detected for the first time in Australia in cultivated triploid Pacific oysters in Georges River, NSW, in late 2010. As at May 2013, POMS is now known to occur in three estuaries in Australia – Georges, Parramatta and Hawkesbury rivers in NSW. OsHV-1 µvar has also been detected in wild Pacific oysters in Brisbane Water, which shares a mouth with the Hawkesbury River, however only Sydney rock oyster cultivation occurs in this estuary.

The POMS situation in NSW, including the challenges and outcomes of management decisions, as well as current research priorities, will be discussed.

## OsHV-1 in Pacific oysters in NSW – Molecular detection and partial characterisation

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Ostreid Herpesvirus (OsHV) was first detected in Australia in late 2010, associated with mass mortalities of Pacific oysters (*Crassostrea gigas*) in NSW. Conventional PCR using the C2/C6 primer set and sequencing of amplicons confirmed the high nucleic acid identity to OsHV-1 µVar. Two real-time probe-based PCR assays are currently used to screen Pacific oysters for the presence of OsHV-1; 1) an in-house assay developed by EMAI and 2) an assay published by Martenot et al (2010) that was included in the OIE Manual for "Infection with ostreid herpesvirus 1" in 2012. Comparative testing of both assays at AAHL, using diagnostic samples and a Proficiency Test panel distributed through the LEADDR network, demonstrated the equivalence of both assays. Regardless of the assay used, the availability of a second equally sensitive assay targeting a different region of the genome is highly desirable.

Conventional Sanger sequencing of variable regions of the genome (ORF 4, ORF 35-36-37-38 and ORF 42-43) identified the ORF4 region as the most variable. Additional sequencing of isolates obtained from various locations in 2011, 2012 and 2013 confirmed the homogenous nature of the viral sequence, as all isolates shared a 100% nucleotide similarity over the ORF4 region. A major difference between the NSW OsHV-1 sequence and those from France, China and New Zealand was a G to A nucleotide substitution at the 3' end of the microvariant region which resulted in Asparagine instead of Aspartic Acid. This region also contained 2 nucleotides common to the OsHV-1 reference sequence and not the OsHV-1 µVar sequence.

Whole genome sequencing was undertaken from nucleic acid extracted from a sample from the original diagnostic submission from EMAI which had a qPCR $C_T$ value of 16. Of 11,106,960 paired end reads obtained using the illumina GAxii, 61,959 read-mapped to OsHV-1 whole genome sequences from France and China. Assembly and analysis of the whole genome sequence of NSW OsHV-1 is ongoing.

Molecular screening and confirmatory detection methods for OsHV-1 and preliminary sequence analysis will be discussed.

## *Ostreid herpesvirus-1* (OsHV-1) in commercially farmed Pacific oysters (*Crassostrea gigas*) in the Hawkesbury River in 2013: Outbreak investigation

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OsHV-1 associated mortalities occurred for the first time in Australia in 2010 in commercially farmed Pacific oysters in Woolooware Bay, which is part of the Georges River-Botany Bay estuary in Sydney. On 21 January 2013 a massive outbreak began 50 km to the north of Botany Bay in the Hawkesbury River system. About 10 million oysters died and the commercial losses exceeded $3M within a few weeks. The mortality event was reported to NSW DPI on January 21 (day 1). OsHV-1 was detected by PCR tests conducted by NSW DPI on day 2 and was confirmed at AAHL, and a state response plan was quickly implemented to contain the disease by quarantine orders.

The Hawkesbury River outbreak occurred during an FRDC research project in which sentinel oysters and environmental monitoring had already been established. An outbreak investigation study commenced on day 1. The aim of the study was to describe the epidemiological features of this natural outbreak of OsHV-1 disease in *C. gigas*. The outbreak investigation was conducted in parallel with a locally determined disease control response which was facilitated by a well-established, coherent local association of oyster farmers. The response consisted of the following steps: immediate voluntary introduction of sanitary measures to limit spread of OsHV-1 by farmers; emergency harvest of adult stock; a survey of healthy stock to identify the geographic extent of OsHV-1 infection in the estuary so as to make recommendations about the risk of movement of oysters between bays; risk-based management of stock for growout and harvest; tracing recent local oyster movements and monitoring the spread of clinical disease; an audit of stock which had been affected by mortality to identify age and size classes affected; identification of risk factors using environmental records and data from sentinel oysters which had been placed in the estuary previously, and; identification of transmission mechanisms.

The key findings of the outbreak investigation included a high likelihood that the epizootic was derived from a point source, and originated from one massive water-borne exposure event, with an incubation period of 2-4 days. Oysters of all ages/sizes were affected. Low level exposure of oysters in the estuary preceded this epizootic by some months. Spread of the clinical disease from bay to bay with movements of oysters was inefficient and did not lead to massive propagating epizootics in destination bays. Currents and tidal flows ensured that oysters on all active leases were exposed and became infected with OsHV-1 within 8 days of the start of the epizootic, or widespread low level infection preceded the epizootic. The environmental source of OsHV-1 leading up to the outbreak was not identified. It is now clear that OsHV-1 is not sufficient to initiate epizootic mortality. Major risk factors for the outbreak are still the subject of investigation.

## Investigating the transmission factors of Ostreid herpesvirus-1 (OsHV-1) within the environment: Wild mollusc species as a possible source of infection?

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Pacific Oyster Mortality Syndrome (POMS) is a highly complex, multi-factorial disease which arises from the interactions of oysters, pathogens and environmental factors. As the name suggests, POMS causes severe mortality in the Pacific oyster (*Crassostrea gigas*), affecting predominantly spat and juveniles with mortalities of 100% and 40-100% respectively. Ostreid herpesvirus-1 (OsHV-1) is the sole member of the viral family *Malacoherpesviridae* within the order *Herpesvirales* and has been identified in Australia and many European countries as one of the key pathogens causing POMS disease. The disease, which first appeared in Australia in the Georges River in November 2010, poses a serious threat to the Australian *C. gigas* industry (2007-08: $53 million), with a potential to cause dramatic economic loss to producers in NSW, TAS and SA. Within the current literature there exists a significant degree of research focused toward essential OsHV-1 diagnostic methodologies, however very little information exists on the equally important topic of OsHV-1 transmission factors. Very little is understood about where OsHV-1 exists within the environment surrounding commercially produced *C. gigas*, even less on how it is transmitted in water to farmed *C. gigas* and what environmental factors trigger these outbreaks. Previous studies performed in Europe have suggested that OsHV-1 can exist subclinically in other species, as well as cause mortality in species other than *C. gigas*, including *Ostrea edulis*, *Ruditapes decussates* and *R. philippinarum* (Arzul *et al.* 2001; Moss *et al.*, 2007). Understanding what species may act as a potential host and/or reservoir of OsHV-1 in Australian *C. gigas* producing waters is thus crucial to understanding the disease cycle of POMS both here and abroad.

A survey of wild mollusc species was conducted in the Georges River, between November 2012 and April 2013. Survey samples were collected on three separate occasions during this period – specifically November, December 2012 and April 2013. Species sampled included wild *C. gigas*, wild Sydney Rock oysters (*Saccostrea glomerata*), and various other mollusc, crustacean and plant species. Sites for collection were objectively chosen based upon observations of existing wild mollusc colonies and included the infrastructure of oyster farming enterprises within Woolooware Bay, sand bars and oyster reefs within Woolooware Bay and sites some kilometres upstream of active oyster leases. Samples were pooled for analysis and tested for the presence or absence of OsHV-1 using a real time Polymerase Chain Reaction technique (qPCR). These data were then compared to data collected from experimental field oysters present in Woolooware Bay during the 2012-2013 POMS outbreaks.

Thorough investigation into where, when and how this virus operates is central to understanding the disease cycle of POMS and ultimately vital to creating practical and economically viable recommendations for producers to prepare for future outbreaks of this disease, and in doing so limit the economic loss to industry.

**References**

Arzul *et al.* 2001. Dis Aquat Org. 46: 1-6.
Moss *et al.*, 2007. Dis Aquat Org. 77: 207-223.

## Effects of husbandry practices to reduce OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas,* and first steps towards integrated management within an infected estuary

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Ostreid herpesvirus-1 (OsHV-1) was first detected in the Georges River (NSW) in November 2010 associated with mass mortality of wild and farmed *Crassostrea gigas.* The virus has subsequently spread to the Hawkesbury River where the first POMS associated mortalities were detected in January 2013. The disease and its potential spread nationwide poses a serious threat to the Australian *C. gigas* industry in SA, TAS and NSW (2007-08: $53 million). While the concepts for management of this disease mainly involve reducing the rate of spread, active surveillance and mollusc breeding programs targeting production of resistant animals, the effects of aquaculture practices on infection and mortality rates are still poorly understood. Overcoming these knowledge gaps might lead to opportunities for oyster farmers to manage production in the face of the disease.

A FRDC funded research project was initiated by the University of Sydney in July 2012 seeking to understand how the virus is transmitted and spreads, and which factors lead to disease outbreaks. This project involves intensive field monitoring in infected oyster leases as well as trials of different husbandry practices involving modification of age, immersion times, rearing structures and water inputs in order to reduce mortality.

Major findings from experiments conducted in the two infected estuaries (Hawkesbury and Georges Rivers) between 2011 and 2013 indicated significant differences in mortality rate related to modification of the immersion time (high *vs.* low growing height), the physiology of oysters (spat *vs.* mature oysters) and some water quality parameters. A significant 30-50% increase in survival of adult oysters was demonstrated by raising the height of intertidal rearing structures. While mortality of spat reared at high height was delayed for weeks in comparison with the low height, the final cumulative mortalities were identical for both groups. This result could be related to an interaction between beneficial lower exposure to viral particles and deleterious physiological impacts of extreme height revealed by differential growth rates (size and weight) and histological observations.

Preliminary findings from a current experiment suggest that simple water treatments may enable survival of post-hatchery spat in land-based facilities in infected estuaries. Combined with better understanding of seasonal factors leading to infection/disease risk (the window of infection) and spat growth potential during winter, an integrated farming strategy can be suggested. It involves "safe rearing" of post-hatchery spat in treated water during summer, placement of spat into the estuary in autumn when the risk of disease risk has past, lifting sub-adult oysters to high growing height when disease risk increases in late spring, and marketing of oysters as soon as they reach suitable size. Some oysters may need a second winter season to reach market size. Many gaps remain in the practical assessment of this strategy, but some of these are researchable issues.

**Experimental infection of Pacific oyster *Crassostrea gigas* using the Australian OsHV-1 strain: mortality, dose-response relationship and inoculum storage conditions**

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Ostreid herpesvirus (OsHV-1) has the potential to devastate *Crassostrea gigas* culture in Australia as it has done in many other countries, highlighting the need for a better understanding of viral transmission and disease expression. As there are no established immortal cell lines for molluscs, the most relevant bioassay to measure in detail particular aspects of viral biology (infectivity, transmission, stability) requires the development of a controlled and repeatable experimental transmission model to infect Pacific oysters. Industry also requires such a model in order to test the susceptibility of different genetic (family) lines of *C. gigas* to meet its objective of breeding for disease resistance.

In July 2012 the first infection trial protocol was successfully developed and tested in a purpose-built PC2 animal facility at the University of Sydney in order to confirm the reproducibility of the disease in laboratory conditions using the Australian OsHV-1 strain. Subsequently, a total of three infection trials were performed to (i) confirm the repeatability of the infection model; (ii) assess the dose-response relationship between the quantity of viral particles injected, the progression of infection and the mortality rates of oysters; and (iii) determine the most appropriate storage conditions for the inoculum to maintain the infectivity of the Australian OsHV-1 strain under laboratory conditions. For each infection trial healthy unexposed Pacific oysters (11 month old) were injected with purified tissue homogenate prepared from either naturally infected oysters sampled from the Georges River (NSW) during the summer 2011/2012, or healthy oysters sampled from a non-infected estuary. Mortality was monitored daily for 10 days and no active sampling was performed on live oysters. Dead oysters and water samples were collected daily from each tank for OsHV-1 quantification by PCR analysis. Additionally, control and surviving oysters were all sampled and sacrificed at the end of each experiment.

Overall, these experiments demonstrated that an experimental infection model can be developed under "local" conditions using the Australian OsHV-1 strain and healthy virus-free Pacific oysters from a non-infected estuary. However some variability in mortality rates among infection trials was observed, highlighting the potential issues that might occur in the development of a reproducible and transferable infection model. These issues concern the conservation/stability of the viral inoculum and variation in the natural host due to the utilization of different batches of recipient oysters. A clear dose-response relationship for OsHV-1 was demonstrated and further research is recommended to determine the most appropriate viral concentration to use for an infection model for genetic selection. Important decisions about the standardization of experimental conditions will also need to be made (age/size of oysters, temperature, salinity, feeding, biofiltration, UV disinfection of water). Finally this experimental work showed for the first time that production of an infective inoculum can be performed successfully by using frozen oysters (-80°C for a minimum of 6 months). While this discovery facilitates the conduct of experiments throughout the year, it raises an issue for AQIS about the potential risks and consequences of importing frozen oysters from infected countries into Australia.

## Design of a detection survey for Ostreid Herpesvirus using hydrodynamic dispersion models to determine epidemiological units

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Since 2010, the New Zealand Pacific oyster industry has been under pressure due to large scale oyster mortalities attributed to infection with Ostreid herpesvirus-1 microvariant (OsHV-µVar). To assess how wide spread OsHV-µVar is in the key oyster growing areas of New Zealand outside of the Northland region, the feasibility of a detection survey was considered. Results of a detection survey could potentially help understand the mechanisms of spread of the disease, in order to inform management decisions regarding reseeding and transfer of live oysters and spat between oyster growing areas.

A detection survey was designed, targeting the key areas of importance for the oyster industry. The sampling methodology used in this survey design incorporated the concept of independent epidemiological units with some element of random selection. The epidemiological units were identified by the connectedness of the areas of coastline /bays/sounds based on modelling the potential spread of a viral particle due to the hydrodynamics of the selected area. Sample site selection was based on the size and complexity of these epidemiological units. Simple random sampling was applied to the smaller, less complex epidemiological units, whilst a 2-stage random sampling regime was used in the larger, more complex interconnected hydrographic areas.

To determine appropriate sample sizes, a 2 percent design prevalence of disease was assumed in all cases and since no diagnostic "gold" standard exists for OsHV-µVar, sampling designs were based on using molecular based diagnostic tests assuming 95% sensitivity and 99 % specificity. Although it was intended that samples would be collected from farms where possible, if the randomly selected sampling sites were located where no farm was present, the samples would be taken from the wild oyster population.

Other factors such as oyster size and time of year (water temperature) were taken into account to maximise chances of detecting the virus should it be present.

This survey design illustrates the potential use of epidemiological units based on areas defined by hydrodynamic dispersion models as a way to divide marine areas into sensible sampling units for surveys of any waterborne diseases.

**Poly I:C induces a protective antiviral immune response in the Pacific oyster (*Crassostrea gigas*) against subsequent challenge with Ostreid herpesvirus (OsHV-1 µvar)**

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*In-vivo* studies were carried out to investigate the protective effect of a synthetic viral analogue (poly I:C) against Ostreid herpes virus (OsHV-1 µvar). Pacific oysters (*Crassostrea gigas*) were immune-primed by intramuscular injection of 240 µg of poly I:C or sterile seawater at 1 day prior to infection with OsHV-1 µvar. Poly I:C injection induced an antiviral state in *C. gigas* as the percentage of viral-infected oysters at 48 hours post infection was significantly lower in the poly I:C treatment (11%) compared to seawater controls (100%) (Figure 1A). In an additional experiment, we demonstrated that the protective role of poly I:C is reproducible and elicits a specific antiviral response as immune-priming with heat-killed *Vibrio splendidus* provided no protection against subsequent viral infection (Figure 1B). In both experiments, genes homologous to a toll-like receptor (TLR), MyD88, interferon regulatory factor (IRF) and protein kinase R (PKR) were up-regulated in oysters immune-primed with poly I:C compared to seawater controls ($p < 0.05$). The MyD88, IRF and PKR genes were also significantly up-regulated in response to OsHV-1 µvar infection ($p < 0.05$), which is suggestive that they are implicated in the antiviral response of *C. gigas*. Our results demonstrate that *C. gigas* can recognise double-strand RNA to initiate an innate immune response that inhibits viral infection. The observed response has striking similarities to the hallmarks of the type-1 interferon response of vertebrates.

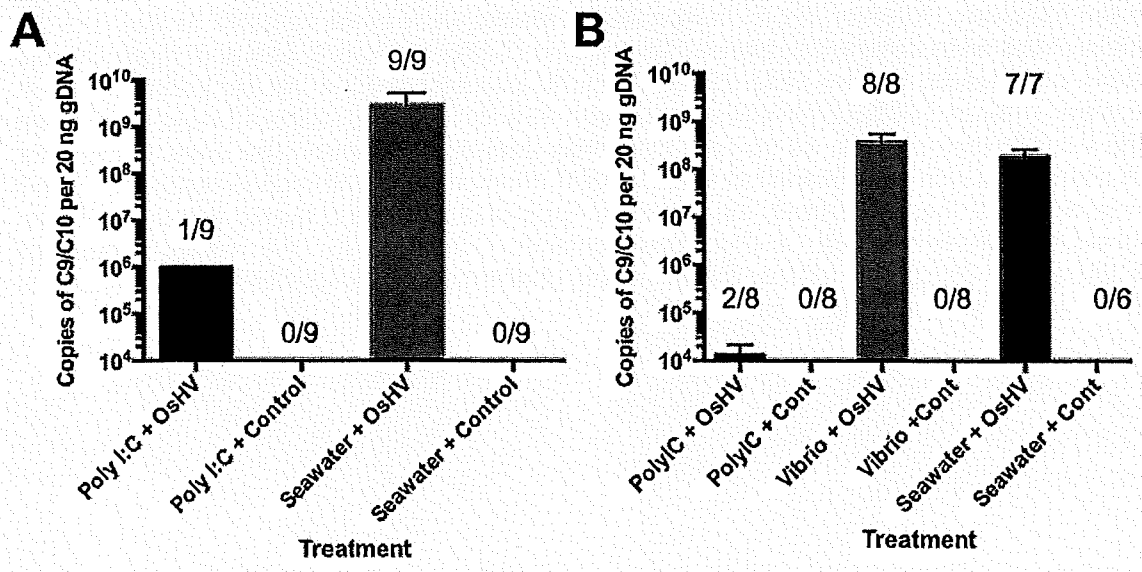


Figure 1. OsHV-1 DNA detection by quantitative PCR in whole spat injected with either poly I:C or seawater and challenge with OsHV-1 µvar or control homogenate. Results of the first experiment (A) and second experiment (B) are expressed as the mean number of C9/C10 copies detected per 20 ng of genomic DNA extracted from whole oyster spat (mean ± SE). The proportion of oysters containing OsHV-1 DNA (34 cycles of qPCR) in each group is also presented.

**Susceptibility of the black-lip pearl oyster, *Pinctada margaritifera,* to Ostreid herpesvirus (OsHV-1)**

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The production of pearls from the Black-lip Pearl Oyster, *Pinctada margaritifera* represents a major source of income for the economies of French Polynesia and the Pacific Islands. The recent spread of OsHV-1 to Oceania is of a particular concern to French Polynesia, as the islands import Pacific oysters from France and New Zealand that have been tested and are known to be positive to OsHV-1. This virus, known to infect different bivalve species, might be transmitted to black-lip pearl oysters through accidental co-habitation with infected Pacific oysters and could lead to disastrous impact on the pearl oysters industry. Therefore, the present study investigates the susceptibility of the pearl oyster to Ostreid herpesvirus, using the Pacific oyster *Crassostrea gigas* as a positive control. Oysters were injected with *C. gigas* tissue homogenates sourced from either naturally infected oysters as the viral source or non-infected oysters as controls. The experimental infection challenge lasted 14 days post-infection during which oyster tissues and haemolymph were sampled at 7 time points. Mortality rates and immune responses were determined to evaluate the susceptibility of *P. margaritifera* to OsHV-1. The experimental challenge was successful, as the infection of *C. gigas* was confirmed by TEM and real-time PCR, and mortality rates were in accordance with the literature. However, for *P. margaritifera*, results were more complex to interpret, as no differences were detected between infected and control animals in terms of mortality and immune parameters, but the virus spread from the adductor muscle via the haemolymph in all organs in around 60% of the animals. Around 40% of the pearl oysters blocked the entry of the virus to their tissues, as no viral DNA was detected. However, the detection of viral DNA associated with visualisation of virus in TEM micrographs in all organs revealed that, in the conditions of the experiment, the virus did not replicate in pearl oyster tissues and was progressively eliminated after 3 days post-injection. Consequently, the results suggested that under the conditions of the experimental challenge, *P. margaritifera* is resistant to OsHV-1 and is not an efficient carrier.

## Gill-associated virus epizootics in Queensland prawn farms in 2012

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Two large prawn farms in north Queensland reported pond-scale crop failures starting in mid-November. Both prawn farms had stocked *Penaeus monodon* fry originating from the same hatchery, at which the same group of wild, east coast *P. monodon* broodstock were being used. On one farm, all eight ponds stocked with the *P. monodon* fry had stopped moulting and started to die before 120 days of culture (DOC). This farm stated that all other stocked ponds remained normal with healthy prawns. The second prawn farm had stocked 20 ponds with the fry produced from the same hatchery using wild, east coast broodstock. Two ponds on this farm failed, with diseased prawns beginning to die before 100 DOC; six ponds had reduced feeding and problems with moulting; and the remaining 14 ponds were normal. A third farm had stocked fry from the same hatchery source in two ponds. No mortalities were seen in those two ponds but they reported slower growth as compared to the prawns in other ponds. The clinically diseased juvenile prawns had signs of reduced feeding, reduced FCRs, no moulting, fouled carapace, soft or rough carapace, reddened bodies and melanised shell disease lesions.

Diagnostic investigations failed to identify any evidence of haemocyte enteritis, septic hepatopancreatitis, vibriosis (one prawn only), gill fouling or any mycoses. Histologically lymphoid organ spheroids were present but at levels less than expected in mid-crop mortality syndrome (MCMS) epizootics. Some of the prawns had unusual cuticular lesions with focal necrosis of epidermis, infiltration of haemocytes and the presence of hypertrophied epithelial-like cells with a deeply basophilic, finely granular cytoplasm. In addition there were swollen branchiostegites and, or swollen latero-tergal plates of the abdominal segments with pooling of haemolymph in the connective tissue spaces created. Details on this histopathology will be presented and contrasted with the changes usually seen in MCMS outbreaks.

Some bacteria were isolated from the haemolymph of some prawns, but this was a light, mixed growth, not considered significant. PCR tests done on clinically affected prawns for the viruses TSV, IHHNV and SMV were negative. GAV was present but some unusual results were seen using the OIE Protocol 2 multiplex RT-nested PCR for GAV/YHV. Prawn tissues were sent to the AAHL Fish Diseases Laboratory for further PCR analysis. That information will be provided in another presentation. These epizootics and future implications for diagnostic approaches will be briefly discussed.

## Detection of a new genotype of the yellow head complex of viruses in *Penaeus monodon*

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The yellow head complex of viruses (Family: *Roniviridae*) consists of 6 genotypes, only one (YHV: genotype 1) of which causes yellow head disease (YHD). YHD, resulting in mass mortalities of farmed shrimp (*Penaeus monodon* and *P. vannamei*) and significant economic losses in Asia, remains exotic to Australia. Gill-Associated Virus (GAV), designated genotype 2, is less pathogenic but has previously been associated with mortalities. GAV has been detected in Asia and Australia whereas genotypes 3-5 have only been detected in Asia and genotype 6 in Africa. Genotypes 3-6 have been detected in healthy shrimp and are not known to cause disease.

In late 2012, farmed *P. monodon* on the North Eastern Coast of Queensland were found to have a reduction in molting, colour-change and increased mortalities. Initial diagnostic testing at the Tropical and Aquatic Animal Health Laboratory, detected the presence of a YHV-complex virus. Ethanol-fixed *P. monodon* were received at the Australian Animal Health Laboratory (AAHL) for confirmation. Molecular screening by real-time PCR, followed by extensive testing by conventional PCR and sequencing detected the presence of GAV. It remains to be determined if GAV was the causal agent of disease in these shrimp.

During the investigation, *P. monodon* broodstock from Northern Territory that had also changed colour and died in Queensland at the end of 2012 were submitted to AAHL. Sequencing revealed the presence of a previously unidentified YHV-complex virus with closest nucleotide similarity (88%) to YHV genotype 1. GAV was also detected in the broodstock, therefore it remains unknown if the new YHV-complex virus identified causes disease in shrimp.

## Detection of a new rickettsia-like organism in wild-caught sand crabs (*Portunus pelagicus*) from Darwin and Bynoe Harbours

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Surveillance to establish baseline aquatic animal health status was undertaken in Darwin Harbour, Bynoe Harbour and the mouth of the Adelaide River in the Northern Territory, Australia. Four sample collection trips were carried out between August 2012 and March 2013 as part of an environmental monitoring program. During this period, a total of 952 crabs [627 mud crabs (*Scylla serrata*), and 325 sand crabs (*Portunus pelagicus*)] were caught and examined in the field, and of these 315 crabs (225 *S. serrata* and 90 *P. pelagicus*) were subjected to further parasitological and histopathological examination.

A range of parasites were detected, but the most notable finding during the course of these surveys was the detection of a previously undescribed rickettsia-like organism (RLO) in the hepatopancreas of sand crabs sampled from Darwin and Bynoe Harbours. Histologically there was moderate to severe destruction of hepatopancreatic tubules which appeared to be associated with hypertrophic tubular epithelial cells containing numerous small pleomorphic intracytoplasmic Gram-negative organisms. Granulomatous inflammatory responses were often present in affected tubules. Field data revealed most of these affected sand crabs were lethargic and weaker than unaffected crabs at capture, but no other gross signs were observed and the colour of the haemolymph of the affected crabs was normal.

The overall prevalence of the RLO infection in sand crabs from all sites detected using histopathology was low (4.44%, n = 90 crabs), but there appeared to be some evidence of seasonal and spatial variation. During the dry season (August-September), the prevalence was highest at 6.89% (n = 58), but no infections were detected (n = 32) during the buildup and wet season (November-March). Spatially, the prevalence was highest at 10.52% (n = 19) at Outer Darwin Harbour, followed by 3.3% at Bynoe Harbour (n= 30) and 3.1% at Inner Darwin Harbour (n = 32). No infected sand crabs were detected from the Adelaide River (n = 9 crabs examined by histopathology).

Exclusion testing performed at the Australian Animal Health Laboratory (AAHL) found that the rickettsia-like organisms did not react with PCR designed for Milky Haemolymph Disease of Spiny Lobsters, nor did they react with PCR designed to detect rickettsia-like organisms from the European shore crab or *Penaeus monodon*.

The results from these surveys have provided useful insights into the baseline health status of populations of wild crustaceans in tropical Australia, and provide further support for Australia's aquatic animal health surveillance and monitoring capability.

## Teaching driving research: A case study using a novel microsporidium found in western king prawns off the Townsville coast

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In 1988 a prevalence study of the Queensland coast identified Microsporidia infections in five species of prawns. Microsporidia were presumptively identified as being either *Thelohania spp.*, which is now *Agmasoma* if found in a marine environment, or *Ameson spp.* (Owens and Glazebrook, 1988). Funding restraints precluded further identification work on these samples at the time. In 2011 Western king prawns were found off the Townsville coast. These presented with signs and histology identical to *Thelohania* spp.-infected prawns found off the Townsville coast 23 years earlier, providing another opportunity to study this infection.

Since the original presumptive identification of these Microsporidia, it has become increasingly apparent that morphological identification of Microsporidia has limitations, such as divergent spore morphologies, that can be overcome by molecular analysis (Stentiford et al., 2013, Vossbrinck and Debrunner-Vossbrinck, 2005). The small subunit ribosomal RNA (ssrRNA) gene has been identified as a useful tool for phylogenetic analysis and identification in Microsporidia (Franzen, 2008). Current identification methods should therefore include molecular analysis for identification and the ssrRNA gene appears to be a good target. However, a lack of immediate external interest in funding further identification and other priorities for research students looking for a project put the samples collected in 2011 into cold storage.

The School of Veterinary and Biomedical Sciences at James Cook University delivers a block mode intensive course to third year and graduate students entitled "Microbial molecular diagnosis and epidemiology". Good quality, highly infected tissues are desired for the course as it is designed to teach students the basics of extraction, detection and analysis of nucleic acids of microorganisms using authentic infected tissue samples. This requirement has provided an opportunity for researchers to be the beneficiaries of sequence data and phylogenetic analysis produced by the course. In 2012, the above mentioned prawns collected in 2011 were examined. Two published primer sets targeting the conserved ssrRNA gene (Fedorko et al., 1995, Lee et al., 2010) were purchased and briefly tested with RNA extracted from prawn tissue to ensure bands were produced on a gel. One set (Fedorko et al., 1995) was then used by students on the course. Plasmid products produced by students were sequenced and the NCBI database searched using tBlastx.

The closest match to recorded sequence was a *Trichonosema* with a 79% similarity (E values 6e-07). Published phylogenetic trees indicate this genus has a freshwater host (Vossbrinck and Debrunner-Vossbrinck, 2005). This molecular course has provided the first sequence evidence for a novel marine microsporidium. Further bioinformatical analysis is being carried out by students in this course in 2013 to update phylogenetic trees and design new primers to expand the sequence data available for this species. This will aid in our understanding of the relationship of this pathogen to other Microsporidia. In addition, this case has shown the potential of teaching subjects to generate research data.

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## Endogenous virus-like elements in redclaw crayfish *Cherax quadricarinatus*

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Viral fragments integrated into host genomes are called endogenous viral elements (EVEs). Endogenisation of viral elements occurs when a double stranded DNA copy of the viral genome is inserted into the germ line of the host. They can potentially be transmitted vertically to the offspring and become fixed in the population of the host. Integration of viral genomes into the host cells involves a broad range of virus families both RNA and DNA viruses and has been reported from various organisms. In decapod crustacea, two endogenous viral sequences have been found including endogenous *Penaeus stylirostris* densovirus-like elements (EPstDNVEs) in *Penaeus monodon* and endogenous white spot syndrome virus-like elements (EWSSVEs) in *P. monodon* and *P. japonicus*. This study reported for the first time the presence of endogenous virus-like elements in freshwater redclaw crayfish *Cherax quadricarinatus*.

Histological examination and polymerase chain reaction (PCR) were conducted. Nine endogenous Brevidensovirus-like elements (EBreVEs) were identified in *C. quadricarinatus* from different sources suggesting the widespread nature of these elements in the redclaw in northern Queensland, Australia. These endogenous virus-like elements shared nucleotide identities (70–100%) and amino acids similarities (34–100%) with infectious hypodermal and haematopoietic necrosis virus (IHHNV), currently referred to as *Penaeus stylirostris* densovirus (PstDNV). They may not have originated from IHHNV genomes, but could be derived from another uncharacterised member of the genus Brevidensovirus that share nucleotide similarities with IHHNV. The most striking feature of EBreVEs was that in each case, the segment (portion) of viral sequences inserted into the host genomes was from the same region of the viral genome and most likely derived from non-structural protein regions of ancestral virus, but they cannot be assembled into one consensus sequence. The EBreVEs may be inserted into the redclaw genomes following chronic or persistent infection by a corresponding virus that may have occurred as multiple independent integration events years ago leading to the accumulation of several integrated elements in their genomes. These insertions may have a protective function to the host.

*Keywords*: redclaw crayfish, endogenous virus-like elements, nucleotides, amino acids, consensus sequences.

## From Research to Development: Pioneering the Use of Aquaculture Vaccines in Australia

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Up until the mid-1980s, farming of salmonids in Australia was limited to small freshwater operations producing plate-sized rainbow trout. This niche industry was transformed in the space of 25 years to become a major agribusiness based on marine farming of Atlantic salmon and rainbow trout. Centred in Tasmania, production in the financial year 2011-12 was valued at over $500m with industry plans to increase production to $1b by 2020.

When marine farming of salmonids commenced in Tasmania, almost nothing was known about the range of endemic aquatic bacterial or viral pathogens that might impact production and the viability of the industry. In 1985, the Fish Health Unit, working closely with the nascent salmonid industry, implemented health surveillance programs as a means of determining and assessing causes of disease. These programs have yielded data that has led to disease management strategies that underpin the sustainability and profitability of Tasmania's salmonid industry.

Aquatic animal health in Tasmania is viewed as a shared responsibility between the salmonid industry and Government. This perspective enabled the Fish Health Unit to place an emphasis on establishing diagnostic capacity in fish diseases and led to a comprehensive assessment of bacterial and viral pathogens of salmonids that occur in Tasmania. With the continued expansion of the industry, finding effective means to control some of the disease agents became increasingly important. The partnership model between industry and Government formed the basis on which a program of research and development was started that has led to the wide-scale use of vaccines by the Tasmanian salmonid industry.

In 1988, Anguillvac-C©, for the control of Vibriosis caused by *Vibrio anguillarum*, was released by the Fish Health Unit and was the first registered, commercial aquaculture vaccine in Australia. This was followed in 1997 with a second vaccine, Yersinivac-B© for controlling *Yersinia ruckeri* and was followed in 2006 by AnguiMonas, developed in collaboration with Merck Sharpe Dohme, for *Aeromonas salmonicida* biovar Acheron and *Vibrio anguillarum*. This vaccine was the first bivalent formulation with an adjuvant and administered by injection.

The success of these vaccines has led to the development of Corrovac for the Tasmanian *Rickettsia*-like organism and is now being evaluated in field trials. Further research is in progress on the development of viral vaccines for the Tasmanian Aquabirnavirus and Tasmanian Aquareovirus.

The collaborative approach to vaccine development by industry and Government has enabled salmonid growers to be supplied with vaccines designed for salmonid pathogens specific to Tasmania and achieve sustainable production of salmonids under challenging conditions.

**Evaluation of an experimental and commercial state-of-the-art vaccine against enteric redmouth disease (ERM) in rainbow trout by waterborne challenge with *Y. ruckeri* O1 biotype 2**

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In recent years, there has been an increase in reported outbreaks of enteric redmouth disease (ERM) in ERM vaccinated farmed rainbow trout in a number of countries, including the United States and a range of European countries such as Denmark, Spain and the UK, which both affect animal welfare and cause considerable economic losses. These reported disease outbreaks have been associated with non-motile, virulent serovar O1 strains of *Yersinia ruckeri*, classified as biotype 2.

A standardised challenge model has been developed based on a newly isolated and highly virulent *Y. ruckeri* O1 biotype 2 strain obtained from an ERM disease outbreak in a Danish trout farm. This waterborne infection model gives us the opportunity to test and evaluate the effect of commercial and experimental vaccines against *Y. ruckeri* O1 biotype 2.

An experimental vaccine containing equal amounts of *Y. ruckeri* O1 biotype 1 and biotype 2 (whole cell bacterin) was developed, based on the most immunogenic strains from our collection. Rainbow trout were vaccinated by immersion (30sec) or bath (5min) in $1 \times 10^9$ CFU/ml, or given an intraperitoneal (i.p.) injection ($5 \times 10^8$ CFU/fish).

The effect of the experimental vaccine has been compared to a state-of-the-art commercial ERM immersion vaccine (AquaVac® Relera™). Un-vaccinated and sham vaccinated rainbow trout were included as controls. Two months post vaccination the rainbow trout were challenged in duplicate with *Y. ruckeri* O1 biotype 2 by bath. No effect of the experimental immersion or bath vaccine was observed in the present study. However, full protection was achieved with i.p. injection of the experimental vaccine ($p<0.0001$, RPS=100%). Bath vaccination with AquaVac® Relera™ induced a significant, partial protection relative to the control groups ($p=0.002$, RPS=58.5%).

It is suggested that the immunity induced by immersion and bath vaccination is inadequate for protection of the rainbow trout fry, since mortalities are still high in the vaccinated groups, which may explain the incidences of ERM disease outbreaks due to high virulent biotype 2 strains in farms, where the fish are primarily immersion vaccinated.

## Effect of inactivation method of *Yersinia ruckeri* on the efficacy of single dip vaccines

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*Y. ruckeri* is a pathogen which causes significant losses in farmed salmonids. *Y. ruckeri* is the causative agent of both enteric red mouth disease in rainbow trout in the Northern Hemisphere and yersiniosis in Atlantic salmon (*Salmo salar*) in the Southern Hemisphere. Currently, Yersinivac-B, a commercial bacterin-based vaccine manufactured by MSD Animal Health and prepared from formalin killed whole-cells, is used for immersion vaccination against *Y. ruckeri* for most Tasmanian Atlantic salmon. Until recently, these fish were vaccinated once by bath immersion at 5g. However, half a million vaccinated juvenile Atlantic salmon died of yersiniosis in a single Tasmanian hatchery in 2007. In this study we evaluated different inactivation methods of *Y. ruckeri* on the efficacy of single dip vaccines including formalin inactivation, ammonium sulphate inactivation, and pH-lysed then formalin treated inactivation.

Fish from each treatment were sampled before vaccination (time 0) and at week 4, week 6 and week 12 post-vaccination for blood collection and were challenged at week 12 post-vaccination. The unvaccinated fish showed a significantly lower survival rate than the three vaccinated fish and the positive control fish. The mortality in vaccinated fish varied from 3.30% to 9.38%, giving the RPS of 93.4% for the fish vaccinated with ammonium sulphate inactivated vaccine , 85.4% for fish vaccinated with formalin inactivated vaccine and 81.3% for fish vaccinated with pH-lysed then formalin inactivated vaccine. Interestingly, serum antibodies measured by ELISA and bacterial agglutination were present only in the injection vaccinated fish.

The results of this study have shown that ammonium sulphate can be used to inactivate *Y. ruckeri* and can replace formalin inactivation that is the most commonly used in vaccine preparations. Ammonium sulphate that is useful to precipitate, preserve and purify protein was successfully used for *Y. ruckeri* inactivation and has a potential for bacteria inactivation for fish vaccines.

## Oral and anal vaccination against enteric red mouth disease protection against yersiniosis

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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 and anally vaccinated fish. The objective for this project was to investigate whether oral and anal vaccination of rainbow trout against *Yersinia ruckeri* O1 (biotype 1) causing Enteric Red Mouth disease (ERM) could protect rainbow trout against a subsequent experimental bath challenge. The rainbow trout were given oral vaccinations with AquaVac™ ERM Oral vet. (MSD animal health) or an experimental vaccine based on formalin killed *Yersinia ruckeri* O1, (biotype 1) bacteria. Eight groups were studied: 1) Control group (no vaccination, no infection), 2) infected control, 3) experimental vaccine, 4) experimental vaccine w/ booster (4 months post primary vaccination), 5) Experimental vaccine w/ 50 times higher dose, 6) AquaVac ERM (as a primary vaccine), 7) AquaVac w/ booster, 8) and one group which received a low bacteria dose of the experimental vaccine anally, as a primary and booster vaccination. 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 groups which received the experimental vaccine anally and at a fifty times higher concentration. These results showed that a low dose of ERM bacterin fully protected rainbow trout when they were vaccinated anally. Oral vaccination could also induce full protection but the dose of the bacterin had to be 100 times higher than if the fish was to be vaccinated anally. This indicates that much of the oral feed bacteria is digested in the stomach of rainbow trout. This work has shown that it is possible to vaccinated orally against ERM, but the bacteria has to be coated in order to avoid digestion. Protection mechanisms will be discussed.

## Use of a recombinant protein from the amoebae *Neoparamoeba perurans* as a vaccine candidate against AGD in Atlantic salmon

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Amoebic gill disease (AGD) is the main disease affecting salmonids in Tasmania, Australia. The aetiological agent is *Neoparamoeba perurans* and the clinical presentation produces severe mortalities if not treated. The current treatment (freshwater bathing) decreases the number of amoebae on the gills, but since it has to be repeated several times it is costly for the industry and stressful on the fish. The development of a vaccine remains a high priority for the local industry.

Previous work identified a Mannose binding–like protein (MBL) in *N. perurans*, similar to attachment factors of other amoebae, suggesting that by interfering with this MBL and blocking attachment of *N. perurans*, the severity of AGD could be reduced.

Fish were immunized with this vaccine candidate using two different delivery strategies. Both systemic and mucosal antibody responses were measured using ELISA for a period of 12 weeks before challenge. Additionally, the production of antibodies by local cells in mucosal tissues was also measured using tissue explants obtained from gills and skin at the end of this period. Both vaccination regimes induced an increase in the production of antibodies against the antigen at the mucosal level during the 12 weeks.

Following this period, fish were challenged with the parasite for 7 weeks. Survival curves did not show any definitive trends and therefore, antibody levels -both systemic and mucosal - were assessed by ELISA. Additionally, gill samples from fish vaccinated using different methods were evaluated for AGD severity - expressed as percentage of filaments affected - and the amoebae were quantified in individual fish using a DNA extraction technique combined with a highly sensitive real-time PCR assay.

This study provided insight into the physiological effects and survival outcomes of a novel vaccine candidate against *N. perurans* and represents an initial step into the development of an efficient vaccine against AGD.

## The comparative susceptibility of four Tasmanian endemic fishes and Atlantic salmon to experimentally induced amoebic gill disease

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Since the first reported outbreaks of amoebic gill disease (AGD) the condition has been primarily of concern to producers of Atlantic salmon, *Salmo salar* (both domestically and now globally). AGD has been reported in some non-salmonid (cultured) temperate species including turbot (*Scophthalmus maximus*), sea bass (*Diplodus pintazzo*), sea bream (*Dicentrarchus labrax*) and ayu (*Plecoglossus altivelis*). Anecdotal and published evidence regarding the susceptibility of Tasmanian endemic species has been somewhat contrasting and lacking systematic investigation. Earlier, unpublished findings had indicated the presence of both *Paramoeba* sp in wet smears and culture plates taken from the gills of couta (*Thrysites atun*), a single bastard trumpeter (*Latridopsis forsteri*), sand flathead (*Platycephalus bassensis*) and mullet (*Aldrichetta forsteri*) displaying gross gill lesions presumptively diagnostic of AGD. Histologically, AGD was detected from one of 12 endemic fish (three species) opportunistically sampled from a salmon cage immediately prior to AGD treatment. However, during 1999, a histological survey of 325 wild fishes (comprising 12 species) collected around and within salmon cages failed to identify AGD or the causative agent within those samples. The authors also suggested that AGD could not be experimentally induced in flounder or seahorses. Striped trumpeter farmed on a trial basis in the Huon Estuary (within AGD prone leases) during 2008/9 did not present with any mortality attributed to AGD, however these fish were not examined directly for presence of amoebae or signs of disease.

For this study, four endemic fish species were captured and exposed to *N. perurans* under experimental conditions in separate trials simultaneously with Atlantic salmon. Four short duration (10 days) screening trials were undertaken with Australian salmon *Arripis trutta*, yellow eye mullet, purple wrasse *Notolus pucicola* and southern sand flathead. A further challenge trial of extended duration (28 days) was subsequently undertaken using yellow eye mullet cohabited with Atlantic salmon.

*N. perurans* trophozoites were associated with hyperplastic changes in the gills of tested native species, however the intensity of infection (as indicated by the relative abundance of lesion-associated amoebae) and subsequent disease expression were substantially lower than Atlantic salmon housed within the same recirculating water. Upon native fish gills, *N. perurans* trophozoites often displayed morphologies consistent with morbidity and were often "detached" from gill lesion surfaces. Eosinophilic leucocytes were often noted within and upon lesions associated with amoebae and were observed in close association with amoebae upon lesion surfaces. In many cases hyperplastic lesions showed signs of resolution as indicated by the recruitment of mucus cells and/or retraction/reduction of hyperplastic tissues proximal to the filamental core. Yellow eye mullet were the least affected by exposure to *N. perurans* where the prevalence and pathological severity of AGD in exposed fish were substantially lower than in Atlantic salmon. Additionally, AGD could not be diagnosed in this species histologically at 21 or 28 days post-exposure to amoebae despite early disease signs appearing at 3, 7 and 14 days post-exposure and an ongoing infection pressure being present for the duration of the experiment.

## A cross-species approach to functional feed development for Amoebic Gill Disease

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Amoebic Gill Disease (AGD) occurs in Atlantic salmon farmed in South-Eastern (SE) Tasmania, a zone responsible for 75% of the industry's current 50,000t annual salmonid production. For 25 years the only commercially viable method of AGD control has been freshwater bathing, a process that is repeated at intervals throughout the production cycle to reset the infection. The labour and infrastructure required for bathing adds $1.50/kg to the cost of every Atlantic salmon produced in the SE, resulting in an annual burden of $50m to the industry's bottom line. AGD has recently emerged as a significant cause of farmed salmon mortality in Ireland, France and Scotland and has also been reported in Norway, Chile, Spain and North America. In the space of a few years AGD has progressed from a problem measured in the millions of dollars in Australia to a potential billion dollar problem affecting the World.

There have been many attempts in Australia over the years to mitigate the effects of AGD by means other than freshwater bathing, and concurrently much effort invested on the part of researchers in understanding the complex biology of the disease. Since 2011 CSIRO has sought to synchonise these fundamental and applied aspects by taking a fresh line of enquiry into the mechanisms of AGD resistance, followed by incorporation of the specific components conferring resistance into experimental AGD treatments.

We report on the search for AGD resistance machinery in a) selectively bred Atlantic salmon, b) a range of native fish species endemic to SE Tasmania and c) in other salmonid species. We report on the development of an *in silico*, *in vitro* and *in vivo* screening pipeline for discovery and rationalization of target compounds. We report on the production of an experimental anti-AGD diet based on these findings and our early experiences with this approach. We discuss future work in this area and related areas through the potential harnessing of other compounds and approaches to ultimately transfer knowledge gained about AGD resistance to the Tasmanian Atlantic salmon industry and the international market.

## Immune gene expression in Atlantic salmon (*Salmo salar* L.) affected by AGD

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The amoebic gill disease, AGD is an ectoparasitic infection of fish gills due to infestation with the marine protozoan *Neoparamoeba perurans* (Amebozoa: Dactylopodida) which causes epithelial hyperplasia, lamellar fusion, formation of interlamellar crypts and an excessive production of mucus in fish farmed in the marine environment. This disease has a negative impact on the production of Atlantic salmon, *Salmo salar*, in Tasmania, Australia, and has been isolated throughout many different places worldwide including Ireland, Chile, Spain, the US and Norway. If the infected fish are not treated, AGD can cause mortalities of over 50%. In Tasmania AGD contributes to approximately 20% of production costs that can be attributed to freshwater bathing, the only effective treatment.

The primary aim of this study is to investigate the immune host response in the Atlantic salmon so as to better understand the mechanisms of the disease. The identification and characterization of numerous fish cytokine and immune-regulatory genes in recent years has facilitated the study of gene expression during disease processes and is now considered to be essential for the elucidation of host immune defense mechanisms.

Atlantic salmon were inoculated with *N. perurans,* previously isolated from the gills of AGD–affected Atlantic salmon, and gill, liver, anterior kidney and spleen tissue sampled at 0, 5, 10 d post inoculation from control (AGD–unaffected) and AGD–affected fish. To investigate the gill tissue a 2 mm biopsy punch was carried out on a selected area of the second gill arch. A qRT-PCR was performed on the punched gill samples to characterise selected immune response genes, particularly to identify gene expression relative to lesions and no lesions areas on the same sample.

## *In vitro* assessment of gill function in AGD-affected Atlantic salmon, *Salmo salar*, gills

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AGD is the most significant health issue affecting the production Atlantic salmon in Tasmania with estimated costs to the industry of up to 20% of total production. Outbreaks of this disease are more frequent in the summer months however with increasing water temperatures, bathing frequency and associated costs appear to be extending into traditionally AGD-free periods such as spring and autumn. Mitigation of these costs is essential to maintain the profitability of the industry. As such, AGD has been noted as a clear research priority.

Although the pathophysiological effects of AGD have been previously investigated, these in vivo based studies were highly reliant on lightly affected fish with minor AGD induced gill pathology and the ability to survive surgical and/or experimental procedures. Similar studies for heavily affected animals have not been successful therefore the physiological cause of AGD associated mortality has remained elusive. For this research an in vitro perfused gill preparation was developed specifically for AGD research. Manipulation of the model to varying external (surrounding medium) and internal (perfusate) conditions then allowed direct examination of gill function in heavily affected gills. Knowledge regarding gill function in AGD affected fish will ultimately provide industry with a more in-depth and thorough understanding of AGD pathophysiology. Understanding the physiological mechanisms of AGD related mortality could offer alternative avenues for AGD treatment. The results of this research will be discussed.

**Culture and trialling of cryopreservation techniques for *Neoparamoeba perurans*, the causative agent of amoebic gill disease in marine-farmed Atlantic salmon, *Salmo salar***

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Amoebic gill disease (AGD) caused by *Neoparamoeba perurans* is rapidly becoming a serious health issue for Atlantic salmon culture industries worldwide. The disease has been reported in virtually all countries where salmon are farmed, with the exception of Canada, which makes the development of techniques to deal with the disease an imperative. Important steps in this process are the successful culture of the virulent amoeba and the application of techniques to cryopreserve the organism.

*N. perurans* has now been maintained in culture at University of Tasmania in Launceston for more than 2.5 years. Initially virulence of the cultured amoeba was shown to be maintained after 5 months and subsequent infection trials have shown that the cells still cause AGD after 2 years in culture. The culture procedure is routine but time consuming in that weekly sub-culturing is required to minimise the risk of overgrowth by contaminating organisms. Incubating cultures at a range of temperatures (10, 14 and 18°C) showed differences in the growth rates of the amoeba. At 10°C growth of the amoeba and contaminants was markedly slower and extended the period between subculture to at least 3 weeks.

Cryopreservation techniques based on the protocol form the American Type Culture Collection (ATCC) have been successfully applied to other species of *Neoparamoeba* but not as yet to *N. perurans*. Previous attempts using the ATCC protocol on cultured and freshly isolated *N. perurans* have not been successful. Numerous attempts using variations to the ATCC and other cryopreservation protocols have been attempted and the results will be discussed.

**Towards the application of RNA interference (RNAi) against Amoebic Gill Disease (AGD) of farmed Atlantic salmon (*Salmon salar*)**

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RNA interference (RNAi) is a natural regulatory mechanism of most eukaryotic cells that uses small double-stranded RNA (dsRNA) molecules as triggers to direct homology-dependent control of gene activity. Since its discovery, RNAi has emerged as a powerful tool for rapid analysis of gene function in non-model organisms and has the potential to identify candidate drug targets against diseases of economic importance to aquaculture.

With regards to amoebic gill disease (AGD) of farmed Atlantic salmon, RNAi could become an invaluable research instrument to unravel the role of proteins essentially involved in amoeba attachment and pathogenicity, as well as to validate important treatment targets by investigating the effect of specific gene knockdown on amoeba survival. Additionally, RNAi technology could greatly assist on the elucidation of possible factors associated with the loss of virulence in certain species from the *Neoparamoeba* genus.

However, before RNAi technology can be employed in *Neoparamoeba*, it is important to consider whether members of this genus possess the required set of proteins involved in the RNAi pathway. As a result, the main purpose of the present study was to use functional and comparative genomics approaches to investigate whether functional RNAi machinery has been retained or lost in species from the *Neoparamoeba* genus. The gene knockdown experiments were performed using the closely-related *Neoparamoeba pemaquidensis*, as the *in vitro* culture of *Neoparamoeba perurans*, the aetiological agent of AGD, has only been achieved recently and was not available for this work.

Using a *N. pemaquidensis* and *N. perurans* transcriptome database we were able to identify putative proteins containing conserved domains of RNAi-related genes, such as Dicer and Argonaute. Their involvement in the RNAi pathway was validated by assessing their levels of expression followed the administration of dsRNA and small interference RNA (siRNA), respectively. The presence of an active Dicer in both species was also corroborated by the RNAse III assay, which showed complete degradation of dsRNA following incubation in amoeba lysate. Further evidence for the presence of active RNAi machinery was also supported by gene silencing experiments where *N. pemaquidensis* specific genes were successfully downregulated by the administration of RNAi-trigger molecules. However, knockdown efficiency was dependent on dose, target gene, delivery method and RNAi molecule.

The results altogether provide strong evidence for the presence of functional RNAi machinery in *Neoparamoeba* spp. and suggest novel means for management of AGD in the future.

## DAFF-FRDC Aquatic Animal Health Training Scheme

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The 2010-2012 Aquatic Animal Health Training Scheme was funded by the Australian Government Department of Agriculture, Fisheries and Forestry (DAFF) and the Fisheries Research and Development Corporation (FRDC). The scheme was administered by the FRDC People Development Program, with a total budget of $237,000.

The scheme aimed *"to improve the knowledge and skills in aquatic animal health management to support Australia's fishing and aquaculture industry, including the aquarium sector".* People directly involved in fishing and aquaculture industries, with a specific aquatic animal health component in their role were eligible to apply for funding under the scheme.

18 applicants were awarded funding under the scheme, with 11 projects for individual training and 7 projects for group training. A total of 142 people received training under these projects. Funded projects provided training on aquatic animal disease diagnostics, epidemiology, outbreak investigation, up-skilling of vocational trainers, aquatic animal health management training for industry, fish pathology, molecular biology techniques, and aquatic animal veterinary medicine. Aquatic animal health sector professionals from industry, government, research and educational organisations participated in training delivered under the scheme.

The 2010-2012 Aquatic Animal Health Training Scheme provided valuable training opportunities for Australia's aquatic animal health professional community and met its aim to improve the knowledge and skills in aquatic animal health management to support Australia's fishing and aquaculture industries. These opportunities have enhanced Australia's collective ability to effectively manage aquatic animal diseases.

Following review of the 2010-2012 Aquatic Animal Health Training Scheme, DAFF and FRDC have agreed to continue the scheme for a further two years. Application dates for the first round of funding will be announced at this conference.

## The Neptune Project - A comprehensive database of Australian aquatic animal pathogens and diseases

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Biosecurity is an increasingly important concern for the growth and productivity of aquaculture. Rapid growth in global trade of aquatic animals and the expansion of aquaculture make the introduction and spread of aquatic animal diseases a major issue in Australia and worldwide. Information on aquatic animal diseases is evolving rapidly, and resources for collating and sharing this information is needed for industry, regulators and scientists.

"Neptune" is a project aimed at enhancing the availability and exchange of biosecurity information. The project accomplishes this by providing a unique web-based knowledge store with information on all known aquatic animal diseases and pathogens reported in Australia. This centralised repository facilitates interactions between industry, biosecurity officials, research laboratories and pathologists. Neptune is available online via the Australian Biosecurity Intelligence Network (ABIN). The current version provides detailed, searchable host and agent information, maps displaying host and agent distribution across Australia, and supplementary information including disease images.

The database also incorporates whole microscope slide imaging to facilitate remote diagnostics, aquatic animal pathology teaching and expert discussions, regardless of geographical location. One hundred and eighty histology slides have being collected from aquatic pathology experts around Australia and digitalized using Ultra-Resolution Digital Scanning, which allows advanced visualization capabilities of true-colour digital whole slide images, where any region of interest can be instantly magnified up to 400x.

The Neptune project was initially funded through the National Collaborative Infrastructure Strategy (NCRIS), with support from the Queensland Department of Agriculture, Fisheries and Forestry. Neptune is now being funded by the Fisheries Research and Development Corporation and the Australian Government Department of Agriculture, Fisheries and Forestry, with contributions from the Queensland Museum. The current funding is being used to improve the facility in several respects. The user interface will be upgraded to streamline data searching, viewing, and data entry. While the digital microscopy slide image library contains examples of endemic aquatic animal diseases, a digital microscopy slide image library for significant exotic aquatic animal diseases is absent, and will be established. A program of user activities and training will also be implemented throughout 2013. Collectively, these features will facilitate learning, training and development of Australia's biosecurity capacity.

## Aquatic Animal Health Technical Forum (AAHTF) - Current Status

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The Aquatic Animal Health Technical Forum (AAHTF) was established to provide aquatic animal health technical staff opportunities to interact, establish networks, exchange knowledge and information, and share expertise to enhance Australian diagnostic laboratories' capability to provide high quality health services to stakeholders in the aquatic sector.

Initially, with funding from FRDC, an inaugural workshop was held at CSIRO-AAHL in 2009. Following the success of this first workshop further funding was obtained for continuation of development of the AAHTF and a second workshop hosted by DPIPWE in Launceston Tasmania in 2011. Recently, a further three-years' funding to continue the AAHTF has been obtained and the 2013 workshop was held in March in Glenelg, South Australia.

The 2013 workshop had 23 participants from various Institutes and organisations in Australia and New Zealand. The workshop was conducted over three days at venues in Glenelg, The University of Adelaide - Roseworthy Campus, and the South Australian Research and Development Institute (SARDI), West Beach. Participants presented aspects of their work during the workshop. Presentations at the Roseworthy Campus and SARDI were made by experts in the aquatic animal health field. These venues were able to provide the group with a tour of both facilities also including a practical session on fin fish dissections.

## Towards the development of a national aquatic animal health curriculum for tertiary institutions

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Trained professionals in aquatic animal health are needed to support the continued development and sustainability of the aquaculture sector within Australia. The productivity and marketability of the aquaculture and fishing industries depend directly on the capacity to promote, monitor, manage, research, and regulate the health and welfare of a wide range of species in a wide range of aquatic environments. Currently training opportunities and resourceful corporate knowledge exist across the country covering only a subset of the core attributes required to meet industry, government and service needs. It is necessary to review available training capacities to develop a standard national curriculum to educate relevant veterinarians and other aquatic health professionals.

This presentation will facilitate the further discussion about a national curriculum by helping define what is meant by a national curriculum and exploring some of the possible outputs and applications of a nationally designed and developed curriculum.

## Gill Diseases of Fish

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Gill diseases are one of the most important categories of diagnoses in a routine diagnostic laboratory, often accounting for approximately 50% of the case load. This talk will provide a general overview of gill diseases in fish including commercially-reared species, in both freshwater and the marine environments. Topics covered will include those areas of basic structure and function needed to understand some aspects of pathophysiology, and therefore what is the best way to approach controlling the disease and limiting any deleterious effects on the host.

Following this will be a section on how the gills respond to injury in a general sense and to a range of insults. Illustrations will include gross, biopsy, routine paraffin sections, as well as scanning and transmission electron microscopy. Diseases used as examples to illustrate various processes will include bacterial infections such as bacterial gill disease (BGD caused by *Flavobacterium branchiophilum*), water quality parameters, viral, and parasitic disease. Particular attention will be paid to BGD, and to the pathogenesis of the experimental disease. I will show that the basis of the disease probably lies in a vasoconstriction of afferent branchial vessels due to a prostaglandin-based mechanism.

## Gill lesions in Murray cod *(Maccullochella peelii)* raised in farm dams

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The Murray cod (*Maccullochella peelii*) is a large perciform fish native to the Murray –Darling basin. Cultivation of these hardy fish in farm dams as a secondary enterprise is growing in popularity but little is known of the relative importance of disease and management factors in the success of these endeavours.

We are undertaking a surveillance project looking at fish health and farm management in a selection of farms across three states. Fish are sampled monthly, or when a disease outbreak occurs, and sacrificed for autopsy. Kidneys and any lesions seen are submitted for bacterial culture and a full range of tissues is examined histologically.

Gills are the most commonly diseased tissue, with parasitism and possibly on farm treatment protocols being the most common suspected causes of lesions. We present here a range of gill lesions seen and discuss their relationship to fish production.

## South Australian marine mortalities, summer 2013: An overview

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South Australia experienced an ongoing and widespread marine mortality event during the summer of 2013. Large numbers of dead fish in Spencer Gulf were reported on 3 March 2013. Over the next 2 months, numerous more reports were phoned in (FISHWATCH hotline) from across the State. These included fish (mostly small-bodied benthic species) and abalone kills, over 30 dead dolphins, large amounts of dead seagrass / macro-algae and species observed outside their normal range (e.g. a leatherback turtle).

An investigation was initiated at the first fish kill report, with data suggesting that the unusually high, fluctuating, water temperatures and evidence of elevated harmful algae concentrations were the likely cause. A similar 'marine heat wave' event was reported off Western Australia during 2010/11 (Pearce et al, 2011). However, public and media speculation fuelled conspiracy theories including desalination plants, underwater volcanoes to Fukushima nuclear radiation. An inter-governmental 'task force' was subsequently formed to ensure the separate investigations (fish, abalone, dolphin & water quality) were coordinated and appropriately resourced.

Fish pathology and diagnostic examinations demonstrated prolonged stress (melanomacrophage aggregation in spleens and kidneys) and physical gill damage (proliferative branchitis, focal gill lesions, mucous cell hyperplasia and haemorrhagic inflammation). These pathologies would have impeded respiratory physiology of fish, compromised the immune system ultimately exposing some (weaker) fish to the observed terminal bacterial septicaemia. Pathology was mostly unremarkable in the abalone examined but pedal abscesses associated with bacterial infection was observed in some abalone. To date, there has been no evidence of exotic infectious diseases in either the fish or abalone examined. Diver and underwater video observations found that only a small percent of fish populations were affected.

The unusually high, and variable, water temperature (3-5°C above the historic average), was likely the primary factor in this marine mortality event. The plume of warm water across the State (satellite imagery) corresponded with the kill locations. Harmful (abrasive) algae (*Chaetoceros coarctatus*) were observed in water samples and likely contributed to fish mortalities. A separate investigation (Biosecurity SA and Department of Environment, Water & Natural Resources) into dolphin mortalities determined a morbillivirus outbreak as the underlying cause of mortality.

## Common problems with fish blood samples, haematology, biochemistry and feed analysis

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It is a simple procedure to sample blood from fish which offers the possibility of providing a huge amount of extra information on the physiology, biochemistry and pathogenesis of disease in fish. However, there are significant issues faced with the handling and storage of blood samples from fish compared with the comparable samples from mammals. Some of the difficulties include:

- Clotting of whole blood, plasma and serum. Time delays between collection and analysis exacerbates the problem. Packed cell volume, leucocrit and plasma protein are useful and easily obtained data when faced with ongoing fish disease or ill thrift. Clotting or partial clotting of samples, however, is a major problem if samples are not analysed the same day or were obtained from a rapid, single venipuncture.
- Modern analytical equipment heats blood to 37°C which may affect the validity of results in fish and also makes publication of results difficult.
- Poor repeatability of biochemistry results. Repeat analysis of the same samples has given vastly different results in our laboratory. Serum was found to be the most stable sample.
- Difficulty in obtaining statistically significant results because of large variations in results from samples of a single population. Creatine kinase results from fish with myopathy will be discussed.
- Mammalian enzyme assay kits may not be valid for the species of fish. Creatine kinase isoenzymes will be used as an example of the potential difficulties encountered with enzyme assays.
- Few reference values are available for fish and published papers often quote vastly different values even for the same species. For example there is huge variability in published yellowtail kingfish haematocrits.
- Methods of analysis used in the mammalian field often cannot be extrapolated to fish. Problems with analysis of rancidity, vitamin E, and taurine content of fish feeds will be discussed.

The presentation will outline some specific problems encountered by this researcher over the years and discuss some lessons that were learned.

## Yellowtail Kingfish (*Seriolae lalandi*) Taurine Deficiency - A Diagnostic Case Study

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In 2009, Yellowtail Kingfish (YTK) stock on a commercial farm experienced chronic elevated mortalities across multiple production sites and all cages. In addition to deaths populations had poor food conversion ratio (FCR) and slow growth rates, causing substantial production loss across the farm.

Diagnostic tests were used to further define the tissue changes associated with the disease in two separate year classes of fish. Initial histopathology findings in 2010 and early 2011, described proliferative lymphocytic enteritis, typhlitis, vacuolation of hepatocytes and occasional areas of necrosis and dysbiota of the gut. Large numbers of moribund fish were examined by histology throughout 2011 which defined a myriad of infectious pathogens to be present in variable intensities including: enteric coccidia-like organisms; enteric myxidia-like organisms; renal myxosporeans; *Paradeontacylix* spp.; *Zeuxapta seriolae*; *Benedenia seriolae;* and *Photobacterium damselae ssp damselae.*

Further diagnostics including viral cell culture and electron microscopy were instituted to determine the presence and extent of involvement of infectious pathogens. All provided negative results. Multiple clinical trials were instituted to target suspected pathogens, which failed to resolve the continuing losses. Non-infectious diseases were also considered. Water quality and toxic algae were ruled out through testing. Nutritional factors were suspected to be involved, associated with two different feed companies.

In 2012, a CRC workshop hosted a collection of international specialists and diagnosticians involved in the case to reviewed the collated data and overlay international experience and published studies. A presumptive diagnosis of taurine deficiency was reached. Further clinical trials were conducted to confirm this hypothesis through top-coating diets with crystalline taurine.

The clinical response to treatment in all affected populations, at two separate locations >100km apart was a spectacular decline in mortality. The response was observed when taurine was top-coated, on either of the feed formulations which had been in use. Other clinical indicators including gross pathology, histopathology, and clinical pathology (haematocrit and liver taurine) were all strongly supportive of the presumptive diagnosis of primary taurine deficiency.

*S. lalandi* appear to have a high essential requirement for dietary taurine, similar to their related species *S. quinqueradiata.* Taurine is considered an essential amino acid for YTK, and is essential for the conjugation of bile acid and endogenous production of cholesterol. Bile acids are made from cholesterol within the fish. A step in the process involves amidation with taurine, which produces a highly soluble molecule at pH 6-7. The molecule is large, and is not readily resorbed, allowing it to accumulate in the small intestine, and perform its digestive functions.

A deficiency in taurine therefore, can result in reduced bile acid production, thereby impairing digestion. Subsequently derangements in lipid metabolism and enteric dysbiosis can be promoted. This can potentially lead to the impairment of uptake of long-chained fatty acids and the precursors for the endogenous production of cholesterol.

Inadequate cholesterol uptake and insufficient endogenous production can compromise the membrane stability of red blood cells, resulting in a haemolytic anaemia, demonstrated by low haematocrits in affected stock. (Takagi, et al., 2006) demonstrated the induction of a haemolytic anaemia due to taurine deficiency in *S. quinqueradiata.*

Taurine deficiency is also linked to several other clinical signs described in the affected population of fish, including the clinical presentation of green livers, thickened intestines, and low body condition. Further nutritional work is necessary to determine an optimal commercial diet for farming *S. lalandi.*

## An unusual keratitis and uveitis in farmed barramundi: History, histopathology and cause

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In the summer of 2012 five thousand juvenile barramundi (9-10 cmTL) were stocked into a freshwater pond at a time of high water temperatures (>32°C). From the onset of stocking around 100 fish died each day, fish presenting with one or two protruding eyes. Some fish had a pale, soft growth on the tongue and some fish had ruptured eyes. Half of the group of juvenile barramundi remained at the submitting facility (hatchery 1). When these fish were checked around 2% of fish were reported with one or two abnormal eyes, but there were few mortalities. These juvenile barramundi had been bred and reared in another facility (hatchery 2); arriving at the submitting facility (hatchery 2) 17 days earlier. Two months later, a second group of 15,000 barramundi fingerlings, originating from the same breeding facility (hatchery 2), were transferred to the submitting facility (hatchery 1) for nursery rearing and on-growing. These fish had a slowly increasing mortality of around 60 per day. At this time the typical abnormal fish had signs of irritated gills and abnormal eyes.

Fresh and preserved barramundi were submitted for diagnostic testing. A description of the histopathological and microbiological results will be presented. The first group of juvenile barramundi from the pond had a gill lamellae epithelial hypertrophy and some bacterial proliferation between gill filaments. There was a deep, granulomatous mycosis in the sub-epithelial tissues of affected tongues. Eye lesions were present in juvenile barramundi from the pond and in hatchery reared juveniles. The eye lesions ranged from a peripheral mononuclear cell infiltration into the corneal stroma with a little capillarisation through to rupture of the cornea and complete disruption of internal eye architecture. Except in the ruptured eye, no bacteria or fungi were apparent from histological examination of the eye lesions. The second group of barramundi fingerlings had eye lesions which involved the cornea and iris; again beginning with a peripheral mononuclear cell infiltration and capillarisation together with the appearance of melanin-containing pigment cells in the outer layers of the cornea. Some of the barramundi fingerlings had a bacterial enteritis and most had a generalised hypertrophy of the gill lamellae epithelium. The development and morphology of the eye pathology present in both groups of barramundi will be detailed. The underlying disease processes present in these fish will be briefly discussed.

## Design and reporting of validation studies for diagnostic assays used for detection of aquatic animal pathogens: Are improvements necessary?

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Accurate diagnostic tests are central to the detection, control and prevention of spread of pathogens in aquaculture. The World Organisation for Animal Health (OIE) specifies that tests in terrestrial and aquatic species should be validated as "fit for the intended purpose(s)". The OIE publishes guidelines for researchers, diagnosticians and test-kit manufacturers interested in designing studies to validate existing or novel tests to stages 1, 2, 3 or 4 of the OIE pathway (http://www.oie.int/international-standard-setting/aquatic-manual/access-online/).

The Standards for Reporting of Diagnostic Accuracy (STARD) statement (www.stard-statement.org) was published to encourage complete and transparent reporting of key elements of test accuracy studies in human medicine. The statement was motivated by widespread evidence of bias in test accuracy studies and the finding that incomplete or absence of reporting of items in the STARD checklist was associated with overly optimistic estimates of diagnostic sensitivity and specificity. Although STARD principles apply broadly to test validation studies in all species, the guidelines do not account for unique considerations in aquaculture such as use of experimental challenge studies, a diverse group of testing purposes, epidemiological units and sampling designs, and lack of a perfect reference tests.

To assess how well tests have been validated for important finfish diseases, recent validation studies published in peer-reviewed journals were evaluated according to the STARD 25-item check list for reporting quality. Based on a Pubmed, Scopus and Web of Science search of papers published between 2006 and 2011, 17 test evaluation studies were identified. Six studies were excluded from further evaluation because they were limited to evaluations of analytical sensitivity and specificity, repeatability, and/or reproducibility without consideration of diagnostic performance. Ten of 11 studies were for viral infections and all studies involved evaluation of PCR (sometimes with other tests) with or without a reference tests. Four authors used latent class analysis to account for imperfect reference tests. We independently reviewed the 11 papers according to the STARD checklist. Important deficiencies included: failure to include sensitivity and specificity in the keywords, title or abstract; omission of critical details on populations and sample collection; lack of a clear definition of test purpose and intended application and hence, selection of samples for the validation process that may have been unrepresentative of the populations in which tests were likely to be applied. Most studies failed to mention blinding, training and experience of testers, and many failed to do side-by-side comparisons of available tests on the same set of field samples.

A modified version of STARD is needed to improve reporting quality of finfish test validation studies These guidelines would be helpful for authors submitting papers, for reviewers and journal editors during the review process, and ultimately for systematic reviews and meta-analysis. Modifications need to address data from both experimental and field studies.

**Development and validation of a specific immunohistochemistry method for the detection and localization of *Streptococcus agalactiae* infections in fish tissues**

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*Streptococcus agalactiae* also known as group-B streptococcus (GBS) cause mortalities in a wide range of wild and cultured fish throughout the world. In Australia since 2008, GBS has been an emerging cause of mortality in several wild marine fish and stingrays including grouper, mullet, catfish, and estuary rays. In many cases, GBS was isolated in pure culture from eyes and internal organs.

In order to understand pathogenicity and to aid diagnosis, specific detection systems that can be used in histological sections are very informative. Two methods were evaluated. Fluorescent *in situ* hybridization (FISH) based on detection of specific sequences of the 16S rRNA and immuno labelling using antibodies to target specific antigens at the sub-cellular level on sections of biological tissue via specific epitopes.

The FISH approach proved to be unsuitable for the detection of *S. agalactiae* on formalin-fixed and paraffin-embedded organs. Gram-positive bacteria such as *S. agalactiae* need an additional enzymatic treatment to open the thick peptidoglycan layer, in order to allow access of the probes to its target 16S rRNA within the bacterial cells. Multiple attempts using various enzyme treatments and varying formamide stringency failed to give satisfactory results.

Alternatively, immunohistochemistry (IHC) was optimised using a readily available commercial polyclonal antibody that reacts with type-specific carbohydrate on the surface of GBS. Due to high level of auto-fluorescence of fish tissues, when excited at wavelengths in the green, yellow and orange portion of the visible spectrum, detection of the primary antibody with minimum background was made possible using a commercial photostable secondary antibody coupled with a far-red fluorescent dye.

The IHC approach for the detection and localization of GBS in fish tissues was specific and highly reproducible. Thus both new and archival histological sections from diseased and healthy Queensland groupers, *Epinephelus lanceolatus,* were analysed using this method. Fluorescence imaging was performed with an upright fluorescent slide scanner ideal for processing large numbers of tissue sections at high-resolution.

Pathological analysis of fish infected with *S. agalactiae* displayed consistent pathology including ophthalmitis, exophthalmos, keratitis, meningitis, branchiitis, splenitis, interstitial nephritis, hepatitis, gastritis, enteritis, pancreatitis, peritonitis, myositis and dermatitis with predominant lymphocytic granulomatous inflammatory response, consisting of aggregations of macrophages containing fluorescent coccoid bacteria. Control fish displayed some very minor pathology, but no GBS bacterial cells were detected by IHC in these samples.

## SYBR, TaqMan, or both: Highly sensitive, non-invasive detection of *Cardicola* blood fluke species in Southern Bluefin Tuna (*Thunnus maccoyii*)

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Three species of blood fluke from the genus *Cardicola* are known to parasitize and cause disease in Bluefin Tunas – *C. forsteri*, *C. orientalis*, and *C. opisthorchis*. Although initially believed to be separated by geography and host specificity, recent identification of at least two *Cardicola* spp. concurrently present within all three Bluefin species has raised questions concerning pathogenicity, relative abundance, and distribution of these parasites within Bluefin populations.

Here, we present sensitive and differential real-time qPCR nucleic acid detection of these *Cardicola* spp. by targeting the ITS2 region of the parasite rDNA for PCR amplification. A limit of sensitivity was achieved to be between 1-5 genome copy equivelents for each of the three *Cardicola* species tested without cross-species or host genomic amplification. Similar sensitivity was further achieved in the presence of up to 20 ng/µL non-target host gDNA using SYBR Green chemistry alone, or in the presence of up to 160 ng/µL host gDNA through the utilization of a TaqMan probe common-reporter detection system. These methods were subsequently used to positively identify both *C. forsteri* and *C. orientalis* DNA in preserved samples of serum, gill, and heart from ranched Southern Bluefin Tuna *Thunnus maccoyii*. Both methods were more sensitive for positively and differentially identifying the presence of *Cardicola* spp. than either histological or heart-flush microscopy techniques previously employed, and also possess the ability to be applied in non-lethal blood sampling of these highly-valued fish.

This is the first report for rapid and differential molecular quantitative detection of *Cardicola*, and opens the potential for effective monitoring of infection in cultured bluefin populations. Further, it is anticipated that the use of SYBR Green for melt-curve analyses in conjunction with a common-reporter TaqMan assay will present a flexible, accurate, and cost-effective approach for differential detection of a variety of other pathogens in future.

## Use of real-time PCR assay for detection of *Yersinia ruckeri* and asymptomatic carriers in Atlantic salmon

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*Yersinia ruckeri* is the causative agent of yersiniosis, it was initially isolated from rainbow trout, *Oncorhynchus mykiss* (Walbaum), in the Hagerman valley of Idaho, USA, in the 1950s and different strains and isolates are now widely found in fish populations throughout North America, Australia, South Africa and Europe. Yersiniosis is a contagious disease affecting aquaculture facilities and implicated in large losses of cultured fish. Infection may result in a septicaemic condition with haemorrhages on the body surface and in the internal organs. Different diagnostic methods have been developed for *Y. ruckeri*, including culturing, serological tests and molecular biological techniques where the PCR assay is based on the selective amplification of the 16S rRNA gene. The PCR assay has the advantage of being able to detect low levels of *Y. ruckeri* and provide the possibility to detect asymptomatic carriers, which is very important in order to prevent the transmission and spread of yersiniosis. We monitored *Y. ruckeri* levels in the blood of Atlantic salmon, *Salmo salar,* naturally infected with yersiniosis in Tasmania and determined changes in the bacterial load of *Y. ruckeri* during and after the outbreak. Groups of ten Atlantic salmon naturally exposed to *Y. ruckeri* were sampled and examined every week, in a period of 3 months post-infection, and the samples analysed using PCR.

## Use of Next-Generation Sequencing during the biosecurity response to the detection of *Flavobacterium psychrophilum* in New Zealand

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*Flavobacterium psychrophilum*, the bacterial agent of Cold Water / Peduncle Disease, was recently found in New Zealand during an outbreak of low-level mortality in a Chinook salmon hatchery.

One of the key questions during the biosecurity response was whether *F. psychrophilum* was a recent incursion to New Zealand. To help answer this question, the genome sequence of one of the bacterial isolates was analysed by Next-Generation Sequencing (NGS). Over 66 million basepairs of sequence were obtained which could be aligned to the reference *F. psychrophilum* genome. These data were used to confirm the isolate's identity as *F. psychrophilum*, to analyse its virulence factors, and to perform multi-locus sequence typing (MLST).

MLST analysis showed that the New Zealand isolate had a highly unique sequence type relative to 160 other typed isolates from across the globe. MLST was more informative than sequence comparisons based on individual genes, as phylogenetic trees built from individual gene sequences varied widely from gene to gene.

NGS data was also used to identify changes in virulence factors relative to the reference *F. psychrophilum* genome, including a frameshift mutation in a secreted protease, and an intact collagenase which is commonly disrupted in rainbow trout isolates. In addition, the data provides a great resource for future analysis of new isolates.

The genomic analysis suggested that *F. psychrophilum* was unlikely to be a recent incursion by any of the sequence-typed global strains. This was supported by the discovery of *F. psychrophilum* in other hatcheries, the low levels of mortality, and the lack of reported disease in wild fish in the rivers connected to the hatcheries. Based on this evidence, *F. pyschrophilum* was considered unlikely to be a recent incursion, and therefore not of significant concern.

## MALDI-TOF for bacterial identification and application to strain typing of *Vibrio harveyi*

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Traditionally, bacteria have been identified by performing a number of biochemical tests and comparing the results to known type strains. The tests are carried out in biochemical media prepared in-house or by using a commercially available kit such as the API 20E. Typically, biochemical methods assess whether a bacterium ferments a carbohydrate, utilizes a carbohydrate as a sole carbon source, or hydrolyses a specific substrate. This methodology is based on the biochemical pathways in a bacterial cell. MALDI-TOF MS (matrix assisted laser desorption ionization time-of-flight mass spectrometry) is a high throughput method for the identification of microorganisms such as bacteria, yeasts and fungi (oomycete fungi are not included at the present time). Identification is achieved by examination of the mass spectrometry of proteins of a bacterial cell; particularly the constantly expressed, high-abundance proteins such as ribosomal proteins, and specifically, proteins between 2-20 kilodaltons. The technique still requires growth of the bacterium but can be performed on an isolated colony from a primary plate. Identification of an unknown bacterium is achieved by pattern matching of the generated protein peaks against a stored library containing spectra characteristic for genera and species. The intensity is correlated and used for ranking results. Thus, an assigned score of >2 identifies the bacterium to species level, whereas a score of 1.7-2.0 identifies to genus level only. To generate spectra, a bacterial colony (~$10^6$ cells) is smeared within a well on a target plate. A 1 µl volume of matrix solution (saturated alpha-cyano-4-hydroxycinnamic acid matrix solution containing 50% acetonitrile and 2.5% triflouroacetic acid) is added to the cells and air-dried before being placed into the machine and analyzed within 1-2 minutes. MALDI-TOF has been used for separation of species and subspecies, strain typing, and the detection of antibiotic resistant genotypes.

A number of *Vibrio* species (51) are in the default MALDI-TOF (Bruker Daltronics) database including *V. harveyi* (4 type strains), however not all *V. harveyi* isolates identified by PCR primers designed to the haemolysin gene were identified through MALDI-TOF analysis. The primers are undergoing validation and detect a range of phenotypes of *V. harveyi*. A library of spectra was developed to incorporate phenotypes of *V. harveyi* and dendogram analysis was used to perform strain typing.

The *V. harveyi* database and dendogram analysis identified three main clusters of *V. harveyi*. One cluster was predominated by sucrose-negative phenotypes obtained from a range of aquatic species and geographical locations. A second cluster contained mainly urease-positive or citrate-positive isolates from oysters and fish. A third cluster was predominated by isolates from a disease outbreak.

Thus a database for the identification of different phenotypes of *V. harveyi* has been developed which can be used in future epidemiological studies of disease outbreaks. The database will continue to be expanded as new isolates are received and will assist in overcoming problems associated with the identification of phenotypes of *V. harveyi*, and may lead to new insights into the pathogenicity of the organism.

## A Risk Analysis of Australia's Marine Ornamental Value Chain Focusing on Biosecurity (Diseases and Pathogens) Concerns

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This presentation shows the progress of ongoing PhD research focusing on the management risk prevention efforts that Australia's implementing to insure imported marine ornamental animals do not establish a permanent while foothold within Australia. Additionally, consideration and monitoring of these imported organisms as pathways of foreign disease transmission is being carried out. Supply/value chain analysis is being used to establish an Australian current practices baseline for the marine aquarium trade. This occurs via a survey of the marine ornamental organisms that are available in Australia to identify the species that are non-native. Determination of the marine species status (native, introduced or cryptogenic) occurs using an established protocol.

From there, the methods of how these imported species enter the country are investigated in the importers current biosecurity measures are recorded. These imported organisms are then followed to the distributor who sends them to various stores around Australia who then passes them on to individual hobbyists. While the organisms are being followed, they are monitored for potential diseases and parasites that may have been imported with them, or that they may have been exposed to when they were in quarantine facilities.

## Risk analysis of the aquarium trade as a pathway for release of dwarf gourami iridovirus (DGIV) in Australia and the risks of exposure of wild native fish

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Deficiencies in national and international regulations on the trade of aquatic animals for the pet industry are implicated in the emergence and spread of exotic pathogens. In Australia around 18 million ornamental fish are imported annually. Findings from epidemiological surveys of populations of imported and domestic ornamental fish showed that despite quarantine measures applied to all imported live ornamental fish, around 20% of ornamental fish in the Australian aquarium trade are infected with an exotic megalocytivirus known as dwarf gourami iridovirus (DGIV). Experimental transmission studies have shown that a number of Australian native fish species, many of which have both a high conservation status and high potential commercial value as food fish, are susceptible to DGIV. The presence of DGIV-infected ornamental fish in the aquarium industry and their distribution through trade across Australia means that DGIV is a significant hazard that requires detailed study of the potential release pathways for wild native fish exposure. The anthropogenic movement of live fish is the most important route of spread for many aquatic pathogens. In Australia, the aquarium trade is recognised as a major pathway for human-assisted dispersal of ornamental fish into natural waterways, with 22 of the 34 alien finfish species with established populations in freshwater habitats originating from this source (Lintermans, 2004). Furthermore, there is evidence that intentional movements of live ornamental fish from the aquarium trade for use as feeder fish or broodstock occur within aquaculture facilities in Australia.

In recognition of the existence of pathways for imported ornamental fish to enter natural waters, the food chain for farmed fish, and ornamental fish aquaculture facilities, a qualitative risk assessment was undertaken to assess the risk of release of DGIV and the likelihood of exposure of wild native fish.

Considering the scale of movements of ornamental fish from the aquarium trade, it was determined that the intentional release of live ornamental fish is a sufficient route for the release of DGIV in Australia and its potential dissemination. As the exposure of native fish to DGIV and the efficiency of pathogen transmission was dependent on the interactions among host, pathogen and environment, risk mapping was used to identify low or high risk environments based on the geographical distribution of known susceptible native fish species. Although the most current information on the hazard and the epidemiology of DGIV in Australia was used to inform the choice of probability estimates, the risk assessment is dependent on the quality and quantity of the information on which it is based. Overall, findings from this study show that additional risk mitigation measures are required to prevent the occurrence of epizootics due to DGIV in the natural environment and the Australian aquaculture industry.

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## Incursions of cyprinid herpes virus 2 in goldfish populations in Australia despite quarantine practices

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One of the potential routes for entry into Australia for significant aquatic pathogens is via the ornamental fish trade, in which fish are imported under a policy based on a formal Import Risk Assessment. Nearly 18 million ornamental fish were imported into Australia in 2007, including approximately 3.9 million goldfish. A further 7 million goldfish were domestically produced from aquaculture to supply the pet fish industry. Despite quarantine regulations during importation, there have been several incidents in Australia where exotic pathogens have become established in farmed or free-living fish species. The exotic virus Cyprinid herpesvirus 2 (CyHV2) was first found in Australia in 2003 from privately owned goldfish. At the time, this suggested that sub-clinically infected goldfish were passing quarantine regulations requiring health certification and three weeks of quarantine. Subsequently in 2008, an opportunistic survey of retail outlets reporting sick or moribund fish in Sydney, Australia revealed that CyHV2 was detected in 17% (15/87) of goldfish. However, since retail outlets contain an assortment of imported and domestic goldfish sharing the same environment, the source of the virus could not be identified. Furthermore, domestic breeders have anecdotally claimed that their stock succumb to diseases when brought into contact with imported goldfish in wholesale and retail premises.

A series of targeted surveys were conducted to determine whether CyHV2 has already established in farmed or wild ornamental fish in Australia. For this purpose, goldfish populations were tested to OIE standard to detect 2% prevalence with 95% confidence assuming a test of 100% sensitivity and specificity. CyHV2 was found at wholesaler premises, farms and notably in several populations of wild goldfish in the ACT and Victoria. The findings of the study demonstrated that CyHV2 was already established in Australia and were used to inform quarantine policy to revoke the requirement for goldfish exported to Australia to be certified free of CyHV2. The findings provided clear evidence that an aquatic pathogen from ornamental fish with quarantine significance can become established in farmed and wild populations. This is of particular significance to Australia as there are many endemic and ecologically sensitive populations of fish that may be severely affected by exotic pathogens. The incursion of CyHV2 in Australia should be considered a case study to inform pathway analysis for pathogen establishment.

## Welfare and Aquaculture: Where we are? Where we go?

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Animal welfare is defined as animals that are "fit and happy". This definition is intentionally vague but includes the two main concepts of "function-based" and "feeling-based" welfare. "Function-based" welfare relates to basic physiological health including absence of homeostatic stress and disease, while "feeling based" relates to the emotional state as perceived by the animal. These two concepts are the foundation of the "5 freedoms" that help assess the welfare of farmed animals.

Because they are evolutionarily distant from terrestrial mammals and have no audible vocalisation, fish seem to be the victims of "speciesism" within the animal welfare debate. Welfare discussions for aquatic species focus primarily in finfish, as vertebrates, and tend to ignore crustaceans or molluscs, that are invertebrates. The capacity of finfish to experience emotions and feelings has been a main point of contention amongst scientists and within the general public. Some believe that finfish are more like 'robots' and only use reflexive or instinctive responses to interact with their environment; while others believe that finfish are sentient and have the ability to consciously experience suffering and well-being (Branson, 2008). Part of this conflict lies in the definition of consciousness and how it applies to finfish. Because of its subjective nature, it might be impossible to directly measure and assess conscious thoughts in finfish. However, welfare scientists have developed and continue to develop, indirect methods to investigate finfish consciousness.

Amongst all emotions, the feeling of pain seems to be the most studied in finfish. Three main areas of investigation for pain perception in finfish are (Sneddon, 2011):

1. *Neural detection and processing of pain,*
2. *Adverse behavioural and physiological responses,*
3. *Conscious perception of pain.*

Most current aquaculture systems are intensive which raises several welfare concerns related to transport, housing, feeding, and slaughter of fish (Fish Physiology & Biochemistry, 2012). Recently, a shift occurred in the perception of fish welfare by farmers moving from conventional reactive response to a disease outbreak towards proactive practices to reflect fish preferences and stimulation. Part of the industry realised that the lack of welfare limits the biological capacity of the fish and that counter actions such as treatment and culling were unsustainable in the long run. Beside mortality and apparent lesions, non-invasive indicators based on behaviour are under development to assess the welfare of farmed fish. According to the cultured fish species, on-farm behavioural markers associated with positive and negative emotions can be monitored and quantified in real-time using video systems. For instance, the degree of exploratory behaviour, feed anticipatory activity, and reward-related operant behaviour are related to the fish well-being; while altered foraging behaviour, increased gill ventilating activities, aggression, changes in individual and group swimming behaviour, and stereotypic behaviour are associated with acute and chronic stress and subsequently poor fish welfare. Ingenious systems are being validated using visual stimuli to harmonize and trigger shoaling behaviour in a fish cage and result in enhanced production.

The future of fish welfare in aquaculture is intimately linked to the ability to induce positive natural behaviour in a confined environment that is associated with competitive fish growth and with better food quality and safety.

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## Parasites of introduced freshwater fish species in Murrumbidgee Basin and their impact on aquaculture development in the region

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There has been significantly less research on parasites from Australia's freshwater fish fauna compared with work on these parasites from marine fishes. Exotic freshwater fishes have been known to cause many changes in Australian rivers and lakes from depletion of native fish stocks to increased turbidity. However, one area that has not attracted much attention is parasite fauna of the introduced fishes. In the current preliminary study, we examined carp (*Cyprinus carpio)* and gambusia (*Gambusia holbrooki*) caught from Murray-Darling Basin in New South Wales (NSW) for infection with parasites. Various parasitic groups, including apicomplexa, monogeneans, cestodes and crustaceans were found. Although some of these parasites, such as monogeneans are known as having the potential to cause serious disease and massive mortalities both in farmed and wild fish, all infected fish in the present study had a healthy appearance. These introduced fish are known to be resilient against many parasitic diseases therefore they can act as reservoirs for dangerous parasites and can be a major threat to the aquatic biodiversity as well as aquaculture development in regional areas. Flooding events in recent years connected several isolated wetlands in the region, either permanently or temporarily and could have facilitated parasite transfer between introduced species and native fish and further distribution of parasites in the region.

Potentially, parasites from introduced fish species could transfer into aquaculture and may already be present in farms. Parasites, causing little apparent damage in feral fish populations, may become causative agents of diseases of great importance in farmed fish, leading to pathological changes, decrease of fitness or reduction of the market value of fish.

## Integrated parasite and disease management strategies for finfish aquaculture in tropical north Queensland: a JCU and QLD Department of Agriculture, Fisheries and Forestry initiative

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Parasites and their associated diseases and pathology are a significant burden on the aquaculture industry in tropical north Queensland through the impact of direct mortalities, decreased production and treatment costs. A number of potentially high-valued tropical fish species have been recently trialed for production in Queensland through Queensland DAFF or private company investment, including: barramundi cod (*Cromileptes altivelis*), common coral trout (*Plectropomus leopardus*), cobia (*Rachycentron canadum*), flowery cod (*Epinephelus fuscoguttatus*), gold-spot rock cod (*E. coioides*), Queensland groper (*E. lanceolatus*) and mangrove jack (*Lutjanus argentimaculatus*). Three of these species, *E. coioides, E. lanceolatus* and *P. leopardus* have now been successfully developed to the point of commercial viability through the Northern Fisheries Centre, although parasite and disease issues continue to cause some problems at each phase of production.

This recently funded collaboration between the Queensland DAFF, James Cook University and a number of industry partners seeks to address and manage the parasite and disease problems currently hindering growth and further development of aquaculture in north Queensland. The goals and outcomes of this collaboration are to describe and characterize the biology, life-cycles and infection dynamics for the most significant parasite problems with the aim of developing treatments and management regimes which are proactive, rather than reactive. Substantial gaps exist in our knowledge of the transmission and development times for many of the known pathogenic parasites in tropical Australia, therefore we seek to gain this critical information in order to effectively manage infections and prevent outbreaks.

Here we present the results of our recent survey identifying and characterizing the significant parasites and pathogens causing problems at each of the various development phases, from brood stock, larval rearing through to farm grow out for the grouper in development at Northern Fisheries and in barramundi and grouper on farm with our industry partners. These include some of the parasite faunas typically found in temperate or cold-water aquaculture, such as monogeneans (Diplectanidae spp.), myxosporeans (*Ceratomyxoa* and *Henneguya*/*Myxobolus* spp.) and ciliated protozoans (*Trichodina* and *Chilodonella* spp.), but which have developmental times that differ markedly from colder-water forms. Despite strict treatment and quarantine protocols, we have discovered some of these parasites (e.g. diplectanid monogeneans infecting grouper) potentially evading initial quarantine (and subsequent treatment) and persisting in these aquaculture systems, requiring us to modify existing treatments and explore new treatment options. We will discuss how our team is tackling these issues and addressing important gaps in our knowledge of the parasite fauna impacting finfish aquaculture with our partners in north Queensland through *in vivo* and *in vitro* experimental studies and via genetic analyses.

## Praziquantel as a control for fluke infections in fish

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Praziquantel is an isoquinolone anthelmintic developed to control flatworm (platyhelminth) infections in veterinary and human medicine. Flatworms, particularly monogeneans, are problematic parasites in aquaculture, and praziquantel is one of the few candidate medicines that treat these parasites in a targeted manner. Investigations into the utility of praziquantel to treat skin (*Benedenia seriolae*) and gill (*Zeuxapta seriolae*) flukes of yellowtail kingfish (*Seriola lalandi*), gill flukes (*Lepidotrema bidyana*) of silver perch (*Bidyanus bidyanus*) and gill flukes (*Lamellodiscus* spp.) infecting barramundi (*Lates calcarifer*) have shown that in-feed administration of praziquantel is problematic and efficacy is variable even when delivery is optimised. Efficacy may be enhanced by improving delivery, increasing frequency, and combining praziquantel with products that impair metabolism of the active product. The data requirements for permitting or registration are substantial, and metabolism and kinetics, residues and environmental studies are incomplete.

## Review of the fish-parasitic cymothoid crustacean 'tongue biter' genus *Ceratothoa* (Fabricius, 1775) in Australian waters

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Cymothoids are crustacean isopods found exclusively on marine, brackish and freshwater fish of both tropical and subtropical areas. The Cymothoidae is the second largest family of marine isopods in the suborder Cymothoida, with 280 species in 34 genera known worldwide. Representatives from this family have a number of morphological adaptations influenced by their site attachment on the host (gills, mouth, external surfaces or burrowed inside the flesh).

Species of *Ceratothoa* have earned the reputation of 'tongue biter', serving as a mechanical replacement for the host tongue after attaching to and consequent atrophy of the tongue. Other known host pathological records from aquaculture are weight loss, buccal malformation, damaged gill lamellae and erosive lesion to the oral mucosa. The World Register of Marine Species lists 29 species of *Ceratothoa* worldwide, six of which occur in Australia. *Ceratothoa* was first recorded from Australia by Haswell (1881), with the most recent records given by Bruce and Bowman (1989). *Ceratothoa* is difficult to identify correctly to species level due to inadequate historical descriptions, lack of type material, lack of identified hosts and localities, and also morphological variability. It is important to resolve the identity of *Ceratothoa* species to understand which of these species affect farmed fish and potential reservoirs for those isopods that do affect farmed fish.

This talk presents a general introduction to cymothoids, their life cycle, importance of new taxonomy and worldwide records of the impact of *Ceratothoa* on fish aquaculture. Our research reveals four species new to Australian waters and we discuss species that are a potential problem (namely *Ceratothoa banksii* and *Ceratothoa imbricata*) for Australian fish aquaculture. We list possible hosts that may be the "mode of transfer" of these parasites from wild to cultured fish that have been affected from museum collections and fresh material from aquaculture. We explain how we resolved nomenclature ambiguities between *Ceratothoa imbricata, Ceratothoa banksii* and *Ceratothoa trigonocephala* using key morphological characteristics and implementing precise quantitative description for each species. We briefly mention the diversity and distribution of *Ceratothoa* in Australia and the development of a user-friendly DELTA key, which will include digital images, illustrations and descriptions for use by future authors and researchers.

## Development of an Australian strain of *Ichthyophthirius multifiliis* infecting rainbow trout under different temperatures

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*Ichthyophthirius multifiliis* (Fouquet), a ciliate parasite, is one of the most common pathogenic parasites of cultured freshwater fish due to its low host specificity and capacity to reproduce rapidly. A number of Australian freshwater fish farms are having trouble in effectively managing the disease under current treatment programs, which were developed based on strains found overseas. The lifecycle of *I. multifiliis* is direct: a trophont, which infects the epidermis of the fish host, grows and develops into a tomont. The tomont exits the host, settles and undergoes rapid binary fission to produce daughter tomites. After a number of divisions, each of the tomites differentiates into a theront, which is the motile infective stage that seeks a new host. The developmental period of each stage in the lifecycle is associated with water temperature. Five different strains of *I. multifiliis* are identified and each strain has different temperature preferences, but it is unknown which strain or strains occur in Australia.

To characterise the lifecycle of *I. multifiliis*, infected rainbow trout were sourced from a farm in Victoria and recently exited tomonts were transferred into individual wells of multidish plate and incubated at 5, 9, 12, 17, 21, 25 and 30°C and regularly observed until theront release. Development under salinities of 0, 1, 3, 5 and 7.5 g/L was also recorded. The growth rate of the trophont on the host from the point of infection was investigated at 12, 17 and 21°C.

The Victorian isolate of *I. multifiliis* was similar to other temperate strains described from Danish and German isolates, however, developed slightly faster at higher temperatures. Development was longest at 5°C with a mean duration of 190 hours, shortening to a mean duration of 12 hours at 30°C. Temperature also had a significant effect on theront production, which was highest at 25°C with a mean of 493 theronts produced per individual tomont. Reports for development under different salinities and trophont development will also be discussed.

The observations reported here will aid in the development of an integrated treatment program for *I. multifiliis* in Australian freshwater fish farms, which will allow for improved management of this problematic disease.

**Research findings from the investigation of *Streptococcus agalactiae* in Queensland grouper *Epinephelus lanceolatus*, and wild fish and crustaceans of north Queensland**

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Giant Queensland grouper (*Epinephelus lanceolatus*) are an iconic, protected, "no-take" species in Queensland and inhabit inshore coastal rivers, reefs and the Great Barrier Reef. Qld grouper feed on a wide variety of prey, including fish, bait, mud crabs, crustaceans (prawns, crayfish), small stingrays and sharks (Lyle Squire, *personal communication*). Between 2007 and 2011, 93 giant Qld grouper were found dead on the coast of Queensland, Australia, most in the Cairns region of northern Queensland. Many grouper died from bacterial infection with *Streptococcus agalactiae* (Bowater et al. 2012).

In 2009, sick wild fish; giant sea catfish (*Arius thalassinus*) and javelin grunter (*Pomadasys kaakan*), were found infected with *S. agalactiae*. A small survey conducted in Trinity Inlet, Cairns during 2009, was done to determine whether other wild fish species may be a source of infection for Qld grouper. *S.agalactiae* was isolated in pure culture from the heart of one healthy wild, diamond-scale mullet (*Liza vaigiensis)* (Bowater et. al. 2012).

Research, funded by the FRDC, was undertaken by DAFF and UQ to; (1) determine the prevalence and distribution of *S. agalactiae* in wild fish along the north Queensland coast; (2) determine the mode of transmission of *S. agalactiae* in Qld grouper; (3) develop a PCR and other diagnostic tools to detect *S. agalactiae* in fish; (4) determine the origin of *S. agalactiae* using epidemiological molecular tracing techniques; and (5) determine the genetic relationship of Australian piscine, human, mammalian and other aquatic animal strains of *S. agalactiae*.

Challenge trials conducted on juvenile Qld grouper showed *S. agalactiae* is transmissible by water, cohabitation and ingestion of infected feed. Pathology studies done on fish from the challenge trials confirmed *S. agalactiae* causes systemic infection and disease, with multi-organ involvement, the brain, eye and heart most commonly affected. Fish displaying clinical signs of infection had meningitis, ophthalmitis and pericarditis, and a range of other pathologies that will be briefly presented.

Surveillance for *S. agalactiae* in 1332 marine and brackish water fish and crustaceans, was done between 2010 and 2012, covering 3300 km of the Queensland coastline. Testing of collected samples used the gold standard techniques of bacterial isolation and PCR. *Streptococcus agalactiae* was not detected in 100 tilapia (*Oreochromis mossambicus*), 828 other fish (over 20 different species), 373 mud crabs (two species) or 45 banana prawns. These surveillance results will be briefly discussed.

**References**

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**Novel Chlamydia-like epitheliocystis agents in Australian farmed yellowtail kingfish *Seriola lalandi*, striped trumpeter *Latris lineata* and barramundi *Lates calcarifer***

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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 has caused 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.

**First report of *Flavobacterium psychrophilum*, the bacterial agent associated with peduncle disease, from Chinook salmon (*Oncorhynchus tshawytscha)* in New Zealand**

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In December 2012, the New Zealand Ministry for Primary Industries (MPI) investigated a Chinook salmon hatchery which had been experiencing low levels of mortality (~0.03-0.06%/day) in its salmon fry. Fish were described as having ulcerated peduncles or were missing caudal fins, but were otherwise of normal body condition. Initial histopathology revealed acute deep tissue necrosis with little inflammatory response and the presence of filamentous gram-negative bacteria. Peduncle disease, caused by *Flavobacterium psychrophilum* but not previously known from New Zealand, was suspected.

More clinically-affected specimens were submitted to the MPI Animal Health Laboratory. PCR for *Flavobacterium* sp. was performed on bacterial isolates and tissues. All fish submitted were found to be positive, with sequencing confirming *F. psychrophilum*. This is the first time *F. psychrophilum* has been detected in New Zealand, despite being otherwise found worldwide in salmonid culture. Samples from two other Chinook salmon hatcheries, one on the same river to the first case, and one on a different river, also tested positive for *F. psychrophilum*.

Genetic analysis of the strain in New Zealand found it is unique from strains reported overseas. It appears to produce low levels of mortalities in Chinook salmon, and may co-exist with other pathogens, making it difficult to detect. It is suspected that *F. psychrophilum* has been in New Zealand for some time, and is probably widespread.

This bacterium was first detected in a volunteer-run hatchery which produces salmon to augment the recreational fishery in local rivers. The detection of this pathogen has highlighted the potential biosecurity risk non-commercial operations like these can pose, if not managed effectively. MPI and the Department of Conservation are carrying out a joint review of land based aquaculture in New Zealand, which is expected to be completed by the end of 2013. As part of this, MPI has initiated a range of projects to improve biosecurity practices within land based aquaculture.

## In vitro use of synthetic antimicrobial peptides against aquaculturally relevant pathogens

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Traditionally, antibiotics and harsh chemicals have been the primary treatment method for controlling most fish pathogens in aquaculture. These treatments are limited in their ability to control the causative agents of disease, becoming ineffective (via pathogen adaptation) and cost preventative overtime. Thus, there is a need for a broad spectrum treatment which proactively conditions the innate immune system of fish against pathogens. Antimicrobial peptides (AMPs) are produced by all forms of living organisms and represent a novel class of antibiotics to treat infectious diseases. These peptides exhibit broad-spectrum activity against a wide range of microorganisms, are relatively small (<10 kDa), cationic, amphipathic, and may be obtained from different biological or synthetic sources.

Based on analysis of previous work on AMPs, several synthetic peptides were evaluated for their minimum inhibitory concentrations, salinity tolerance/stability, host cytotoxicity, and hemolytic activity against known fish pathogens *in vitro*. These synthetic peptides have shown higher target specificity (K6L2W3 and K7LW3), lower cytotoxicity to the host (P9-4, P11-5, and P11-6), lower minimum inhibitory concentrations (P9-4, P11-5, and P11-6), and improved stability in saline environments (Protegrin-1, P9-4, P11-5, and P11-6) in previous trials.

Antimicrobial peptides represent an emerging group of broad spectrum, multidimensional tools for pathogen control. Currently, little is known about their effectiveness as immunostimulants or adjuvants. Results of minimum inhibitory concentration assays, haemolytic assays, and salinity tolerances will be discussed.

## Detection of bacteriophage-related chimeric marine virus in cultured abalone in Taiwan

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In the past years, a low-rate but persistent mortality of cultured abalone was noted in the farms. This disease was first record to inflicted 80 to 100-day old juvenile abalone reared in the regular ponds on land, and resulted in an average of 80 percent cumulative mortality in the farms. Later on, similar mortality was reported in the intertidal grow-out ponds and intensive culture ponds on land. No virions were observed via the electron microscopic examination using direct negative staining of pooled tissues. Moribund abalones collected from one batch in an affected farm were performed DNA extraction, purified and DNA sequencing. These DNA sequences were compared using the NCBI blast program and have 3370/3378 (99%), 5666/5704 (99%) and 5945/5961 (99%) identity to Abalone shriveling syndrome-associated virus (AbSV) reported in China. These results suggested that Abalone shriveling syndrome-associated virus was isolated from chronic mortality of abalone in Taiwan. Primer sets derived from these sequences was able to amplify a 693 bp fragment from moribund abalone. A retrospective study revealed a 25% (12/48) of PCR-positive cases in the chronic mortality of cultured abalone. The histopathology study of this disease in moribund abalone is undergoing. Further study between low-rate of abalone mortality and AbSV will be done.

## CSIRO AAHL / IFREMER: Collaborative studies on mollusc pathogens

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For the past decade the CSIRO AAHL Fish Diseases Laboratory and the IFREMER OIE Reference Laboratory on Mollusc Diseases (France) have collaborated on projects related to mollusc diseases. Of particular interest is work undertaken on detection and identification of *Bonamia* spp. More recently, a collaborative study on the development of molecular tools specific for the ostreid herpes virus-1 (OsHV-1) and study of the viral gene expressions in the Pacific oyster (*Crassostrea gigas*) was undertaken.

This presentation summarises the outcomes of past projects and highlights the on-going work carried out at IFREMER on OsHV-1 infections in Pacific oysters.

## A longitudinal study of winter mortality disease in *Saccostrea glomerata* (Sydney rock oysters)

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Winter mortality is a poorly studied disease affecting Sydney rock oysters (*Saccostrea glomerata*) in estuaries in New South Wales (NSW), Australia where it can cause significant losses. Winter mortality is more severe in oysters cultured deeper in the water column and appears linked to higher salinities, however significant gaps exist in our understanding of the causes of disease. Current dogma is that winter mortality is caused by the microcell parasite *Bonamia roughleyi*, however evidence linking clinical signs and histopathology to molecular data identifying a bonamiasis is lacking. We conducted a longitudinal study between February and November 2010 in two estuaries where winter mortality has been reported to occur (the Georges and Shoalhaven Rivers). Results from molecular testing of experimental oysters for *Bonamia* spp. were compared to clinical disease signs and histopathology. Available environmental data from the study sites were also collated and compared. Oyster condition declined over the study period, coinciding with decreasing water temperatures, and was inversely correlated with the presence of histological lesions. While mortalities occurred in both estuaries, only oysters from the Georges River study site showed gross clinical signs and histological changes characteristic of the disease. Lesions were prevalent in these oysters and intralesional microcell-like structures were sometimes noted. PCR testing for *Bonamia* spp. revealed the presence of an organism belonging to the *Bonamia exitiosa-B.roughleyi* clade in some samples; however the very low prevalence of this organism relative to histological changes and the lack of reactivity of affected oysters in subsequent *in situ* hybridization experiments led us to conclude that the *Bonamia* sp. is not responsible for winter mortality. Another aetiological agent and a confluence of environmental factors are a more likely explanation for the disease.

## New Zealand's Salmon Export Testing Scheme

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Over the past 40 years, New Zealand aquaculture has grown into a significant primary industry with annual revenue in excess of $400 million. In 2011 New Zealand exported over 5 tonnes of Chinook salmon (*Oncorhynchus tshawytscha*) to 30 countries, around a quarter of which was exported to Australia. The Ministry for Primary Industries Animal Health Laboratory (AHL) provides diagnostic testing services for the exporting of Chinook salmon to Australia. Diagnostic testing includes culture and molecular testing for the rule-out of the following pathogens: Bacterial Kidney Disease (*Renibacterium salmoninarum*), Whirling Disease (*Myoxbolus cerebralis*), *Yersinia ruckeri, Aeromonas salmonicida* and cytopathic viruses. Numerous clients from salmon farms throughout the country participate, facilitating surveillance of these important diseases and meeting the requirements of health certification for export to Australia. This presentation will provide an overview of this testing scheme since its inception.

## A regional aquatic animal health laboratory proficiency testing program in Asia

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DAFF is funding a regional proficiency testing program for aquatic animal diseases in Asia. The program is being implemented with the participation of DAFF, the Network of Aquaculture Centres in Asia-Pacific, the Australian National Quality Assurance Program and CSIRO-AAHL. Seafood production, export trade and food security are dramatically affected by aquatic animal diseases. More timely and reliable detection of disease could lead to better management of disease outbreaks. Benefits of the program will include increased confidence of trading partners (including Australia) that countries within the region have the ability to certify the disease status of aquatic animal commodity exports, meet quarantine requirements, and thus ensure the sanitary safety of trade through appropriate pre-border measures. Improved diagnostic capability for aquatic animal diseases will improve the ability of trading partners to accurately certify the disease status of aquatic animal commodities, strengthening Australia's pre-border quarantine risk management measures. The project aims to improve capability within Asia to detect important transboundary diseases that have the potential to devastate industry sustainability and productivity, thereby reducing their spread.

The first project activity was a workshop in July 2012 in Bangkok with participants from across the region, to explain the mode of operation of the program and decide upon the diseases to be included in the program. Representatives decided on a panel of 10 pathogens of significance for inclusion in the program. The program will include 45 laboratories in 13 countries across Asia. Four rounds of testing are to be run over 2013–14; the first round commenced in May 2013. Similar proficiency testing in Australia has demonstrated benefits in improving proficiency of testing for aquatic animal diseases.

Six diseases of crustaceans and four diseases of fish are included in the program. All are highly significant in the region and are listed for reporting in the regional aquatic animal diseases reporting system. Six of these diseases are considered to be exotic to Australia. Diseases included in the program are:

White spot virus (WSV)
Yellowhead virus (YHV)
Taura syndrome virus (TSV)
Infectious myonecrosis virus (IMNV)
Infectious hypodermal and haematopoietic necrosis virus (IHHNV)
Megalocytiviruses (RSIV, ISKNV, GIV etc.)
Nervous necrosis viruses (NNV)
Koi herpesvirus (CyHV-3)
*Macrobrachium rosenbergii* nodavirus (*Mr*NV and XSV)
Spring viraemia of carp virus (SVCV)

## International Proficiency Testing for Viral Pathogens of Finfish

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Viral pathogens of aquatic animals pose a significant threat to finfish populations, both farmed and wild, worldwide. Disease outbreaks, particularly in finfish aquaculture, can have major economic implications and cost millions of dollars. Australia is free from the majority of significant viral pathogens that affect finfish worldwide. To assess capability to respond to an aquatic emergency animal disease the AAHL Fish Diseases Laboratory (AFDL) participates in an annual proficiency test for exotic and enzootic finfish viruses.

The European Union Reference Laboratory for Fish Diseases, located in Denmark, organises an annual inter-laboratory proficiency test for national reference laboratories for fish diseases. AFDL has participated in this proficiency test since 2000. The viral disease agents of major concern and that have been included in the proficiency test are IPNV, SVCV, IHNV, VHSV, EHNV, ISAV, KHV – most of which are exotic to Australia. This proficiency test is one of very few opportunities to assess AFDL's capability to detect and identify these exotic viruses.

This international proficiency test provides a key performance indicator regarding AFDL's primary role as the national diagnostic laboratory for exotic diseases of aquatic animals. An outline of the proficiency test will be provided and results from AFDL discussed.

**Infestation of Isopod parasite *Catoessa boscii* (Cymothoidae) on Malabar trevally *Carangoides malabaricus* (Carangidae), Southwest Coast of India**

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Parasitic isopods are typically marine and usually inhabit the warmer seas. They are blood-feeding; several species settle in the buccal cavity of fish, others live in the gill chamber or on the body surface including the fins. Isopods can cause morbidity and mortality in fish populations. Infestation of cymothoid isopod *Catoessa boscii* (Bleeker, 1857) in the commercially important marine fish Malabar trevally *Carangoides malabaricus* (Carangidae) was investigated at Colachel coast along the southwest coast of India. The prevalence and intensity of infestation during different months from November 2010 to October 2011was investigated. It was maximum in January (49.1) and minimum in July (7.5). In males the prevalence was maximum in January (38.1) and minimum in August (8.3).The intensity ranged from 0.3 in May and 1.8 in February. In females the prevalence was maximum in January (56.2) and minimum in October (10.5). The maximum intensity (2.2) was recorded in June and minimum (1) was recorded in July and October. Effect of infestation, the morphometric data and the host specificity were analysed. From a single host, four pairs of isopods (one male with one female) were recorded and each pair was attached on the tongue of buccal cavity. Gross lesions were observed in the buccal cavity of infested fish and observed small pin-holes in the tongue region, through which dactyls of periopod claws dig and penetrated into the host tissues. The maximum weight loss was found in females (6.73%) than males (4.12%) of *C. malabaricus.* The rate of growth in weight was found to be higher in uninfested female fish than infested fish. It is revealed that the weight gain is faster in uninfested female fish. The infestation of *C.boscii* did not cause any immediate death of *C.malabaricus*, however, it could easily affect the normal growth of the host. Infested fish exhibited histopathological anomalies such as tissue reactions, primarily associated with the formation of granulomas consisted of macrophages and epithelioid cells, which are occasionally surrounded by a thin rim of fibroblasts. A marked increase in the size of the parasite is associated with the development of marsupium full of juvenile parasite. The infestation usually pressure atrophy often accompanies the presence of larger parasites. They may lead to economic losses in commercial species of fish.

## Survival strategies of an insidious fish ectoparasite, *Neobenedenia* sp.

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Parasites use sophisticated life history strategies to ensure their survival in contrasting and unpredictable environments. Evolutionary strategies of parasites include multiple reproductive mechanisms, high fecundity, predator avoidance and host detection mechanisms that enhance infection success. These strategies evolved in wild populations where the likelihood of encountering a host is low but in captive populations, where host organisms are confined in high densities, it can lead to parasite epizootics.

This study examined the reproductive biology of *Neobenedenia* sp. (Capsalidae: Monogenea), a commercially important fish ectoparasite, in a series of laboratory experiments. The ability of hermaphroditic *Neobenedenia* sp. to self-fertilise was evaluated by infecting individual, isolated barramundi (*Lates calcarifer*) with a single oncomiracidium (larva). Simultaneously, other individual fish were challenged with 10 oncomiracidia each, to examine whether parasites with the opportunity to cross-fertilise exhibit similar reproductive trends as isolated parasites. Single, isolated parasites attained sexual maturity at day 10 post-hatch and laid embryonated eggs. Isolated parasites were fecund and laid an average of 3,229 ± 37 eggs over one week. Egg production commenced on day 10 post-infection and rapidly increased from 58 ± 15 eggs (day 10) to 496 ± 68 eggs on day 15 before gradually decreasing. There was no significant difference in fecundity between isolated and cross-fertile parasites ($p = 0.83$). Eggs laid by isolated parasites were incubated (25 °C, 12:12, LD cycle) and emerging individual oncomiracidia were used to infect more isolated *L. calcarifer* for three continuous self-fertile generations.

Adult *Neobenedenia* sp. exhibited a distinct egg laying and egg hatching rhythm. Parasites laid eggs continuously, but egg production increased in periods of darkness (65.7%), with production peaking between midnight and 0300, while approximately 81% of oncomiracidia emerged from eggs in the first three hours of light.

This study shows that self-fertilisation is a viable method of reproduction for a fish monogenean. *Neobenedenia* egg laying and egg hatching rhythms are likely to be a predator avoidance strategy and increase the likelihood of oncomiracidia encountering a suitable host fish. The numerous reproductive strategies exhibited by *Neobenedenia* (i.e. self-fertilisation, high fecundity, egg laying rhythms and hatching rhythms) ensure the persistence of this notorious parasite in captive and wild fish populations.

**Effects of temperature and salinity on the life cycle of *Neobenedenia* sp. (Monogenea: Capsalidae) infecting farmed barramundi (*Lates calcarifer*)**

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Increasing water temperatures and extreme salinity fluctuations as a consequence of climate change are predicted to exacerbate the frequency and intensity of parasite outbreaks in aquaculture. Effective parasite management strategies of farmed stock require comprehensive knowledge of the influence of environmental parameters on parasite life cycles. Bathing stock needs to be temporally coordinated in order to break the parasite's life cycle. Accurate determination of life cycle parameters (e.g. egg embryonation period, larval longevity/infection success and time to sexual maturity) at a variety of temperatures and salinities is required in order to identify accurate bathing timetables. This research examined the life cycle parameters of *Neobenedenia* sp., a harmful ectoparasitic fluke (Monogenea) that infects farmed barramundi (*Lates calcarifer*).

We examined the effects of current and predicted temperatures (22, 24, 26, 28, 30, 32 and 34°C) and salinities (0, 11, 22, 35 and 40ppt) on the life cycle of *Neobenedenia* sp. Egg embryonation period was shorter in high temperatures (4 days at 30 and 32°C compared to 6 days at 22°C). Hatching success increased with increasing temperatures (22-32°C) and with increasing salinity (22 to 40ppt). However, hatching was significantly reduced at the warmest temperature tested (34°C; $p<0.0001$) relative to cooler temperatures. Eggs did not hatch in low salinities (0 and 11ppt). Infection success decreased with increasing temperature and decreasing salinity. Time taken to reach sexual maturity was shorter at warmer temperatures (6 days at 26, 30 and 32°C compared to 12 days at 22 and 24°C) and higher salinities where it took longer to reach maturity at 22ppt than at 35 or 40ppt in each temperature. Warmer temperatures reduced the time taken to complete the life cycle (10 days at 30°C; 18 days at 22°C).

Although timed treatments eradicate adult parasites from stock, they do not completely eliminate infection sources (eggs and larvae) in a system. Our results indicate that low salinities inhibit egg hatching, which may be an effective treatment options for retained eggs. Further experiments revealed that desiccation and heat are also effective control methods for inhibiting *Neobenedenia* sp. egg hatching. Desiccation for as little as 2 minutes completely inhibited egg hatching. Immersion of eggs in 60°C tap water for 30 seconds was also found to completely inhibit egg hatching.

Knowledge of the life cycle parameters of *Neobenedenia* sp. at a variety of temperatures and salinities can be used to formulate strategic bathing timetables. These in turn can be used to advise fish farmers on the temporal treatment of their stock in varied environmental conditions in order to effectively manage this parasite in aquaculture.

**Differences in epithelial pathology of fish microhabitats infected with the ectoparasitic monogenean *Neobenedenia* sp.**

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Capsalid monogeneans in *Neobenedenia* are known ectoparasites of ornamental and farmed marine fishes in tropical/subtropical environments. Parasites graze on epidermal and mucous cells of the fish by secreting digestive enzymes and reabsorbing the digested tissue. *Neobenedenia* spp. have two attachment organs located anteriorly, and one larger posterior attachment organ, the haptor. The haptor acts as the principal anchoring organ of the parasite to the host. This organ has chitinous structures that provide mechanical attachment and a marginal valve that creates suction on the host. *Neobenedenia* infections have been linked to acute inflammation, haemorrhage, lesions and ulcerations of the host's epidermis, resulting in increased morbidity.

The epithelial pathology of farmed barramundi (*Lates calcarifer*) infected with *Neobenedenia* sp. was examined in four separate microhabitats. Fish were experimentally infected with *Neobenedenia* sp. in the laboratory and skin samples with attached parasites were sampled from the cornea, lower jaw, operculum and mid-body of infected and uninfected fish. Histopathology of infected fish epithelium showed evidence of extracellular vacuole formation, inflammatory cells, cellular debris, and ruptured epithelial and mucus cells at the site of parasite attachment.

Infected *L. calcarifer* exhibited significantly greater epithelial thickness on the mid-body (56±4mm) and operculum (66±4mm) compared to uninfected fish (34±2mm and 50±3mm, respectively). A significantly greater number of epithelial layers were observed on the mid-body (9±0.7mm) and operculum (14±0.4) of infected fish compared to uninfected fish (6±0.4; 7±0.4, respectively). No differences in the number of mucous cells were found in the mid-body and operculum tissue between infected and uninfected fish.

In contrast, epithelial thickness in the lower jaw and corneal epithelium of infected fish (38±3mm; 0.7±0.4mm, respectively) was lower compared to uninfected fish (82±3mm; 31±3mm, respectively). The number of epithelial layers were also lower in the lower jaw and corneal epithelium of infected fish (5±0.4;0.3±0.2, respectively) when compared to uninfected fish (11±0.6; 5±0.3, respectively). The number of mucous cells was significantly lower in the lower jaw of infected fish (4±0.7) compared to uninfected fish (20±3). The pronounced epithelial degradation in the lower jaw and cornea compared to mid-body and operculum indicates higher infection susceptibility in anterior regions of the fish. It is possible that anterior microhabitats exhibit lower immune responses than posterior microhabitats, or take longer periods of time to heal. Therefore, anterior microhabitats of the fish could be considered immunologically privileged sites for the parasite.

**Efficacy of Garlic (*Allium sativum*) Extract in Managing the Fish Ectoparasite *Neobenedenia* sp. (Capsalidae: Monogenea)**

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Development of an effective preventative treatment for managing infections by Monogenea (Platyhelminthes) in aquaculture remains elusive. Present treatment methods offer only temporary respite and are either labour intensive, harmful to fish welfare or environmentally destructive.

Research is now being directed towards the vast unexplored source of plant-based antimicrobials and immunostimulants for disease management, many of which are without the negative side effects associated with synthetic chemotherapy. The value of garlic (*Allium sativum*) extract for bacterial disease control and immunostimulation has previously been demonstrated for a number of cultured fishes. This study examined the efficacy of garlic extract administered as a therapeutic bath and dietary supplement for management of *Neobenedenia* sp. infecting farmed barramundi, *Lates calcarifer*.

The effects of garlic extract bath exposure on attached *Neobenedenia* sp., oncomiracidia (larval) longevity, infection success, egg development and hatching were examined and compared to freshwater and formalin exposure *in vitro*. Garlic extract was found to reduce oncomiracidia longevity to 33, 14, 6 and 2 h for the 1, 2, 10 and 20 $mL*L^{-1}$ garlic treatments, respectively, compared to 37 h in the seawater control. Freshwater and formalin (100 $mL*L^{-1}$) treatments were comparable to 20 $mL*L^{-1}$ garlic extract treatment. Garlic extract reduced *Neobenedenia* sp. infection success of *L. calcarifer* from 55±7% among controls to 25±4% and 11±4% in the 1 and 2 $mL*L^{-1}$ treatments, respectively. Hatching and development of *Neobenedenia* sp. eggs were unaffected (> 90% hatching success) by garlic extract at host tolerable conditions while acute (1 h) 100 $\mu L*L^{-1}$ formalin exposure substantially prohibited hatching (4% success). Survival of attached *Neobenedenia* sp. were also unaffected by acute exposure to garlic extract.

Oral delivery of garlic extract was achieved through formulating a pellet extruded feed. Two garlic supplemented feeds of different concentrations and a non-supplemented control diet were fed to *L. calcarifer* for 10 and 30 days before challenging fish with *Neobenedenia* sp.. Long-term (30 day) supplementation with garlic significantly reduced host susceptibility to infection by up to 70% compared to controls and did not negatively affect specific growth rate, food conversion ratio, or palatability. Host susceptibility was not influenced by short-term (10 day) supplementation suggesting a delayed host response must occur to improve resistance to infection. Incorporation of garlic into a pressure-extruded pellet was found to be an effective method of delivery as only minimal leaching of garlic's active component, allicin, from the diet occurred (< 3% of allicin present) during the interval of water contact between delivery and consumption.

Garlic immersion (bath) therapy offers potential in preventative management of *Neobenedenia* sp. but not as a treatment for immediate respite. However, administered as a dietary supplement, garlic is one of the most practical methods to prevent *Neobenedenia* sp. infection in mariculture.

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