Research Exchange to the University of Edinburgh and University of Stirling

Barbara Nowak

Project No. 2009/719

ISBN: 978-1-925982-58-9

2009/719 Research Exchange to the University of Edinburgh and University of Stirling

PRINCIPAL INVESTIGATOR: Associate Professor Barbara F. Nowak
ADDRESS: NCMCRS, University of Tasmania
Locked Bag 1370
Launceston 7250 Tasmania
Telephone: 03 63243814 Fax: 03 63243804

OBJECTIVES:

1. to investigate gene expression in early amoebic gill disease (AGD), including gene expression in different cell types to investigate AGD pathogenesis using metabonomics and assess further applications of metabonomics to fish health research
2. to compare *Neoparamoeba perurans* strains in Scotland and Tasmania by obtaining *N. perurans* from Scotland to further study distribution and strain differences for this pathogen
3. to develop collaboration with Dr Steve Feist and other CEFAS staff through joint research and investigating potential development of a research proposal to investigate host-parasite interactions in fish.
4. to develop collaboration with Intervet Schering Plough and University College Hospital Medical School in London in particular access to novel immunostimulants
5. to develop collaboration with Dr Ted Hupp, University of Edinburgh

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE

This activity advanced my research skills, collaborative skills and improved potential for research funding. The main benefits were: continuation of AGD research on a global scale (including research on gene expression in different types of gill cells and the applications of metabonomics), investigation of the application of metabonomics in fish disease research, preparation of manuscripts for publication and further development of international collaboration in the area of aquatic animal health. I have developed new collaborative relationship with Marine Harvest Scotland, Stirling University, University of Aberdeen, Imperial College London and CEFAS. I gave three invited lectures, which all provided information on Australian Seafood CRC and acknowledgement of Seafood CRC funding.

I visited three salmon farms in Scotland – two Marine Harvest sites and one Scottish Salmon farm. A number of salmon were examined, however none of the farms had Amoebic Gill Disease (AGD) outbreak at the time of the visit. I established collaboration with Marine Harvest to ensure availability of infected material from future AGD outbreaks.

I visited Dr Jeremy Griffin at Cambridge University and Dr Jake Bundy at Imperial College London to discuss our preliminary results for metabonomics from Amoebic Gill Disease infected salmon. These meetings were very fruitful, provided a lot of technical information and will most likely result in long term collaboration with Dr Jake Bundy. Preliminary results suggest that AGD infected fish can be differentiated from controls on the basis of their metabolite profile, at least in the later stages of the disease, we are currently analysing earlier samples.

Future collaboration in the area of the use of immunostimulants in aquaculture was established with Dr Chris Gould and Dr Patrick Smith from Intervet Schering Plough and Prof Sandra Adams and Dr Kim Thompson from Stirling University. Dr Patrick Smith is following up similar testing of novel immunostimulants developed by University College Hospital Medical School in London.

It was not possible to complete the research on gene expression in different cell types and early AGD lesions due to the equipment failure (laser dissecting microscope at CEFAS), however I established collaboration with Dr Steve Feist from CEFAS and we will complete this project in the next few months.

I established collaboration with French researchers (Dr Philippe Sourd's group) investigating health of farmed seabream and seabass, we have diagnosed AGD in some of their fish, this research will be a joint presentation at EAFP conference and should lead to a publication.

KEYWORDS: **Amoebic Gill Disease, immunostimulants, metabonomics**

Acknowledgements
I would like to thank Australian Seafood CRC for travel grant. The Australian Seafood CRC is established and supported under the Australian Government's Cooperative Research Centres Programme. Other investors in the CRC are the Fisheries Research and Development Corporation, Seafood CRC company members, and supporting participants.
I am grateful to Dr Graham Mair for his support as well as helpful comments and advice, which significantly improved this project. I would like to thank Emily Downes for all her help.

Background and Need

Our group has discovered that Amoebic Gill Disease is caused by *Neoparamoeba perurans*, a species which we have described (Young et al 2007). While we confirmed (using in situ hybridisation) the presence of this species on gills of fish affected by AGD in all countries affected by this disease (Young et al 2008a a) we do not know if there are differences between strains from different geographical locations. We have also developed PCR to detect the amoeba on the gills (Young et al 2008b), this method has been further improved during my sampling in the USA and Canada (September 2008, Nowak et al., manuscript in preparation). The PCR will be used to determine fish positive for *N.perurans,* as well as for the investigation of the presence of the amoeba in environmental samples from Scotland. If there is an AGD outbreak I will isolate *N. perurans* for further strain comparisons.

We have described differential gene expression in the AGD lesions compared to normal tissue from the same individual (Young et al 2008c), however as the lesions were dissected under dissecting microscope it was only possible for investigations of the late stages of AGD when the lesions are grossly visible. Laser dissection microscopy facilities are available at CEFAS and will be used to dissect microscopical lesions from salmon gills to study gene expression in AGD affected fish at an earlier stage of infection than it was previously possible. This means that we can understand the initial stages of the infection. So far we have mostly information about host response in the later stage of the disease. The understanding of host response in the early stages of the disease should offer better potential to control the infection. We will also dissect different cell types from normal gills (epithelial cells, mucous cells and chloride cells) to see the differences in their gene expression to confirm that the changes in the gene expression in lesions are not due to changes in cell types only.

While our research on genomics of AGD is advanced, it is challenging to translate the changes in gene expression to effects on fish physiology or immune response. This is at least partly due to the lack of functional assays for fish and the need to select specific assays, which means making assumptions about the effects. More relevant information could be obtained from metabonomics. Metabonomics aims to measure the global metabolic response of living organisms to biological stimuli, such as diseases, focusing on understanding systemic changes (Nicholson and Lindon 2008). This is done through characterisation and quantifications of molecules present in body fluids or tissue extracts and comparison between normal and diseased individuals (or control and treated). Metabonomics has been used in human diseases to evaluate drug toxicity, identify disease markers, assess disease risk, define individual susceptibility and assess recovery from a disease. We want to determine the potential applications of the metabolic profiling for parasitic disease investigation in fish.

Objectives

1. to investigate gene expression in early amoebic gill disease (AGD), including gene expression in different cell types
2. to investigate AGD pathogenesis using metabonomics and assess further applications of metabonomics to fish health research
3. to compare *Neoparamoeba perurans* strains in Scotland and Tasmania by obtaining *N. perurans* from Scotland to further study distribution and strain differences for this pathogen
4. to develop collaboration with Dr Steve Feist and other CEFAS staff through joint research and investigating potential development of a research proposal to investigate host-parasite interactions in fish.
5. to develop collaboration with Intervet Schering Plough and University College Hospital Medical School in London in particular access to novel immunostimulants
6. to develop collaboration with Dr Ted Hupp, University of Edinburgh

Results

Objective 1
to investigate gene expression in early amoebic gill disease (AGD), including gene expression in different cell types

I established that it was possible to capture individual cells as long as their morphology was distinctive. The initial preservation was snapfreezing in cryoprotectant and then sectioning using cryomicrotome. Unfortunately, it was not possible to complete the research on gene expression in different cell types and early AGD lesions due to the equipment failure (laser dissecting microscope at CEFAS was sent to the manufacturer's due to technical problems, this resulted in a complete replacement of the equipment), however I established collaboration with Dr Steve Feist and Dr Tim Bean from CEFAS and we will complete this project in the next few months. In particular we are going to determine gene expression in mucous and chloride cells of seawater adapted Atlantic salmon and use this knowledge for our interpretation of gene expression in salmon affected by Amoebic Gill Disease. We are planning to submit a scientific paper early next year.

Objective 2
to investigate AGD pathogenesis using metabonomics and assess further applications of metabonomics to fish health research

I visited Dr Jeremy Griffin at Cambridge University and Dr Jake Bundy at Imperial College London to discuss our preliminary results for metabonomics from Amoebic Gill Disease infected salmon. These meetings were very fruitful, provided a lot of technical information and will most likely result in future collaboration with Dr Jake Bundy. The technical detail has been already forwarded to the collaborators at CSL (Dr James Horne). Please see Appendix 1 for our preliminary results. We are hoping to submit a manuscript to a scientific journal early next year.

Objective 3
to compare *Neoparamoeba perurans* strains in Scotland and Tasmania by obtaining *N. perurans* from Scotland to further study distribution and strain differences for this pathogen

I visited three salmon farms in Scotland – two Marine Harvest sites (both system cages) and one Scottish Salmon farm (in Oban – polarcircle cages). A number of salmon were examined, however none of the farms had Amoebic Gill Disease (AGD) outbreak at the time of the visit, however I established collaboration with Dr David Cox through Marine Harvest (Ray Waddell) to ensure availability of infected material from any future outbreaks. Current standard health checks include 12 fish from each site sampled for antibodies against PD virus and PD virus (blood) and gill histology from the same fish. We have discussed use of Davidson's fixative to improve gill histology and freezing or fixing in analytical grade ethanol samples for sequencing for the fish suspected of Amoebic Gill Disease. Currently, despite two outbreaks, Amoebic Gill Disease is not considered a problem for Atlantic salmon farming in

Scotland. However, sea lice is an issue. The sea lice theraputants which are available are:
1. Slice (oral)
2. Alphamax (deltamethrin) (bath)
3. Excis (cypermethrin) (bath) - generally losing efficacy and being superseded by Alphamax
4. Salmosan (azamethiphos) (bath)

The sea lice treatment is strategic and the applications are planned for a whole area whenever possible. I also discussed seal control (Seal Management Group – the farm in Oban is based only 100 m from a seal colony – mostly common seals but also some grey seals) and salmon egg supply (Marine Harvest does not produce their own eggs).

Furthermore, while I was in UK I established collaboration with French researchers (Dr Philippe Sourd's group) investigating health of farmed seabream and seabass, we have diagnosed AGD in some of their fish, this research will be a joint presentation at European Association of Fish Pathologists conference later this year and should lead to a publication in a scientific journal.

Objective 4
to develop collaboration with Dr Steve Feist and other CEFAS staff through joint research and investigating potential development of a research proposal to investigate host-parasite interactions in fish.

I developed collaboration with Dr Steve Feist in the area of gene expression in different types of cells in gills and AGD lesions. While the equipment was not available during my visit we are now progressing the sample processing at CEFAS. Some will be analysed there (gene expression in cell types) and others (presence of proteins in the different types of gill cells) will be done at University of Tasmania using immunohistochemistry. Dr Steve Feist has already visited Tasmania since my visit to CEFAS. During his visit he gave lectures (attended by undergraduate and postgraduate students as well as staff) at NCMCRS. We discussed potential research proposals and funding sources for further collaborative research. We are hoping to apply for funding to support future collaborative research.

Objective 5
to develop collaboration with Intervet Schering Plough and University College Hospital Medical School in London in particular access to novel immunostimulants

My meeting with Dr Chris Gould and Dr Patrick Smith from Intervet Schering Plough was very successful, we discussed our research on Amoebic Gill Disease, yersiniosis vaccine and immunostimulants and agreed to investigate future collaboration. In particular, Dr Chris Gould will most likely visit Tasmania later this year and we will obtain research samples of Ergosan for testing on AGD and potentially other fish species. Dr Patrick Smith is following up similar testing of novel immunostimulants developed by University College Hospital Medical School in London.

I have also discussed future collaboration in the area of immunostimulants with Prof Sandra Adams and Dr Kim Thompson from Stirling University. We are investigating sources of funding for research on fish immune response and use of novel immunostimulants in aquaculture. Furthermore I visited University of Aberdeen and discussed the potential use of cytokines as immunostimulants with Prof Chris Secombes.

Objective 6
to develop collaboration with Professor Ted Hupp, University of Edinburgh
I have held preliminary discussions to collaborate with Professor Ted Hupp, we are currently investigating sources of funding for the use of AGD as a model of hyperplastic disease.

Extensions

I gave three invited lectures – one at Institute of Aquaculture at Stirling University (Parasitic diseases in Australian fish farming), and two at CEFAS (Parasitic diseases in Australian mariculture and Amoebic Gill Disease in marine environment), they were all well received, attracted large audience and were followed up by discussions. In my presentations I have promoted Seafood CRC and acknowledged the travel grant.

I have assisted with the organisation of European Association of Fish Pathologists conference (September 2009), in particular I reviewed abstracts for oral presentations and organised the order of presentations in the sessions. There are six oral presentations co-authored by NCMCRS University of Tasmania staff and students, which will be presented at EAFP conference in Prague in September 2009, this will result in a noticeable presence of Australian aquatic animal health research at that conference.

Planned Outcomes

This activity advanced my research skills, collaborative skills and improved potential for research funding. It was consistent with my performance management goals. The main benefits were: continuation of AGD research on a global scale (including research on gene expression in early AGD lesions and the applications of metabonomics), obtaining material for further investigation of *N. perurans* (in particular investigating strain differences between Tasmanian and Scottish isolates), investigation of the application of metabonomics in fish disease research, preparation of manuscripts for publication, providing professional development activities to Australian fish parasitologists, development of international collaboration in the area of fish parasitology. Publications arising from this exchange will target high impact factor journals. We are planning to submit at least two manuscripts based on the AGD research covered by this application (one on metabonomics and one on gene expression in different types of gill cells).

The proposed research travel will result in increased knowledge about Amoebic Gill Disease which will significantly contribute to the progress of our Seafood CRC research projects which focus on the improvement of AGD vaccine. In particular, we are hoping to identify host response in the early stages of the disease at genomic and metabonomic level, which will assist with the development of alternative control and treatment strategies for AGD. This understanding of early host responses will also contribute to the new areas of investigation within existing Seafood CRC projects, including new ways to investigate disease progress and efficacy of experimental vaccines. Investigations into phylogenetics of *N. perurans* will increase our knowledge of the strain differences, distribution of the pathogen on a small and large scale and assessment of the relationship between the presence of the pathogen and the risk of AGD outbreak. That will have practical applications, including assessment of AGD risks at new sites.

The proposed research travel resulted in a better understanding of the biology of *Neoparamoeba perurans* and Amoebic Gill Disease and will contribute to future development of sustainable control strategy of this disease and farmed salmon welfare. Furthermore I established new contacts and collaborations aiming at developing new grant applications to address parasitic diseases in aquaculture. So, this activity has now a potential to improve the existing Seafood CRC projects through variations and develop new project proposals.

My new skills, knowledge and opportunities have been discussed directly with my research group, and will be discussed with our CSIRO collaborators, salmon industry and other Seafood CRC participants. This will be done through presentations at meetings and conferences, written reports and publications. Results will be presented at salmon industry seminars (October 2009) and Aquatic Animal Health FRDC conference in Cairns in July 2009. Furthermore I have passed any relevant information to other Seafood CRC participants, for example Dr Craig Hayward (SARDI, Seafood CRC project 2008/711), for whom I am acting as a mentor. This will ensure that the information can be applied in other finfish industries including Yellowtail Kingfish and Southern Bluefin Tuna (hatchery).

References

Nicholson, J.K., Lindon, J.C. (2008) Metabonomics. Nature 455, 1054-1056.

Young, N.D., Crosbie, P.B.B., Adams, M.B., Nowak, B.F., Morrison, R.N. (2007) *Neoparamoeba perurans* n. sp. an agent of amoebic gill disease in Atlantic salmon (*Salmo salar* L.) International Journal for Parasitology, 37, 1469-1481.

Young, N.D., Dyková, I., Snekvik, K., Nowak, B.F., Morrison, R.N. (2008a) *Neoparamoeba perurans* is a cosmopolitan aetiological agent of amoebic gill disease Diseases of Aquatic Organisms, 78, 217-223.

Young, N.D., Dyková, I., Nowak, B.F., Morrison, R.N. (2008b) Development of a diagnostic PCR to detect *Neoparamoeba perurans*, agent of amoebic gill disease (AGD) Journal of Fish Diseases. 31, 285-295

Young, N.D., Cooper, G.A., Nowak, B.F., Koop, B.F., Morrison, R.N. (2008c) Coordinated down-regulation of the antigen processing machinery in the gills of amoebic gill disease-affected Atlantic salmon (*Salmo salar* L.), Molecular Immunology, 45, 2581-2597.

Appendix
Applications of metabonomics to AGD
Barbara Nowak and James Horne (University of Tasmania)

Introduction

The following short report outlines the methods used to discriminate between infected and control salmon populations on the basis of metabolomic profiling using NMR spectroscopy on blood plasma samples. The data herein is sufficient for proof of concept but requires further work-up to obtain better data reliability and coverage of the metabolome.

Experimental

1H-NMR spectra were recorded with presaturation of the residual water signal at 400 MHz and 4C on a Varian wide-bore spectrometer fitted with a z-axis gradient inverse probe in a 5 mm Wilmad 527-PP NMR tube. 128 transients of FID data were acquired over a spectral width of 4802 Hz and digitised into 32 K data points. A relaxation delay of 5 s was applied between each transient. Data were Fourier transformed with zero filling to 64K data points. FID's were multiplied by a 0.3 Hz line broadening exponential multiplication apodisation, manually phase corrected and baseline corrected. Data were processed using VnmrJ 2.1 and MNova 3.1 software. Chemical shift referencing was performed relative to the literature residual water peak shift at 4C. Figure 1 shows an example of a processed spectrum with labelling of some characteristic metabolite peaks.

Processed spectra were binned using MNova 3.1 over a 0.04 PPM spectra width and the calculated area under each data curve was normalised with respect to the

total volume integral for the entire dataset. The aliphatic region of the binned spectrum (~ 0 – 4.5 PPM) was then analysed using the statistical package “The Unscrambler” (CAMO software). The control and 21-day infected sample group are described here as these gave the best clustering. The two groups were assigned a binary value, 0 for control and 1 for infected, and were then subjected to a PLS1 regression analysis. In Figure 2 panel a) shows a PLS-components score plot which shows clear discrimination between the control and 21-day infected fish plasma samples. Panel b) shows the residual variation that is described by the PLS components across the range of spectral frequencies modelled (from which variation of specific components can subsequently be identified). Panel c) shows the proportion of variation described by the first six PLS-components. Panel d) shows the predicted vs. actual binary values used to describe the two groups, demonstrating that the model accurately predicts membership of the appropriate variable grouping.

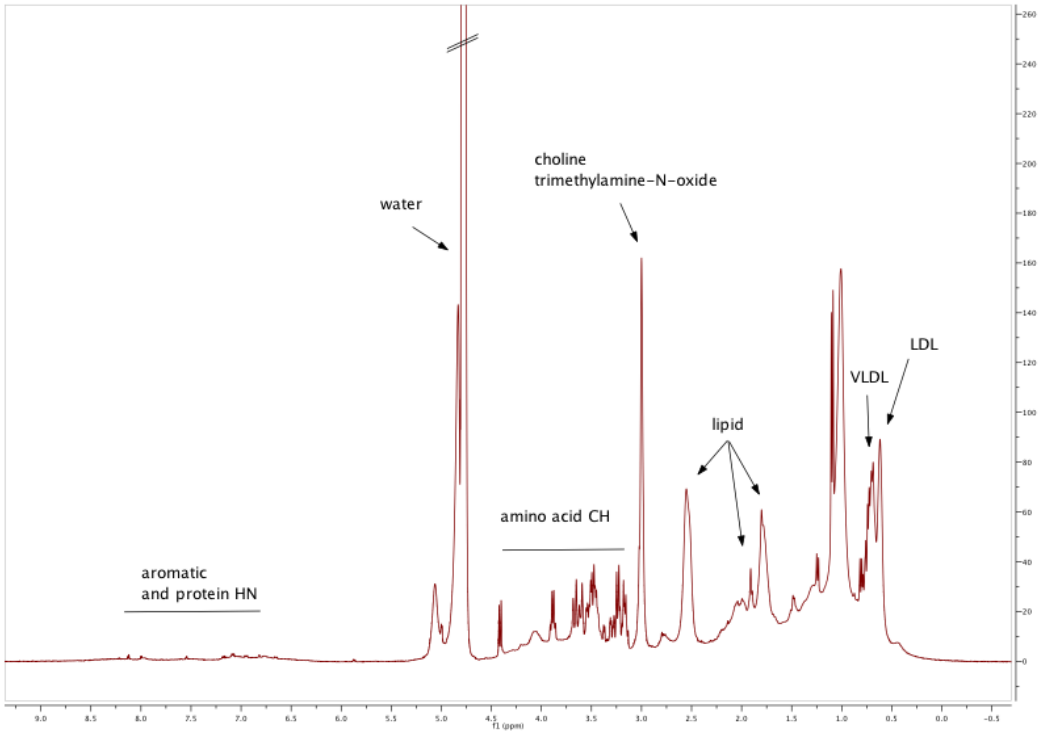

Figure 1. ^{1}H-1D spectrum of salmon plasma (150uL in 300uL D2O) acquired at 400MHz and 4 C. ^{1}H chemical shift values are shown on the horizontal axis and signal intensity on the vertical axis. Characteristic chemical shifts for some example metabolites are indicated.

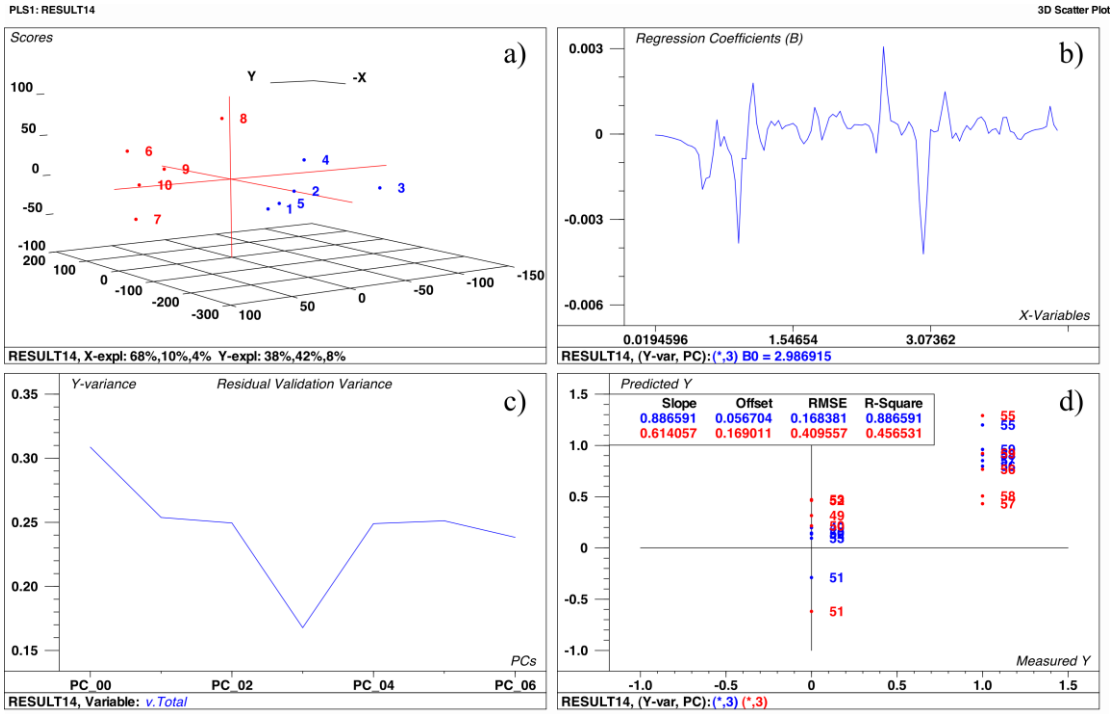

Figure 2. PLS component score plots for control vs. 21 day infected fish populations: a) PLS components score plot (control – blue, infected – red; b) residual variation in regression coefficients that is described by the PLS components across the spectrum; c) proportion of variation described by the first six PLS-components; d) predicted vs. actual binary values used to describe the two groups.