# Investigations on the immunology of stressed abalone

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# Investigations on the immunology of stressed abalone

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By

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**OBJECTIVES:**

1. To establish the most useful immunological parameters of abalone haemolymph (blood), and the best methods to use to assess immune function in abalone on abalone farms.
2. To determine the effects of a series of common on-farm stresses by monitoring the changes in immune function and histology (micro-structure of body tissues).
3. To establish a list of repeatable laboratory tests on abalone haemolymph and see if these indices correlate with the development of histological changes.

**OUTCOMES ACHIEVED TO DATE**

1. A set of well-described tests that can be applied on farms is now available, to assess the immune status of abalone. These methods can be employed to compare different management procedures and how much stress they are likely to cause to their stock.
2. The project has led to a better understanding of the nature and effects of summer heat stress, and the stress that occurs during stock movements, as a result of experiments conducted on a farm.
3. These methods to evaluate stress and model designs of on-farm experiments and analyses have established a process for future trials of stress reduction strategies. We provide a pathway for many more outcomes to come.
4. Farmers have attended a training session to set up a surveillance program. Several Victorian and South Australian farms now use diagnostic pathology services set up as a result of this project. The response at Abalone Growers Association workshops where the work was presented has been very favourable.

**NON TECHNICAL SUMMARY:**

In aquaculture, as in other kinds of stock production, outbreaks of disease can damage profits both due to large die-offs of stock and due to reduced growth during periods with low grade disease. Because abalone farming is relatively new, little is known of how abalone protect themselves – their immune sytem, or of how stresses, such as warm water in summer or being moved from one tank to another, might reduce their capacity to resist infection.

This project set out first to find the best ways to measure how well an abalone's immune system is functioning, and to develop and define these assays so that they can be used to monitor the effects of procedures on abalone on farms. We have shown that small samples of blood can be drawn from abalone and small amounts

used in three reliable assays that measure different aspects of stress or disease resistance. Together these provide a good assessment of immune function.

Next, experiments and monitoring were done using these assays, to discover how much abalone immune defences are affected by some common stressors on farms, namely elevated summer temperatures and stock movement. Moving abalone from one tank to another when the tanks become too full as the abalone grow is a stressful process. Part of the movement procedure is anaesthetizing the abalone to release their grip on the tank floor. We showed that one of the commonly used anaesthetics was the most stressful part of the movement process on the farm studied, although handling during movement is also important. This will prompt farmers to find less stressful ways to move abalone.

In summer, when water temperatures rise, farms often experience increased mortality. We found that abalone are only affected by a severe elevation in temperatures and they apparently can adjust to heat stress. This suggests that the summer mortality problem involves not just elevated water temperatures but other factors are likely to be involved that were not part of this investigation. Other possible problems not covered in this project include water quality problems such as decreased dissolved oxygen and increased numbers of bacteria in the water. Further work on these factors, by farmers via management experiments, or in a further project, is recommended.

Our work also defined and trialed a large number of laboratory tests, as well as the on-farm assays; and we examined the ways in which the tissues of abalone are damaged by stress, which could affect the long-term recovery of the abalone. While the assays used on the farm showed different results with different stressors, the tissue damage found was similar for various types of stress, although quantitative scoring might reveal differences.

We have designed experiments to work on the farm and have shown how to use powerful methods to analyse the information. Others can now use our designs, methods and assays to investigate other situations on farms where stress reduces stock survival or growth.

We have also provided farmers with a review of what was known about how abalone immune systems work and our subsequent results, plus a glossary of technical terms, so they can build on this knowledge. Further, the publication of our review and experiments on farms provides a stimulus and a foundation for more work elsewhere that will further help farmers to grow abalone more productively, and with fewer disease problems.

**KEYWORDS: abalone, aquaculture, immune function, stress assays.**

## ACKNOWLEDGMENTS

This work would not have been possible without the extensive support and facilities provided by Great Southern Waters abalone farm (GSW). We wish to thank in particular Anton Krsinich, Adam Clark and Matt Van der Graaff from GSW. A number of other GSW staff helped over the years in different ways. We acknowledge also support from Coastal Sea Farms, Southern Ocean Mariculture, and the Australian Abalone Growers Association. The support and encouragement of Ann Fleming was pivotal in setting up this study. We thank also Gribbles Veterinary Pathology, Flinders University, and The University of Melbourne for the use of facilities, and especially John Ahern and Bruce Abaloz (Zoology Department, University of Melbourne) for help with aquarium experiments and histology, and Dr Jeremy Carson (DPIWE, Launceston) for assistance with the preparation of bacterial cultures. We are grateful to Julian Goulias, who came to Australia from France to undertake a 'stage' in his Masters Degree with Rob Day, and who set up some of the biochemical assays used in France. We are grateful also to a number of volunteers who assisted with setting up experiments and immune assays, including particularly Matthew Wassnig, Nicole Currie and Athol Whitten.

## BACKGROUND

Commercial aquaculture, like all farming, involves raising animals at relatively high densities. High densities however, increase the likelihood of transmission of diseases and other parasites between the animals, and the provision of plentiful food to the tanks can encourage opportunistic bacteria. Under these conditions it is important that the animals are in good physiological condition and able to resist infections. Resistance to infectious disease is an important role of the immune system.

Knowledge of the physiology and immune systems of the species in culture is essential for maximising health and productivity in aquaculture. The immune system provides the principle line of defense against a broad spectrum of pathogens, so that studies on immune functions can provide an indication of the potential to resist disease under different culture regimes (Benkendorff, 2003). This applies to any aquacultured species, but abalone have not been cultured for long and are not closely related to other species that are extensively cultured, so we have little knowledge of their immune systems or the diseases that may affect them. A national survey of diseases in abalone has been undertaken and a range of bacteria and protozoans were identified as likely infectious agents (Handlinger et al., 2003).

Disease will typically be manifested in a range of physiological and histological changes to the animal that can be monitored directly. In the abalone industry however, the characterization of disease problems and growth rates under different culture conditions is still in its infancy. When for example a water quality problem results in mortality and poor growth rates, the problem has not been characterised in the abalone itself. This has led to ad hoc improvements in husbandry rather than systematic improvements based on an understanding of the animal and its responses to stress. We need to understand how various stresses that arise on abalone farms will affect the immune function of abalone, and thus their resistance to disease.

The stress response is the mechanism by which animals, including abalone, maintain homeostasis after exposure to physical or biological changes. Part of this response is to divert energy away from non-essential processes, such as growth, toward bioenergetic processes, which will help the animal to survive the threat (Malham et al., 2003). In all animals investigated, it appears that stress will also have a direct effect on immune functions and consequently stress can alter the animal's susceptibility to disease. Previous studies have shown that stresses such as mechanical handling and high stocking densities can lead to increased mortality due to bacterial infections (Wells and Baldwin, 2000; Vanderpeer, 2003). More recently, Malham et al. (2003) demonstrated a direct link between handling stress and immunity in the European abalone *Haliotis tuberculata*. More research is required on *Haliotis* spp., particularly those used in Australian aquaculture, in order to assess the links between immunity and various conditions of stress.

An abalone aquaculture health monitoring program that began in South Africa before this project commenced, characterized some of the histologic changes seen in abalone with differing stresses, including nutritional problems, water quality problems, overstocking and acute severe stress e.g. due to cessation of water flow (Mouton, 2003a,b). Histologic changes commonly have associated biochemical changes, which frequently precede the morphologically visible changes. Thus, it would be valuable to link Mouton's work on abalone histology with work on the underlying physiology and immunology.

A range of in vitro assays are available for monitoring the immune system of invertebrates (Benkendorff, 2003). These have the advantage of non-destructive sampling, as only a small sample of the haemolymph is required. Because the amount of abalone blood needed is small, the abalone survives and can be returned to the tank. Immune assays have been

previously applied to for example prawn aquaculture, to determine the optimal conditions of pH, temperature and salinity for maximal immune function (Cheng and Chen, 2000), as well as for assessing the value of introducing dietary additives that increase immunity, such as b-1,3-glucan into aquaculture feeds (Chang et al., 2000; Li and Shiau, 2001).

The work by Malham et al. (2003) demonstrated that immune assays can also be applied effectively to studies on abalone immunity. Their simulated mechanical handling of abalone was found to result in transient immune changes, with altered haemocyte density, migratory activity, phagocytosis and intracellular super oxide production. But Malham's work was done in a laboratory, where the shaking stress may be different from that which occurs on farms, and where complex laboratory analyses could be undertaken easily. Further work is required to determine which assays are optimal for routine health monitoring on Australian abalone farms, and how the actual stresses that occur on farms can best be studied, using convenient assays that are based on haemolymph extracted at the farm.

It is also important to have a range of measures of immunity and stress. The literature has demonstrated that different stressors evoke different responses and these responses may or may not impact on immunity. If a parameter is influenced by stress but does not reflect immunosuppression, then we need to know its limitations, as it may give farmers false information on the risks of farm processes. For example if we carry out an NRR assay and find it indicates a rise in temperature is stressful, then we need to see if other tests also indicate the immune function of abalone has been reduced. Further, some immune function measures may relate to immunity from bacteria, while others may indicate immunity from viruses.

This project was developed in consultation with the Abalone Aquaculture Subprogram Steering Committee and industry representatives to address the problems mentioned above. The approach to investigating the problem was discussed in a meeting at the 10th annual Abalone Aquaculture Subprogram Workshop in Pt Lincoln. The industry representatives included Steve Rodis and Anton Krsinich from Great Southern Waters, Mark Gervis from Southern Ocean Mariculture, Megan Russell from Southern Australian Seafood, and Shane McLinden from South Australian Mariculture Abalone. Also present were and Adam Butterworth, Ann Fleming, Judith Handlinger, Kirsten Benkendorff, Anna Mouton, Matt Landos and Celia Hooper.

## NEED

Disease is a significant issue for abalone aquaculture: significant mortalities result in substantial financial setbacks on some farms (Fleming, 2003). In southern Australia, mass mortalities due to summer high temperatures are of serious concern. In all Australian farms some mortalities result during handling and transportation. As a result, health is identified as a strategic research area required to develop a profitable industry in the Abalone Aquaculture Subprogram Strategic Plan. Particular needs identified were to "increase and apply knowledge of programs to survey the health status of stock on farms" and to "increase and apply knowledge of stress, its effect on production performance and strategies to minimise stress during production".

This project will contribute directly towards these aims, first by establishing methods to measure parameters of immune function in abalone that can be conveniently used on farms, so that future investigations or monitoring on farms can use these methods. Also, we will determine how stressors, nominated by farm managers as common in normal abalone farm processes, affect the immune system. This should lead to specific recommendations for minimizing stress or maximizing health under stressful conditions.

This research will pave the way to maintain health and provide for more secure productivity in Australian abalone aquaculture. By trialing various physiological and immunological assays we can find the most efficient and reliable set of parameters for future studies, and establish the best methods for use on farms. We will also establish the natural variation in these, and thus provide background information on normal ranges of parameters, needed when investigating any aspect of abalone husbandry.

Another goal of the FRDC subprogram strategic plan is "To improve the profitability of production", through increased growth and survivorship. By testing the effects on abalone immunity of stresses that occur due to husbandry such as stock movement, or environmental stress such as summer heat, we should be able to make recommendations for maximizing survivorship and productivity, as well as provide a process to evaluate what changes will most reduce stress and reduce disease risk and future growth. Currently husbandry practices are monitored via mortality and poor growth. These are very crude measures of problems on the farm, and occur well after the event, which prevents any proactive response.

## OBJECTIVES

1. Establish the best methods to use to measure immune function in abalone on abalone farms.

Many methods have been used, including hemocyte counts, phagocytic ability, migratory activity, serum antibacterial activity, agglutination, intracellular and extracellular reactive oxygen species (superoxide anion production), lysozyme activity, prophenoloxidase activity, and serum biochemistry tests; including levels of protein, glucose, iron, calcium, potassium, LDH, HCO3, sodium and chloride. Many of these methods are very sketchily described in the literature, as well known methods used on vertebrates have simply been adapted for use in invertebrates. What is most needed is a range of well-defined methods that can conveniently be used on farms, and that will reliably assess various components of immune function.

The first objective was originally proposed as establishing the normal range of immune parameters in abalone haemolymph. In vertebrates the normal ranges of immune parameters are narrow and well known, and used to assess samples from diseased or stressed animals. But we had not appreciated how little was known in abalone of which assays might be closely related to immune function, and that immune parameters in abalone may vary widely over time and between farms, for reasons that we do not yet understand. Thus we subsequently gained approval for objective 1 as written above and we have achieved this. We have used the controls in our experiments to assess the ranges of useful immune parameters.

2. Determine the effects of a series of controlled stressors by monitoring the immunologic and histologic changes. The stressors will include; temperature changes, anaesthetics, mechanical stress and time out of water.

This objective has guided most of the work in this project. The stresses we have focused on are those that appear most important to farmers. These are heat stress, which occurs as pumped water in tanks heats up over summer, and the stressors associated with stock movements between tanks, which include both anaesthetics and the combination of mechanical stress and time out of water during collection and movement.

3. Establish a list of repeatable laboratory tests on abalone haemolymph and see if these clinical pathology indices correlate with the development of histologic changes which have already been observed (Mouton's South African work).

This objective has been achieved by establishing and trialling a large number of laboratory tests, as shown in the Methods, but we heard from growers that they wanted convenient on-farm tests, and realised too that experiments done in the context on farms would provide far more relevant information than laboratory based tests, and thus would add considerably to the knowledge gained from laboratory studies. We have investigated the relation of on-farm assays to subsequent histologic changes in abalone, but many of the histological changes were fairly nonspecific.

## METHODS

As the methods used in research on abalone around the world vary, and are often incompletely described, we provide here details of all aspects of the methods used. For example, haemolymph can be extracted from abalone in many ways: by inserting syringe needles into the cephalic sinus behind the head, into the pedal sinus in the middle of the foot, into the pedal sinus at the rear end of the foot; or even by slicing the head region. Our preference was extraction from the pedal sinus because we found it was the easiest method.

We trialed a large number of methods, such as flow cytometry and numerous biochemical assays such as the acid phosphatase and phenoloxidase (L-DOPA) assays. Previous experimental work by Cheng *et al.* (2004c) and Shuhong *et al.* (2004) have specifically related haemocyte counts, phagocytosis, phenoloxidase, acid phosphatase and lysozyme assays to survival rates after infection with pathogenic bacteria. This indicates these assays reflect the immune status of abalone, and can be used by or for farmers to monitor stocks. The MTS assay measures directly the suppression of pathogenic bacteria by the haemolymph after the cells have been extracted (Vakalia and Benkendorff, 2005).

The neutral red retention assay and some enzyme assays still require validation in abalone by comparing results to mortality rates in infection trials. Without validation, there is no guarantee that a change in these parameters translates into altered immune function in abalone. In the assays described below we have selected and used convenient methods for on-farm use, preferably validated assays. Importantly, these include measures of both cellular and non-cellular immune functions. Cheng *et al.* (2004c) validated the phagocytosis assay using pathogenic bacteria, not zymosan, which is derived from killed yeast, but phagocytosis assays using zymosan have been developed in other species (Ordas et al. 2000) and used in abalone immune studies (Chen et al. 2005), so we are confident that this assay, which is much more easily used, reflects immune function.

### *Bleeding Abalone*

Abalone were usually removed from tanks by 'chipping': sliding a flat plastic blade beneath them quickly, so that they had no warning and had not clamped to the surface. Abalone were bled as soon as possible after chipping, using 1 ml plastic syringes with 25 gauge half-inch needles. Haemolymph was routinely taken from the middle section of the pedal sinus.

Repeated needle sticks and slow suction of haemolymph into the syringe was commonly found to be associated with clumping of the haemocytes, so that we took note of the speed and ease of bleeding. We only used samples if the haemolymph was obtained within 1 minute with less than three needle stick entries into the pedal sinus. Abalone that were difficult to bleed were discarded. As the numbers of these were very small, we are confident this selection could not lead to any significant bias towards more or less healthy abalone in our experiments.

### *Temperature control incubator*

To carry out the two slide-based assays described below on a farm, a simple incubator was used to keep the temperature of the slides close to that of the water that the abalone were in, so as not to shock the haemocytes after removal from the abalone. The incubator was a polystyrene box with a perforated polystyrene shelf 2 inches below the top of the box. Slides are placed on the shelf with a thermometer to allow easy monitoring of the temperature in the incubator. The base contained ice and fresh water. The amount of ice added depended on the temperature needed. To keep the incubator within 2 degrees of the temperature of the farm's tank water, more ice was added on hot days and only water on cold days.

***Balanced Salt Solution used for assays***

A balanced salt solution is required for the phagocytosis and NRR assays. A number of published recipes for saline and also seawater were tested using the very sensitive NRR assay (method described below), to determine which one maintained haemocyte lysosomal membranes better (and thus affected the haemocytes least). The oyster saline recipe given in Zhang et al. (2006) Aquaculture 256: 502-509 was the best of the published saline recipes (Table 1). This recipe was then tested against sterile (autoclaved) filtered fresh seawater in which the abalone were stocked and the seawater was found to be better.

**Table 1: Recipe for Oyster Saline from Zehu Zhang et al. (2006).**

| | |
|---|---|
| 0.48 g | $CaCl_2$ |
| 1.45 g | MgSO4 |
| 2.18 g | MgCl2.6H2O |
| 0.31g | KCl |
| 11.61g | NaCl |
| 0.35g | NaHCO3 |

Add the ingredients into 1 litre of distilled water and keep at $4^0$ Celcius.

Oyster Saline, Abalone haemolymph and Filtered Seawater were compared using Gribbles Veterinary Services biochemistry equipment. The abalone haemolymph was taken from abalone that were acclimatized to the seawater that was tested. Abalone are osmoconformers, which explains why the seawater had closer NaCl levels to abalone haemolymph than the oyster saline recipe (Table 2), and presumably for this reason was associated with better membrane stability as demonstrated by the NRR assay.

**Table 2: Analysis of alternative salt solutions for assays.**

| | K mmol/L | Na mmol/L | Cl mmol/L | Cu umol/L | PO4 mmol/L | CO2 mmmol/L | Ca mmol/L | Mg mmol/L |
|---|---|---|---|---|---|---|---|---|
| **Oyster saline** | 4.3 | 210 | 237 | 3.11 | 0 | 4 | 3.2 | 6.8 |
| **Filtered Seawater** | 11 | 457 | 598 | 3.34 | 0.02 | 2 | 10 | 7.79 |
| **Abalone Hemolymph** | 12 | 520 | 656 | 208 | 0.11 | 6 | 8.3 | 4.13 |

***Haemocyte Counts***

Haemocytes are the central cell of the immune system in abalone and other invertebrates. They are involved in phagocytosis, production of antibacterial factors, and the stress response. A count of the haemocytes in a constant volume (using a haemocytometer) shows the density of these cells in the blood, and has been used in other animals to measure immune responses. Note however, that the circulatory system in molluscs is open, meaning that the haemocytes and fluid move through tissues and organs, and the sinuses are simply larger channels where the fluid can move rapidly. Thus when haemolymph is extracted from the sinus of a mollusc, the density of cells represents the relative numbers that are circulating rapidly, as opposed to lodged within the tissues.

We found that the haemocyte count varies with the age of abalone and probably species, so that controls are needed to interpret the counts. Nevertheless the counts can provide very useful information: for example abalone infected with the ganglioneuritis virus (AVG), were found to have very reduced haemocyte counts. In this case, we know that the haemocytes accumulate in and around the ganglia (Hooper et al. 2007), where the virus that replicates in the nerve cells causes severe damage.

Fresh hemolymph was used to count haemocytes. The cells were counted within 5 minutes of the hemolymph being removed. The hemolymph was added into both sides of a standard

Neuerbach Hemocytometer Counting Chamber. The cells in 5 squares of the chamber were counted on both sides. The mean was taken and the total count multiplied by 5 x $10^4$ to give the cell count per ml.

One way in which haemocytes respond to foreign objects (not from the abalone itself) is to adhere to those items and to each other, forming a clump. In the abalone, this would lead to haemocytes surrounding and isolating the foreign object. The surface of a syringe or haemocytometer is such a foreign object, and over time, haemocytes begin to clump. Some researchers conduct assays on haemocytes mixed with anti-coagulation agents, such as Alseviers solution. We preferred to examine the cells in as natural and fresh a state as possible.

An assessment of clumping was recorded as well as the count itself. The assessment was subjective. We used 1+ to refer to clustering of cells without adhesion to the surface and with no interference with counting. 2+ was used when there were small clumps which did not interfere with the ability to count the cells. 3+ records dense clumps of up to 20 cells that could not be counted accurately. Any samples containing large dense clumps with greater than 50 cells were discarded.

***Phagocytosis Slide Based Assay***

The aim of this assay is to assess *in vitro* (in the fluid on the slide) the capacity of haemocytes to phagocytose foreign particles. Phagocytosis is a key mechanism of immune defense in invertebrates. The *in vitro* tests that have been published include slide-based methods and methods using haemocytes suspended in an anticoagulant solution and counted via flow cytometry. Both involve artifacts, but the slide-based system interferes less with the haemocyte's ability to recognise and engulf a foreign body. On the other hand, slide-based methods require great care to ensure the methods are accurate and repeatable.

Systems holding haemocytes in solution need to use anti-coagulant additions to stop the haemocytes clumping. The cellular systems involved in clumping are similar to those involved in phagocytosis, so that anti-coagulants will diminish the haemocytes' ability to engulf foreign organisms. The slide-based system used in this series of experiments may alter the arrangement of receptors on the cell surface, because the cells recognise the glass surface as foreign and adhere to it, but there is little other interference with the cells.

The slide-based assay involves adding fresh haemolymph containing haemocytes to a glass slide, allowing the haemocytes to adhere to the glass surface, then adding a foreign body such as yeast in solution onto the slide to mix with the adherent haemocytes. After a fixed incubation time the slides are fixed in formalin-seawater, stained, and examined under light microscopy. The percentage of haemocytes that have phagocytosed the yeast is measured.

We added a yeast solution called zymosan, which is a commercially available yeast, commonly used to incite phagocytosis in vertebrate and invertebrate phagocytic cells (Sigma #Z4250, Zymosan A from *Saccharomyces cerevisiae*). Zymosan particles were counted in a standard Neubauer Haemocytometer Chamber to obtain a concentration of 1 x $10^8$/ml.

Each abalone was removed from the tank with a spatula and immediately bled. Approximately 40µl of haemolymph was placed onto a Poly-L-Lysine$^R$ glass slide and the slide was then placed within the incubator for 10 minutes. After 10 minutes, 40µl of the zymosan solution was added onto the haemolymph. The zymosan was added in several small drops placed multifocally over the haemolymph droplet in order to more evenly distribute the zymosan and increase contact of individual zymosan particles with individual haemocytes. The smears were incubated for 30 minutes then placed into seawater formalin

to fix for 10-20 minutes. The smears were then stained with May Grunwald Giemsa stain and coverslipped. Two smears were made for each abalone sampled.

100 haemocytes were counted in each smear and the number of haemocytes that had phagocytosed zymosan was recorded, and expressed as a percentage. The sites on the smear chosen for counting were those areas with a monolayer where the cell margins were distinct. Single cells and small clusters were both selected for counting. Thick clusters were excluded from the count. Only those clusters where the cell margins were reasonably clear were included in the count.

***Neutral Red Retention Assay***

The NRR assay measures the retention time of neutral red dye in the lysosomes of haemocytes. Lysosomes are organelles that store reactive enzymes in the cell that may be used to destroy foreign organisms. They are involved in immune responses such as phagocytosis. The membranes around the lysosomes appear to become more permeable when the abalone is stressed, so that water enters and the lysosomes swell, then the dye leaks out. The assay has been evaluated in other invertebrates as a measure of stress that may be related to immune function. For example in oysters there is published data on the effect of various stressors such as temperature and salinity changes, on the stability of lysosomal membranes. The method below is a modification of methods in the literature based mainly on the method described by Zehu Zhang and Xiaoxu Li in their work with oysters (Zhang et al. 2006).

The assay has been done in various ways. One method is to record the time for more than 50% of haemocytes to have swollen lysosomes. Another method is timing when the dye is lost from the lysosomes into the surrounding cytosol. When trialled on-farm, this latter method could take 2-3 hours, and this was deemed too long for a useful on-farm test. In this series of experiments, the time it took for lysosomes to swell to > 7-8 times their normal size was used as the endpoint of the assay.

Neutral Red Stock Solution composed of 2.28 mg of neutral red powder was dissolved in 1ml of dimethyl sulphoxide (DMSO). The working solution was 20µl of the stock solution added to 1ml of filtered sterilised seawater.

Fresh haemolymph was drawn from an abalone and approximately 40µl was added to a Poly-L-Lysine$^{R}$ glass slide. The slide was put in the incubation chamber for 10 minutes to allow the haemocytes to adhere. Excess haemolymph was poured off, 40µl of working solution was added, a coverslip was placed on top, and the slide was returned to the incubation chamber. The smear was checked every 15 minutes until > 50% of the cells had swollen lysosomes. The time at the last 15 minute check was taken as the endpoint.

***MTS antibacterial Assay***

This assay assesses antibacterial levels in the haemolymph plasma (the fluid without the cells) (Valkalia & Benkendorff, 2005). It is based on the reduction of the MTS tetrazolium compound to a red formazan product by dehydrogenase enzymes in metabolically active bacterial cells. The multiscan plate reader works on a similar principle to a spectrophotometer. The filters needed are 492nm for antibacterial assays, and 592nm for protein. An increased absorbance on the spectrophotometer indicates a higher level of active bacteria present. Dilutions of the haemolymph are used to obtain a good range of absorbance, from which antibacterial activity in the haemolymph can be calculated. The assay requires a well-equipped laboratory, but abalone blood samples can be kept on ice until they can be frozen at -80ºC, then kept frozen on dry ice during transportation, and defrosted immediately before use. We used Kirsten Benkendorff's laboratory at Flinders University of South Australia. The assay provides a direct, relevant measure of non-cellular

immunity: the ability of the plasma to kill pathogenic bacteria. The recipes are explained below:

Agar
1. 25 grams Oxoid Nutrient Broth No. 2 CM0067.
2. 12 grams Agar.
3. Add to 1000ml of distilled water.
4. Autoclave at $121^0$C for 15 minutes (fluid cycle).
5. Keep the agar warm until poured into petri dishes or it will set in the bottle.
6. The agar plates can be kept in a fridge for several weeks.
- put them in a plastic bag closed with sticky tape and label with the date.
- check for contamination and drying out (shriveling and cracking).

Nutrient Broth
1. 25 grams Oxoid Nutrient Broth No. 2.
2. Add 1000ml distilled water.
3. Autoclave as above.
4. Keep in a flask with cotton wool and foil.
5. Label the date on the flask. Use within a week.

MTS Reagent
Cell titre 96 Aqueous One Solution Reagent (Promega).
Keep frozen at $-20^0$C.
As it is light sensitive, aliquot small samples (~1ml) into eppendorf tubes wrapped with foil, and use these.

Bacterial cultures
Bacteria pathogenic to abalone must be used; typically for abalone *Vibrio harveyi* or *Vibrio anguillarum*, which have been isolated from disease outbreaks on abalone farms. These are ordered from Dr Jeremy Carson, and delivered freeze dried to an institution with import approval from the state Chief Veterinary Officer.
1. Add the bacteria to 10ml nutrient broth in a McCartney bottle to grow them.
2. Incubate overnight at 25ºC (30ºC can be used for faster growth) on an orbital mixer shaker at 200rpm.
3. Add 15% glycerol to the densely growing cultures
4. Subdivide into 1ml batches in cryogenic vials, freeze at $-80^0$C.
5. Keep these stock solutions at $-80^0$C, subculture for assays as described below.

Preparation of bacterial broth for assay
You need to check the culture is uncontaminated by plating out a loopful of frozen culture onto marine agar, using the streak culture method to isolate a pure colony. Also, the bacteria must be growing exponentially for the assay, because metabolically active cells are required for the assay. Some antibacterial factors kill any bacteria whether in the growth phase or not, but other antibacterial factors kill only growing bacteria, and these will appear not to be working in the MTS assay if the bacteria are not in growth phase. Bacteria in the McCartney bottle will be in stationary phase after incubating overnight because they have used up all their nutrients. When these bacteria are put into new broth, they will grow exponentially again, then will reenter stationary phase when they have run out of nutrients.

1. At least two days before, transfer a stock culture of bacteria from the $-80^0$C freezer to the biosaftey cabinet in an esky of dry ice (if possible, otherwise use ice).
2. Streak plate a loopful of the bacteria onto Nutrient agar (or Hastings or Marine agar); incubate at $30^0$C until clear colonies are visible on the plate (overnight may be sufficient but usually 2 days growth required for *Vibrios*).

3. Take a single colony from the plate and put it into ~ 10ml fresh broth in a McCartney bottle to make the working solution for the assay. Incubate at $30^0$C overnight on an orbital mixer shaker at 200 rpm.
4. Read the bacterial broth culture in a spectrophotometer at λ 600nm.
- To zero the machine prior to reading the turbidity of the sample, place broth alone with no bacteria in a cuvette into the spectrophotometer first.
- Put 1ml culture into a cuvette and read.
A well growing overnight culture should have an absorbance reading of > 1.
5. To get the culture back into exponential growth phase, dilute to an absorbance (optical density) of approximately 0.1 with fresh broth.
6. Then return the McCartney bottle with fresh broth and bacteria to the incubator for 0.5-2 hours until the culture has an absorbance reading of 0.2 at 600nm. This will ensure the bacteria are in exponential growth phase when used in the MTS assay.
7. If the absorbance exceeds 0.2, dilute with more broth until a reading of 0.2 is achieved. This equates to approximately $1.3\times10^9$ cells $mL^{-1}$ for use in the antibacterial assay.

Checking Bacterial Contamination of the Haemolymph

1. Make one plate per abalone. Each agar plate is labelled and divided in 2 to provide a replicate count.
2. Vortex each abalone sample to re-suspend any bacterial cells.
3. A loop of haemolymph or 10µl is taken - plated out onto an agar plate routinely.
4. Incubate at $25^0$ or $30^0$C and check for bacterial colony growth each day for a few days.
5. Count the bacterial colonies. Circle each colony that grows to aid the count.
Count colonies in each replicate and take the mean (= #colonies / 10µl haemolymph). Then multiply by 100 to get the number per ml of haemolymph (CFU/ml; CFU is a Colony Forming Unit).

Testing of Haemolymph

1. Spin the haemolymph samples down and pipette off the plasma. Keep the cell pellet in a fridge in an eppendorff vial.
2. As shown in Table 3, pipette the haemolymph samples from each abalone into the plate wells. As explained below, 90% haemolymph concentrations were used, so that 90µl of haemolymph was added to each treatment well and negative control (haemolymph incubated with broth alone). Positive bacterial controls had 90µl of broth added.
3. 10µl of bacterial culture was then added into each treatment well and positive bacterial control well. The negative control wells had 10µl of broth added.

**Table 3: Example arrangement of haemolymph samples and other components in the MTS plate wells. (Treatments: 90µl haemolymph sample+10µl bacterial cell culture, BC control: broth and bacterial cell culture positive control, with 90µl of broth +10µl bacterial cell culture).**

| | *1;* | 2; | 3; | 4; | 5; | 6; | *7;* | 8; | 9; | 10; | 11; | 12; |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Treatment 1, Ab#1 | Treatment 1, Ab#2 | Treatment 1, Ab#3 | Treatment 1, Ab#4 | Treatment 1, Ab#5 | | Treatment 2, Ab#1 | etc | etc | | |
| A | *BC Control* | replicate A | replicate A | replicate A | replicate A | replicate A | *BC Control* | replicate A | | | | |
| B | *BC Control* | replicate B | replicate B | replicate B | replicate B | replicate B | *BC Control* | replicate B | etc | | | |
| C | *BC Control* | replicate C | replicate C | replicate C | replicate C | replicate C | *BC Control* | replicate C | etc | | | |
| D | *Broth and Haemolymph negative Control: 10µl broth and 90µl haemolymph - whole of line D* | | | | | | | | | | | |
| | | Treatment 3, Ab#1 | Treatment 3, Ab#2 | Treatment 3, Ab#3 | Treatment 3, Ab#4 | Treatment 3, Ab#5 | | Treatment 4, Ab#1 | etc | etc | | |
| E | *BC Control* | replicate A | replicate A | replicate A | replicate A | replicate A | *BC Control* | replicate A | | | | |
| F | *BC Control* | replicate B | replicate B | replicate B | replicate B | replicate B | *BC Control* | replicate B | etc | | | |
| G | *BC Control* | replicate C | replicate C | replicate C | replicate C | replicate C | *BC Control* | replicate C | etc | | | |
| H | *Broth and Haemolymph negative Control: 10µl broth and 90µl haemolymph -whole of line H* | | | | | | | | | | | |

4. The haemolymph plasma and broth are pipetted out first. Adding the bacterial culture last ensures that each well in the plate has a more equitable time in which bacteria are in contact with haemolymph antibacterial activity.
5. As an example, each plate will take 4 treatment groups of 5 samples from individual abalone, with 3 replicates of each sample, as in Table 3. Another arrangement might use 2 abalone per treatment and 10 treatments, again with 3 replicates of each sample.
6. Leave the plates 40 minutes minimum in the incubator at $37^0$C, then add 20µl MTS to each well.
7. Return to the incubator and check periodically for colour change.
8. Read on the plate reader with a 492 nm filter after about 1 hour, or after a visible colour change is seen. Background absorbance from the haemolymph negative controls is subtracted from the absorbances in the respective haemolymph samples (or broth controls) in treatment wells.

Calibration of Haemolymph concentration

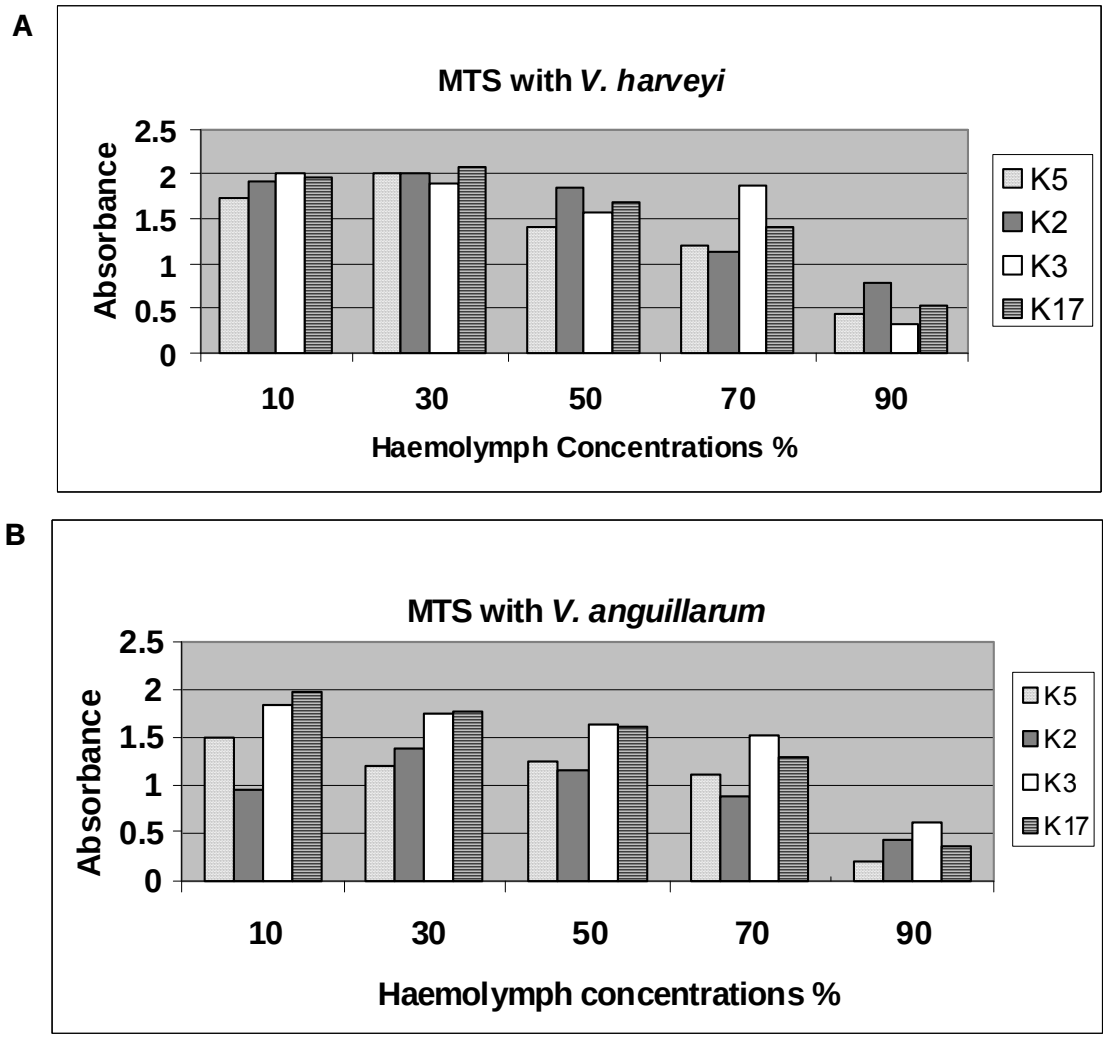
In order to calibrate the method and decide which concentration of abalone haemolymph to use routinely in this assay, a set of trials were run on haemolymph samples from an experiment on the effects of Flow Rate and Density on 2 year-old hybrid abalone at the Great Southern Waters farm in July 2006. The haemolymph antibacterial activity was tested against both of 2 standard pathogenic bacterial types encountered on farms; *Vibrio harveyi* and *Vibrio anguillarum*.

1. Haemolymph was sampled from 8 abalone per tank, from 4 different treatments (1 tank / treatment): Tank K5 - most stressed (low flow, high density); Tank K2 - least stressed (high flow, low density); Tanks K3 and K17 – intermediate.
2. For these trials, the haemolymph samples from each tank were pooled into two samples, A and B, from 4 abalone each. These corresponded to MTS plates A and B – one bacterium species was tested in each plate.
3. In place of the different abalone samples shown in Table 3, 10%, 30%, 50%, 70% and 90% haemolymph plasma concentrations were tested in each column of the plate. 10, 30, 50 etc. µl of haemolymph, plus appropriate volumes of nutrient broth were used, so that each well contained a total volume of 90 µl. Then 10µl of bacterial culture was added into each treatment well and bacterial controls. Negative controls include haemolymph incubated with broth alone.

The haemolymph antibacterial activity at each concentration was measured against the two bacteria by spectrophotometry as described. Figures 1A and B below show the reduction in absorbance against increasing haemolymph concentrations. The reduction in absorbance reflects decreased bacterial cell viability with increasing abalone haemolymph concentration. 90% haemolymph produced decreased absorbance (i.e. decreased bacterial growth) for all treatments, and therefore this concentration was used for all subsequent experiments. Note that this is a pilot, not a full experiment to investigate the stress treatments. For a full experiment replicate tanks of each treatment would be required.

The stress due to low flow and high density reduced antibacterial activity, but the differences between treatments were not consistent. This may be an effect of the tanks sampled, or due to variation between individual abalone. Later experiments have also shown that antibacterial activity of abalone haemolymph varies over time, and perhaps with the history of conditions in tanks.

**Figure 1: Absorbance of wells containing: A) *Vibrio harveyi* , and B) *Vibrio anguillarum*, with haemolymph at 5 concentrations, from 4 treatments.**




***Histopathology***

Histopathology has been one of the main methods used to examine mortality episodes and to monitor health, as it is useful in characterising some clinical disease. Infections with *Perkinsus*, *Vibrio* species and the AVG virus give distinct histopathologic lesions that are commonly diagnostic. The methods are well known, but require experienced histopathologists to identify and interpret lesions.

Abalone were dissected and sectioned after hemolymph had been withdrawn and the abalone weighed. The viscera, mouth-parts, and a transverse section of foot muscle were placed in 10% seawater formalin and left to fix for at least 24 hours. Sections of these tissues were then embedded in paraffin wax, placed in cassettes and processed routinely to make 4µm thick tissue sections using a microtome, which were stained with Haematoxylin and Eosin and coverslipped.

***Biochemical assays***

We describe below methods for biochemical assays that were evaluated in this project, but not used in experiments because they require complex laboratory equipment or expertise, while the methods we have described above appear more useful for on-farm work.

Nevertheless, it is often hard to find good descriptions of such methods in published papers, so we present them here to assist future work.

Protein determination:

The protein determination on centrifuged (plasma) and uncentrifuged haemolymph was used to calculate the level of activity of the different enzymes (described below) as a proportion of the haemolymph protein level. Haemolymph was centrifuged at 10G for 10 minutes at $4^0$C in a Heraeus Frisco 17 centrifuge. 10µL samples of haemolymph or plasma were placed in 96-well microplates (Flat Bottom Microtiter Plates, PathTech). 200µl of substrate was added, made of 1:51 ratio of Bicinchoninic Acid:Copper Sulfate (Bicinchoninic Acid SIGMA B9643-IL, Copper Sulphate SIGMA Aldrich C2284). The total protein concentration present in the haemolymph and in the plasma was measured spectrophotometrically using a Multiskan EX microplate reader (Thermo Electron Corporation) at 595nm. Bovine serum albumin (Sigma A7906) (0.0625 mg/ml - 10 mg/ml) was used to prepare the standard curve and from this curve, protein concentrations were estimated based on absorbance readings in the haemolymph and plasma samples.

Phenoloxidase activity:

Phenoloxidase (PO) activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA)[2] as modified from Asokan *et al*. (1997). Haemolymph was frozen and thawed three times prior to use in the assay, so as to burst open the cells. 100µl was then transferred in duplicate to 96-well microplates and 100µl of L-DOPA (30mM, L-3,4-dihydrophenylalanine, Sigma D9628 in HCl 0.2M, pH 8) added to each well. The microplate was rapidly mixed before each plate reading. Optical density at 492nm was recorded every 5min at 20°C over 30min, using a Multiskan EX microplate reader. A control, without haemolymph, but containing L-DOPA and TrisHCl buffer, was run in parallel; and the values subtracted from test values to correct for possible auto-oxidation of the L-DOPA. One unit of enzyme activity represents the change in absorbance $min^{-1}mg^{-1}$ protein.

Lysozyme activity:

Lysozyme is an enzyme found in lysosomes and among other roles has antibacterial activity. The method used is similar to Shuhong *et al*. (2004). 10µl of haemolymph was placed in duplicate into a microplate and 200µl of 0.02% *Micrococcus lysodeikticus* bacterial suspension (Sigma M3770) in 0.1M $KH_2PO_4$ pH6.24, buffer was added to each well. Lysozyme from chicken egg white (Sigma L6876) was used to create a standard curve (0.625 - 20ug/ml). The decrease in absorbance was measured at 30sec intervals for 15 min at 450 nm using a Multiskan EX microplate reader. Negative controls, without haemolymph, but containing 0.02% *Micrococcus lysodeikticus* bacterial suspension in $KH_2PO_4$ buffer, were run in parallel and these background absorbance values were subtracted from the relevant test values. Results are expressed in µg $mg^{-1}$ protein.

Leucine amino peptidase (LAP) activity:

LAP is a protease enzyme found in lysosomes.100µL of centrifuged haemolymph (10G, 10min, 4°C), was placed in 96-well microplates and LAP activity measured using the procedure described by Pfleiderer (1970), modified by Guerard *et al*. (1998) and used by Ford *et al*. (2006). To each well we added 75 µl of TrisHCl (0.2M, pH 8.0), then 25 µl of substrate; 10 mM leucine p-nitroanilide (SIGMA L2158, in TrisHCl, 0.2M, pH 8.0). The plate was then rapidly mixed before optical density was monitored at 4-min intervals for 20 min at 405 nm using a Multiskan EX microplate reader. The rate of colour change per ml per mg protein in the haemolymph plasma was considered a relative measure of LAP activity. Negative controls, without haemolymph, but containing leucine p-nitroanilide substrate and TrisHCl buffer, were run in parallel and the background absorbance subtracted from the test values.

Acid phosphatase activity (ACP):

ACP is an enzyme found in lysosomes. 20µl of haemolymph plasma was placed in each well, then 20µl of Sodium Acetate buffer 0.1M pH 4.5 (Sigma S8625) was added. The substrate p-Nitrophenyl phosphate disodium hexahydrate (SIGMA Aldrich N22002) was then added and the microplate was incubated for 30 min at 37°C. p-Nitrophenol (Sigma 1048) was used to create a standard concentration curve (2µM - 80µM). Acid phosphatase activity was spectrophotometrically measured at 405nm using Multiskan EX microplate reader. The control wells had 20µl of an inhibitor, Sodium Tartrate Dihydrate (Ajax Firechem A 513), which directly targets ACP instead of the Sodium Acetate buffer.

## RESULTS AND DISCUSSION

The first output of this study was a review of what was known about stress and immune responses in abalone, both from the very limited previous work on abalone and from work on related animals. We realized that much of this work simply assumed similar biochemical processes in invertebrates to the well studied system in mammalian vertebrates, so that it was important to work out which tests were known to provide information on immune function. Published work often ignored the fact that invertebrates are evolutionarily as different, or even more different, from each other than they are from vertebrates. Thus the review focused on which tests were useful and which other species were likely to have similar immune systems. The review guided our choice of tests used in stress experiments, which are described the Methods section of this report. The review was distributed to farmers, and is reproduced in Appendix 4 of this report. We then revised it for publication in the journal "Fish and Shellfish Immunology" (Hooper et al. 2006). As part of the review we prepared a Glossary, explaining technical terms for farmers. This is now expanded so that it covers all technical terms in this report, and is attached in Appendix 3.

The second result has been the development of methods, which are described above in the Methods section. These should provide the detail required for future researchers to readily undertake further work evaluating stressors on farms.

Further, we describe below results of experiments to evaluate the effect of specific stressors commonly found on farms. These cover heat stress, which occurs in summer because the water heats in the pipes and shallow tanks of farms, and many farms have reported summer mortality. The other major form of stress on farms occurs during stock movements, when abalone are either anaesthetised or chipped from tanks, and then handled as they are moved into other tanks at lower biomass densities or harvested. Movement sometimes involves grading to sort animals into size categories. The experiments described below attempt to separate and compare the effects of anaesthesia, of chipping, and of the normal movement process on the farm. All data were screened for outliers. We analysed the heat experiment results using ANOVA, followed by Tukeys test to compare means, at a 5% significance level. More complex comparisons of means are described for the Movement stress experiments.

Previous work overseas has tended to be in laboratories, where various stressors were simulated, but these may not match the stress situations that occur on farms. We have focused on on-farm experiments, undertaken at the Great Southern Waters Limited (GSW) farm. In the case of heat stress, however, one of our experiments used the recirculating aquarium system in the Zoology Department of the University of Melbourne, where experimental application of heat could be set up easily. All experiments used the GSW 'Jade Tiger' hybrids of *Haliotis rubra* and *H. laevigata*. Most experiments and monitoring involved abalone on the farm kept in concrete flow-through 'slab' tanks, 20 metres long and 1.8 metres wide, in which the stock were fed and the tanks cleaned following the usual routine on the farm. All experiments use the methods described above.

### Heat Stress – experiment and on-farm sampling, 2007.

Abalone were kept in water that was heated from 16º to 26ºC and left at this temperature for a week to see what effect prolonged severe heat stress has on a series of immune and physiologic parameters. The lower temperature was the ambient temperature of the water at the time the experiment commenced. The maximum temperature of 26ºC was selected because the farm on which the experiments were conducted often record temperatures of 24 - 25ºC during summer and on rare occasions, temperatures up to 26ºC are recorded. A

monitoring experiment was done on the farm in which abalone were sampled on cooler and hotter days during the summer of 2007 to see if the same effects were apparent.

*Animals and experimental design*

In the laboratory experiment, two year old hybrid abalone from GSW were used (mean weight = 53.89g, se ± 8.39). Abalone were transferred into 12 tanks containing 16°C water for 10 days to acclimatise before the experiment and fed with artificial food obtained from GSW. The tanks used recirculating seawater. Each 8 litre tank contained 8 abalone and had a flow rate of 10 litres per hour. At the start of the experiment, feeding ceased and the tanks were cleaned of feces and excess food. In 6 tanks, the water temperature was increased progressively from $16^0$C at 9.40am until 3.15pm when it had reached 26°C. The temperature was kept at this level for 7 days. The 6 control tanks were kept at the ambient $16^0$C. Abalone were first sampled on day 1 when the water had been heated, then subsequently on day 2 and day 7. On each of these days two abalone were removed from each tank, weighed, bled and then sacrificed; and samples were then put into formalin for histopathology.

The extra two abalone per tank were to provide extra animals in case some abalone were difficult to bleed. In two treatment tanks the heaters malfunctioned and the temperature elevated to $29.5^0$C in tank 1 and $28.7^0$C in tank 5. Tank 1 malfunctioned between day 1 and day 2 and was corrected back to $26^0$C. The remaining abalone in Tank 1 survived for another 5 days, but were all dead on day 6. Tank 5 malfunctioned on day 4 and was not fully corrected. The temperature was $28.7^0$C and $27.2^0$C on day 5 and 6; and the abalone were all found dead on day 6. On day 7, abalone that would have been sampled from tanks 1 and 5 were taken from tanks 2, 3, 4 and 6.

Abalone that were difficult to bleed were discarded. Haemolymph was collected within 1 minute in less than three needle stick attempts. The haemocyte counts, smears for phagocytosis and NRR assays were done the same day as the collection of haemolymph. An extra 2-3mls of haemolymph was collected from each animal and frozen in eppendorf tubes at –80°C for work on enzyme activities, biochemistry and the MTS assay.

On-farm, two year old hybrid abalone (63.7g, se ± 1.2) were sampled on a range of days, selected on the basis of water temperature (Table 4), to gain data on a variety of cooler and hot summer days, typical of the variable weather. The temperatures became consistently high in mid to late February. 13-25 abalone from one cohort were sampled randomly from 24 tanks, bled and assayed as for the laboratory experiment.

**Table 4: Sampling dates, temperatures and number of abalone collected from Great Southern Waters Ltd for the farm-based experiment.**

| Dates | 10.1.07 | 30.1.07 | 5.2.07 | 17.2.07 | 28.2.07 | 3.4.07 |
|---|---|---|---|---|---|---|
| Water Temp pm $^0$C | 22 | 20.4 | 23 | 24.5 | 22.5 | 19 |
| Air Temperature $^0$C | 9-37 | 25-26.9 | 27-34 | 28-38 | 24-26 | 22 |
| Number of Abalone | 15 | 13 | 15 | 13 | 15 | 25 |

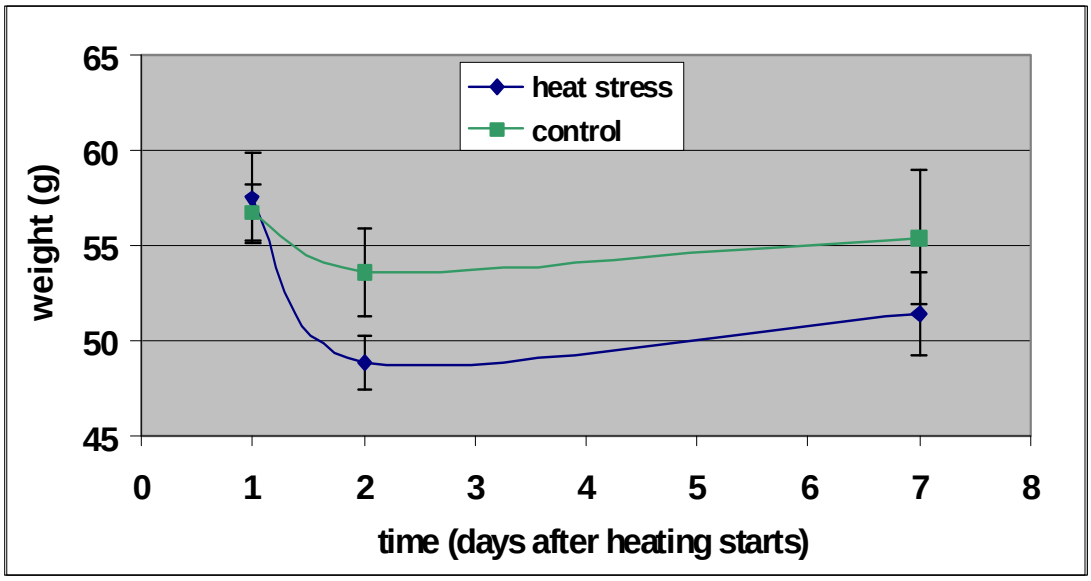
*Observations of laboratory animals*

On day 1, within the first few hours after the temperature started to elevate, there was more mucus in the water in the 6 heat treated tanks and the abalone defecated. They were initially more mobile, but this had ceased by the time they were sampled 5 hours later. When removed, they moved very little, and became immobile when put on their backs to be weighed. The control abalone moved as normal when put on their backs. On day 1 the heated abalone were far easier to remove as their foot grip was weaker, but this difference was not detectable on day 2 or day 7.

*Weight*

In the laboratory experiment heat stressed abalone lost weight on day 2, followed by a slight increase by day 7 (Figure 2A). Control abalone also showed a slight drop in weight on day 2, but not as much as in the treatment group. The drop in the control group may reflect the cessation of feeding on day 1. The range of individual weights in the farm sampling was wide, and non-normally distributed, so that larger sample sizes are needed for definitive conclusions on the effect of heat on weight. There were a few large abalone in the 22.5 and 24.5$^0$C samples, and an increase is expected in later samples. The sample at 19$^0$C in April may simply have lacked heavier abalone by chance.

**Figure 2: Mean weights (g ± SE) of abalone in laboratory tanks heated to 26$^0$C (heat stress) and controls kept at 16$^0$C (n= 6 tanks with 2 abalone per tank)**

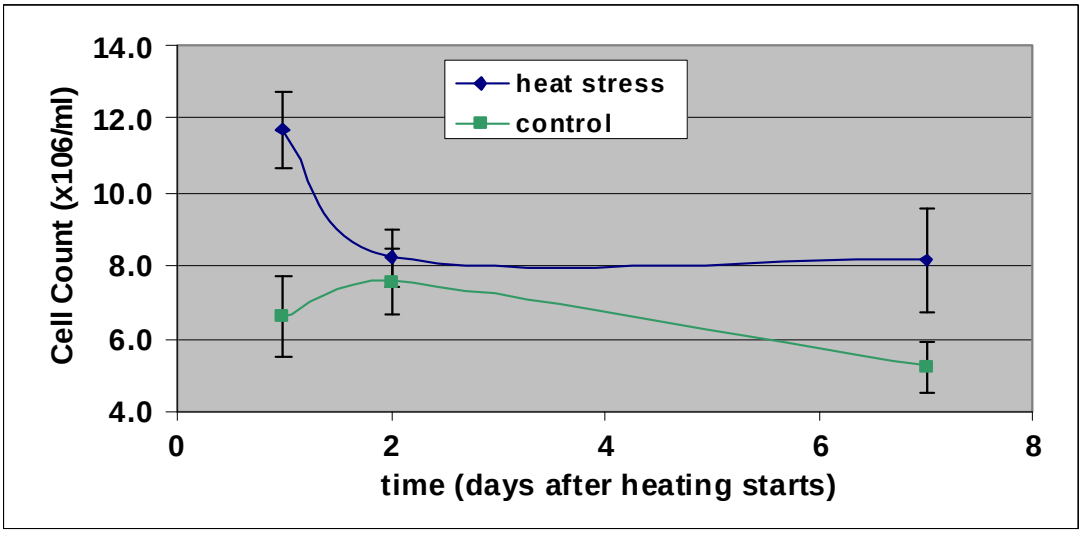



*Haemocyte Count*

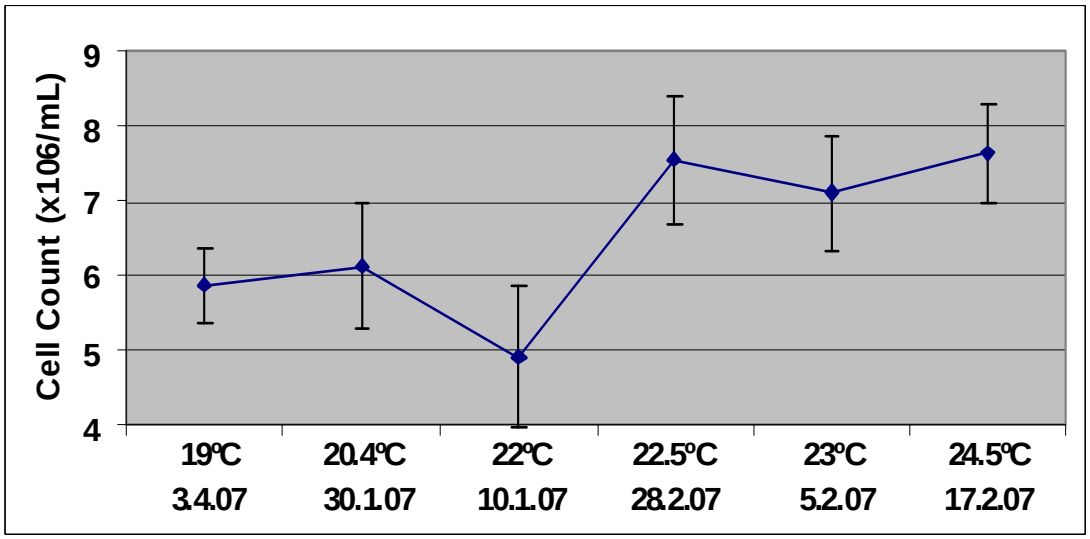
Heat stress affected haemocyte densities in both the laboratory and on-farm samples (Figure 3). In the laboratory experiment there was a significant elevation of haemocyte count in heat stressed abalone versus the controls on the first day after the temperature rose to 26$^0$C (Figure 3A). The effect of heat on the haemocyte counts happened during the first 5 hours of heating, because when the counts for each abalone sampled on day 1 were examined, there was no correlation of the counts to the sequence in which the abalone were sampled. There was a marked decrease in counts in heated abalone by day 2, and counts then remained at similar levels to the controls until day 7. The changes in control means are not significant, but if the trend in the means is real, it may reflect the cessation of feeding from day 0. The results suggest increased haemocyte count is a short term response to temperature increase. Haemocyte counts on the farm increased overall with the temperature (Figure 3B). The drop after a sudden increase to 22°C is not significant. These would be short term responses as temperatures increase each day, after cooler nights.

**Figure 3: Mean haemocyte counts (x$10^6$ $ml^{-1}$ + / - SE): A) in laboratory heat-stressed abalone at 26°C from 1-7 days with 16°C controls (n = 12); and B) in abalone in farm tanks on days with a range of temperatures during summer 2007 (n = 13-25).**

A



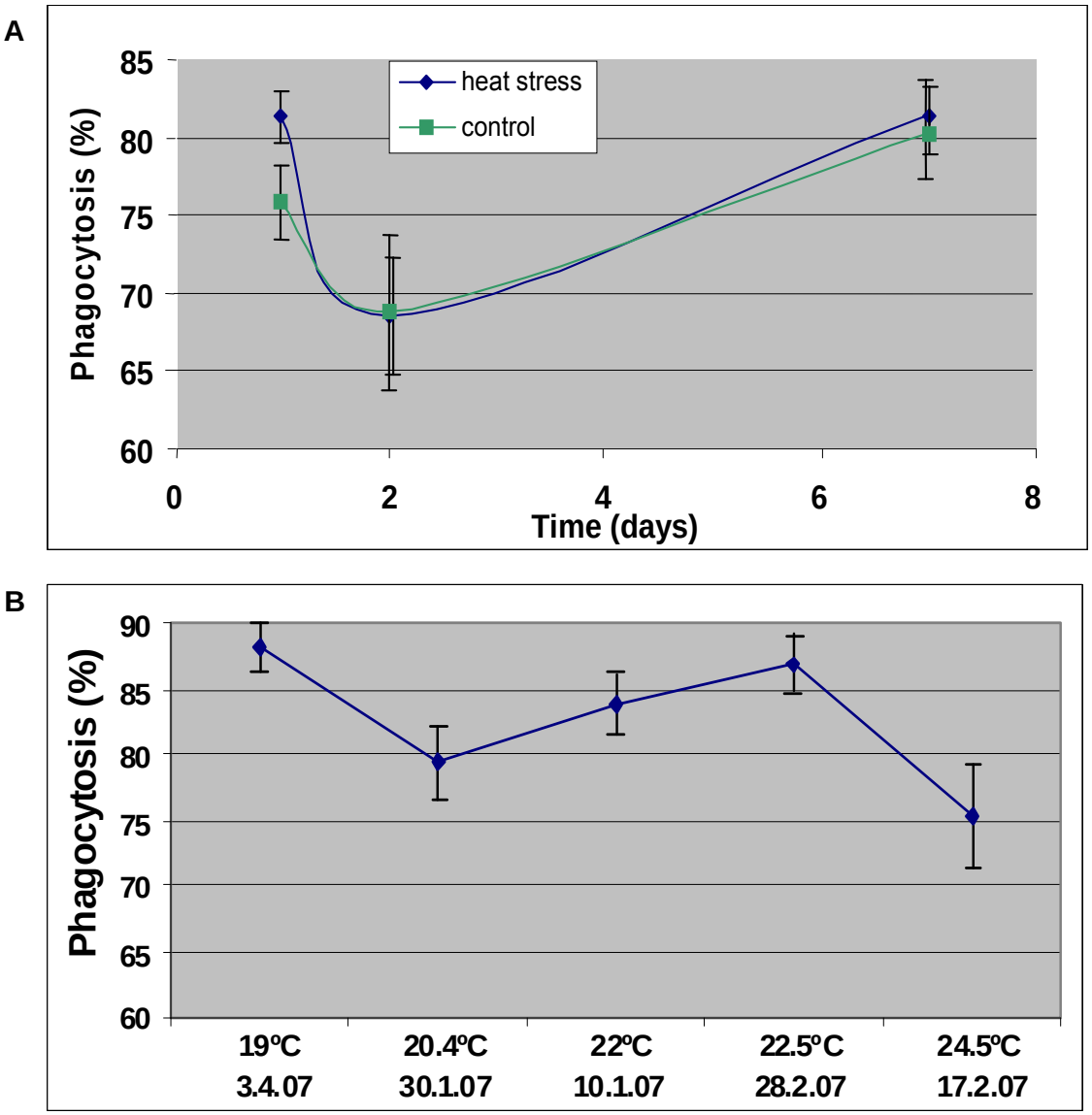

B




*Phagocytic Rate*

Substantial temporal variation was seen in the phagocytic rates of abalone haemocytes (Figure 4). On day 1 in the laboratory experiments, the heat stressed abalone showed significantly higher rates (Figure 4A). Beyond the first day there is no evidence of an effect of heat on phagocytic rate. Unexpectedly, the rate dropped in both the controls and treatments between day 1 and 2. In both it returns to the level of day 1 on day 7. These changes over time are significant, and several other assays (see below) also show this pattern. This may be an artefact, an effect of some unforseen factor (perhaps the cessation of feeding on day 1, or lights were turned on without our knowledge). In the on-farm abalone, there was no overall relation to temperature, but a significant drop in phagocytosis during the highest water temperature conditions (24.5°C) (Figure 4B).

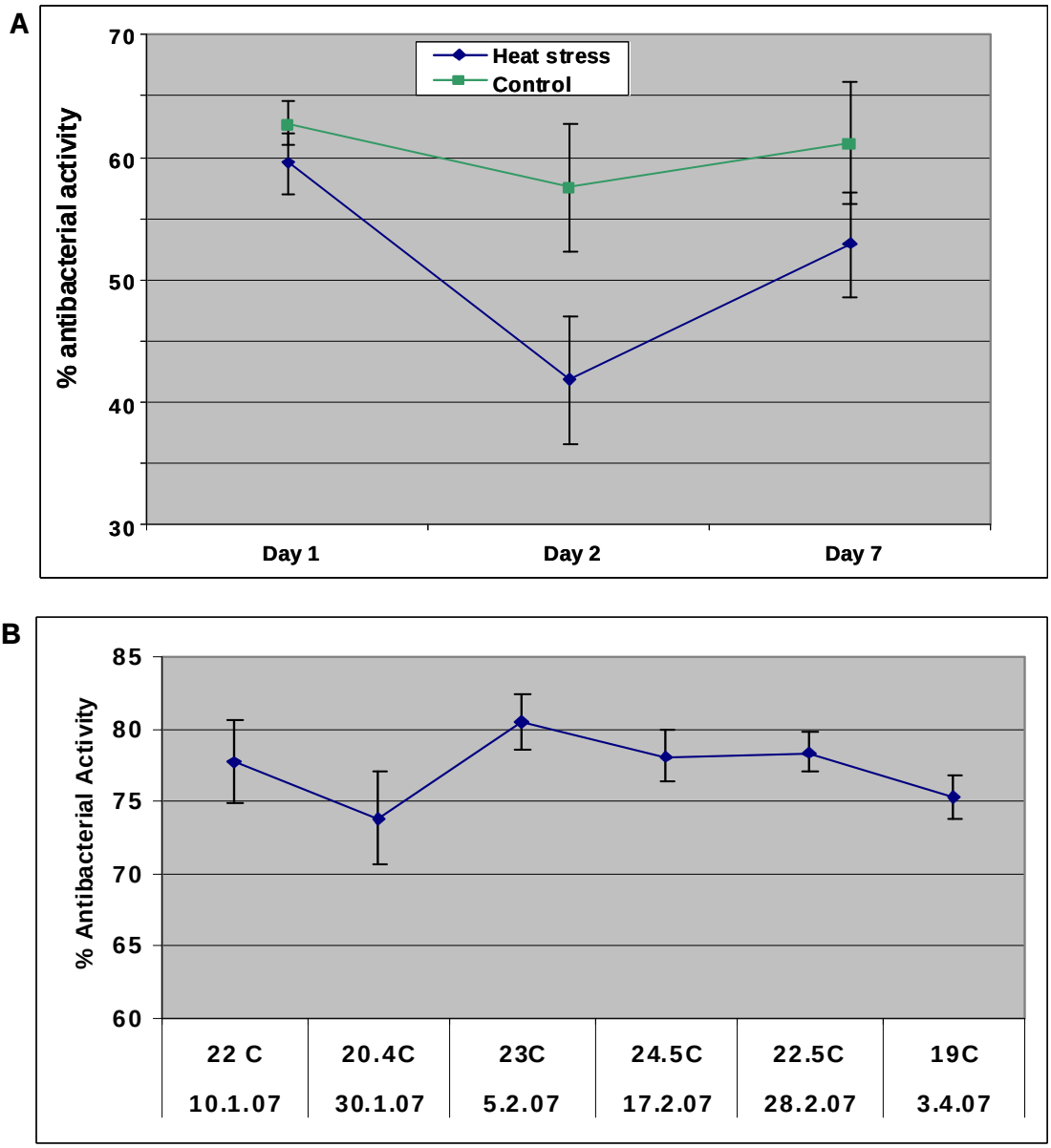
**Figure 4: Phagocytosis (mean percentage of haemocytes that had phagocytosed zymozan +/- SE) in A) laboratory heat stressed abalone held at 26°C for 1-7 days, with 16°C controls (n = 12); and B) abalone in farm tanks on days with a range of water temperatures. Data from 5.2.07 (23°C) is missing because the zymosan preparation was contaminated.**




*MTS Antibacterial Assay*

In the laboratory controlled experiment (Figure 5A) the antibacterial activity was consistently lower with severe heat stress (continual 26ºC water temperature), as compared to the controls held at 16ºC. The differences were significant on days 2 and 7. This indicates reduced antibacterial activity in the heat stressed abalone. The results for the MTS assay from 4 samples taken on the farm during last summer are presented in Figure 5B. There is some trend for higher antibacterial activity on warmer days, but the changes are not significant. Here again, the on-farm results differ from the laboratory results, perhaps due to the rate and severity of heat stress, or perhaps due to other on-farm conditions (flow rates, feeding, etc.).

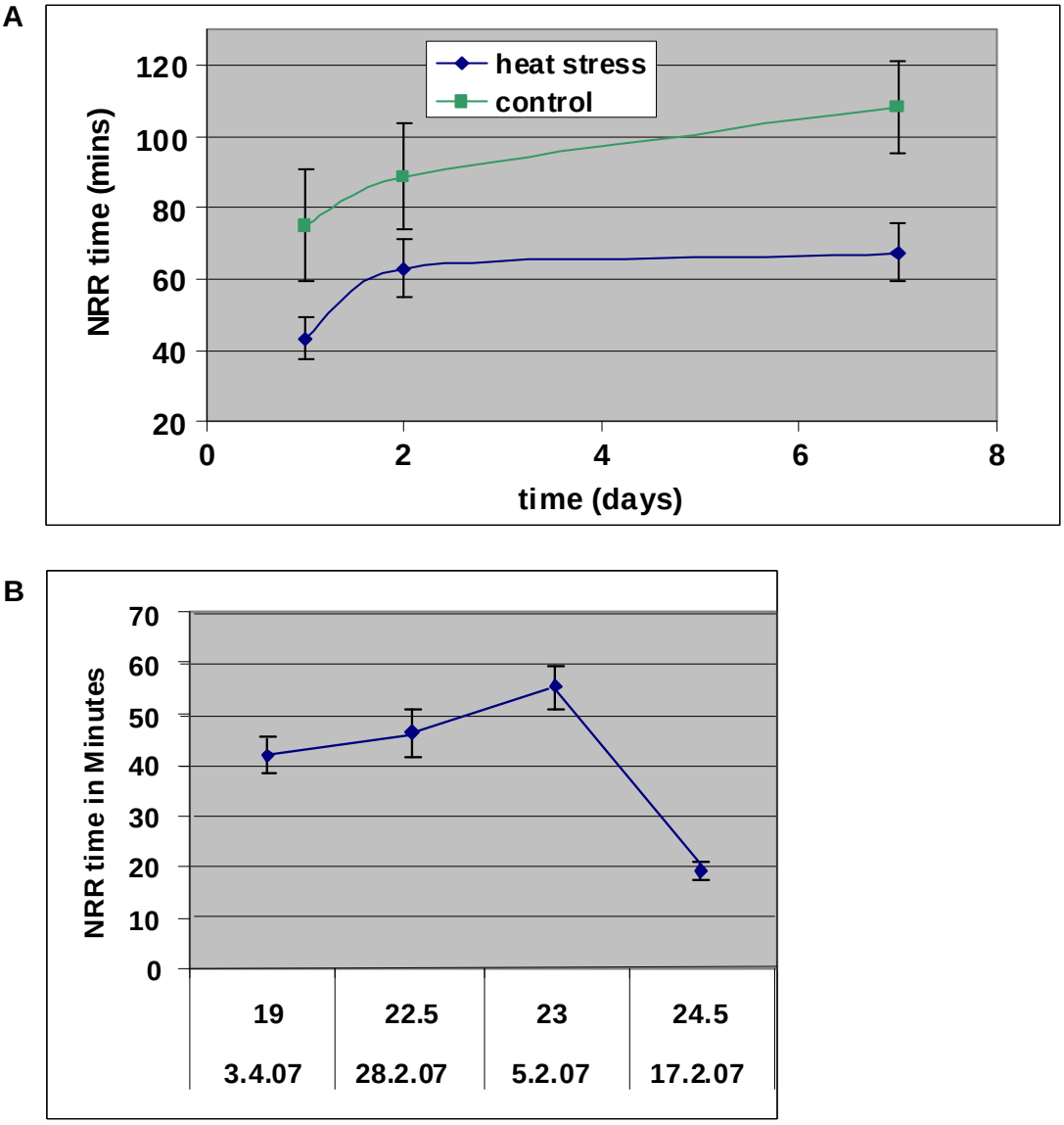
**Figure 5: MTS assay (mean % antibacterial activity +/- SE) based on wells containing *Vibrio harveyi* with 90% haemolymph. A) for the laboratory heat stressed abalone held for 1-7 days at 26°C, with 16°C controls (n = 12); and B) for abalone in farm tanks on days with a range of water temperatures.**




*Neutral Red Retention Assay*

While the assay was not carried out until February on the farm, both the on-farm and laboratory data show a decreased Neutral Red Retention time with elevated temperature (Figure 6). The overall difference was significant in the laboratory. There was also a parallel increase in retention times in both controls and heated from day 1 to day 2, consistent with the phagocytosis result.

**Figure 6: Neutral red retention times (mean +/- SE for minutes until >50% cells have swollen lysosomes) in A) the laboratory heat stressed abalone held at 26°C for 1-7 days, with 16°C controls (n = 12); and B) in farm tanks on days when the assay was done, with a range of water temperature conditions.**




*Haemolymph biochemistry*

Total protein and the concentrations of Sodium, Potassium, Calcium, Magnesium, Copper, Chloride and Phosphate were analysed, but none of these showed significant effects of the experimental treatments over time (Table 5). Magnesium rose and Phosphate fell on day 7 in both the control and treated abalone. On the farm mean protein levels rose from 6 to 6.65 g/l as the temperature rose above 22 in the first week of February, then remained above 6.2 as temperatures fell to 19ºC in April (Table 6). Sodium, Calcium and Chloride rose as the temperatures fell. There was no significant change in other ion concentrations in the haemolymph.

Note that the ion concentrations in the experiment and on the farm differ markedly. This may partly reflect the fact that abalone are osmoconformers: their tissues take up the overall concentration of ions in the seawater, though not necessarily for each type of ion. We can

assume that the salinity of the water varied between the farm and the experiment, carried out in a recirculating seawater system. The differences show too, that there is no closely defined 'normal range' of these ions for abalone.

**Table 5: Mean values (+/-SE) of haemolymph protein (g/L) and ions in the laboratory heat stress experiment**

| | Day 1 | | Day2 | | Day7 | |
|---|---|---|---|---|---|---|
| | **Hot** | **Cold** | **Hot** | **Cold** | **Hot** | **Cold** |
| **CL mmol/L** | 500.6 (9.9) | 509.3 (16.3) | 518.6 (6.4) | 509.9 (4.5) | 525.6 (14.4) | 520.9 (6.1) |
| **K mmol/L** | 10.3 (0.2) | 10.1 (0.2) | 10.7 (0.2) | 10.4 (0.1) | 10.4 (0.3) | 10.2 (0.2) |
| **Na mmol/L** | 399.7 (6.2) | 396.3 (11.6) | 408.3 (4.6) | 401.6 (3.1) | 409.8 (9.6) | 412.5 (3.6) |
| **Ca mmol/L** | 9.47 (0.12) | 9.44 (0.24) | 9.91 (0.12) | 9.68 (0.08) | 9.84 (0.21) | 9.76 (0.07) |
| **Cu umol/L** | 209.9 (10.9) | 232.5 (8.3) | 220.8 (9.9) | 235.4 (8.4) | 230.6 (6.6) | 217.6 (12.9) |
| **Mg mmol/L** | 33.0 (0.2) | 32.9 (0.5) | 32.7 (0.1) | 32.5 (0.1) | 34.6 (0.3) | 34.7 (0.2) |
| **PO4 mmol/L** | 0.480 (0.063) | 0.367 (0.050) | 0.622 (0.13) | 0.413 (0.048) | 0.223 (0.035) | 0.209 (0.083) |
| **Protein g/L** | 6.00 (0.73) | 5.57 (0.57) | 6.92 (0.30) | 6.75 (0.43) | 6.00 (0.50) | 7.00 (0.50) |

**Table 6: Date, temperature and mean values (+/-SE) of haemolymph protein (g/L) and ions during 2007 monitoring of farm tanks.**

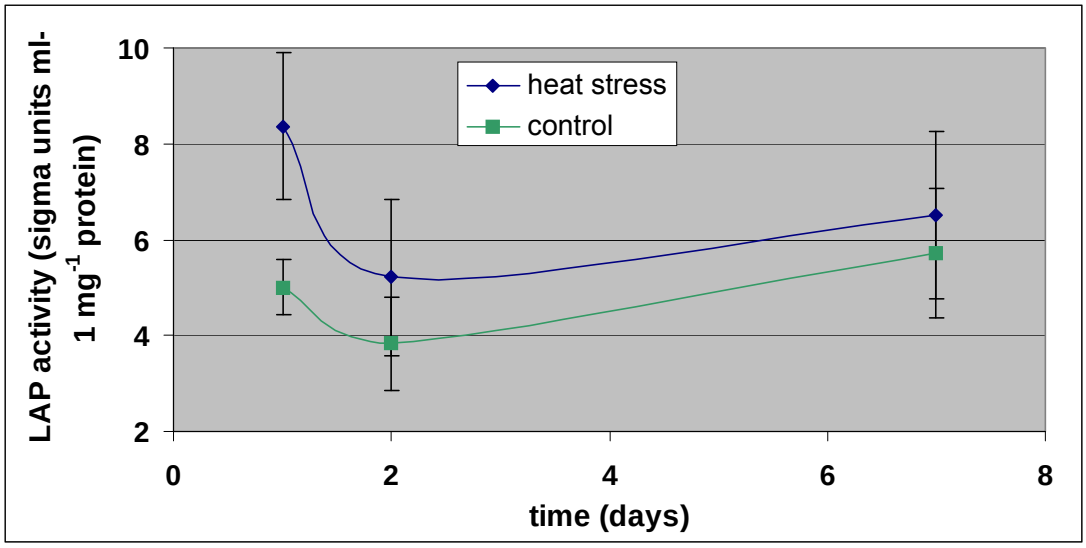
| **Date** | **3.4.07** | **30.1.07** | **28.2.07** | **5.2.07** | **17.2.07** |
|---|---|---|---|---|---|
| **Temp.** | **19ºC** | **20.4ºC** | **22.5ºC** | **23ºC** | **24.5ºC** |
| **Cl mmol/L** | 634 (2.9) | 637 (5.3) | 623 (4.1) | 641 (5.8) | 660 (5.8) |
| **K mmol/L** | 11.94 (0.14) | 11.71 (0.23) | 11.80 (0.10) | 11.76 (0.11) | 11.73 (0.15) |
| **Na mmol/L** | 485 (3.6) | 481 (3.4) | 478 (1.1) | 499 (2.3) | 496 (3.6) |
| **Ca mmol/L** | 10.05 (0.04) | 9.72 (0.26) | 10.16 (0.04) | 8.17 (0.04) | 10.07 (0.06) |
| **Cu umol/L** | 212 (3.3) | 211 (8.1) | 218 (4.2) | 207 (5.5) | 213 (8.1) |
| **Mg mmol/L** | 34.34 (0.04) | 34.85 (0.05) | 34.94 (0.05) | 34.83 (0.22) | 34.34 (0.05) |
| **PO4 mmol/L** | 0.257 (0.026) | Insuff sample | 0.256 (0.035) | 0.273 (0.034) | 0.297 (0.042) |
| **Protein g/L** | 6.36 (0.16) | 6.00 (0.22) | 6.44 (0.15) | 6.67 (0.17) | 6.57 (0.20) |

*Leucine Amino Peptidase*

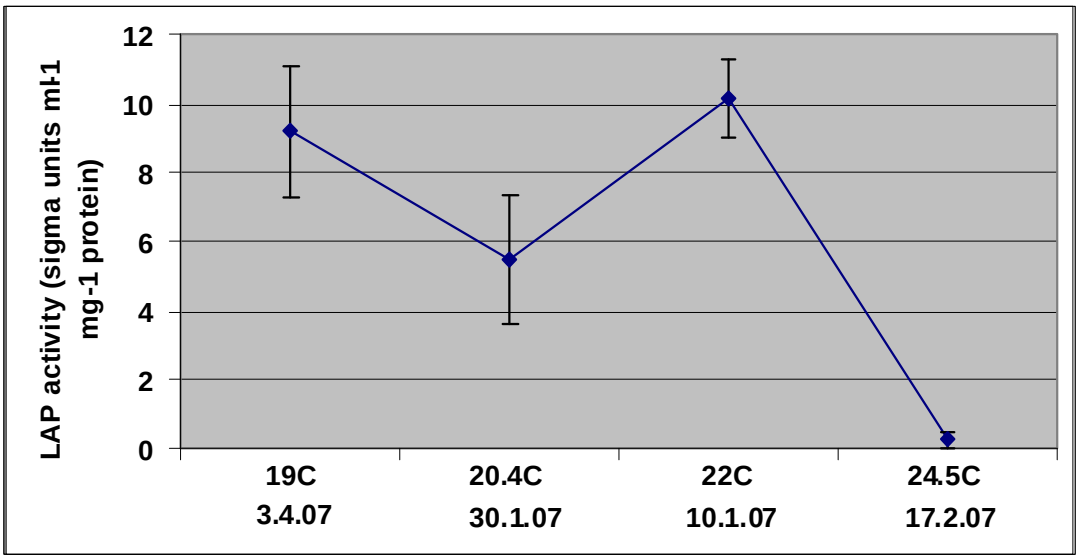
There was no consistent effect of temperature on LAP activity (Figure 7). In the laboratory experiment LAP activity was significantly higher in heat stressed abalone than controls on the first day, but this converged to the control level by day 7 (Figure 7A). As for Phagocytosis and NRR, there is a parallel change in controls and heated on day 2, then recovery. In the on-farm data there are no apparent differences at lower temperatures, but LAP activity dropped severely from 22 to 24.5$^0$C (Figure 7B).

**Figure 7: Leucine amino peptidase activity (mean + / - SE ) in A) the laboratory heat stressed abalone held at 26°C for 1-7 days, with 16°C controls (n = 12); and B) in farm tanks on days with a range of water temperature conditions.**

A



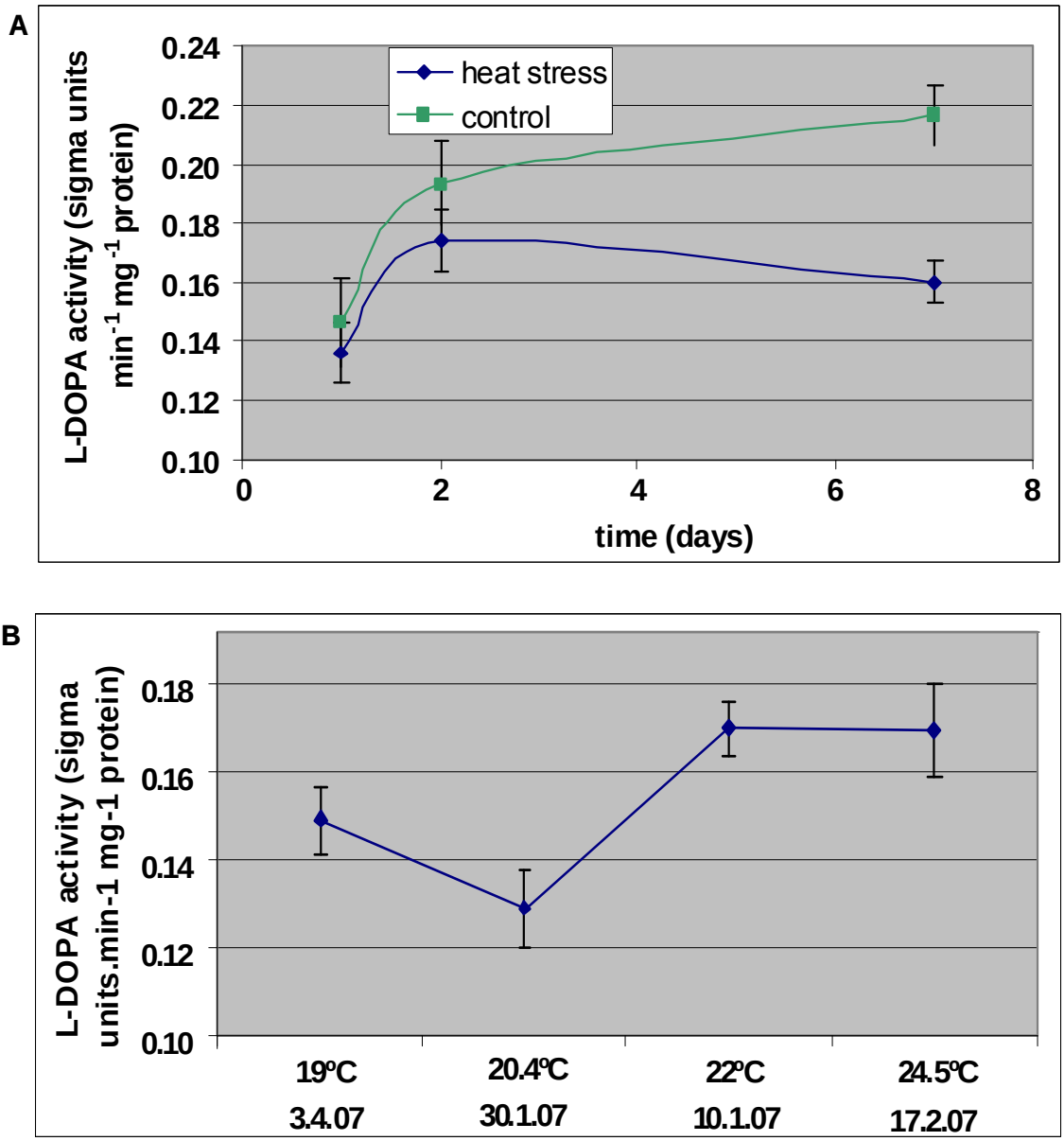

B




*Phenoloxidase Activity (L-DOPA)*

As for several previous assays, there is a parallel change in both controls and heated abalone from day 1 to day 2 in the laboratory data, with significantly increased phenoloxidase activity over time (Figure 8A). Heat-stressed abalone then diverge from the controls so that there is significantly reduced phenoloxidase activity compared to controls on day 7. This suggests long term supression of this aspect of immune function by continued severe heat stress. The on-farm data (Figure 8B) suggest a possible increase with temperature associated with shorter term, less severe heat stress.

**Figure 8: Phenoloxidase activity (mean +/- SE sigma units $min^{-1}.mg^{-1}$ protein) in A) the laboratory heat stressed abalone held at 26°C for 1-7 days, with 16°C controls (n = 12); and B) in farm tanks on days with a range of water temperature conditions.**

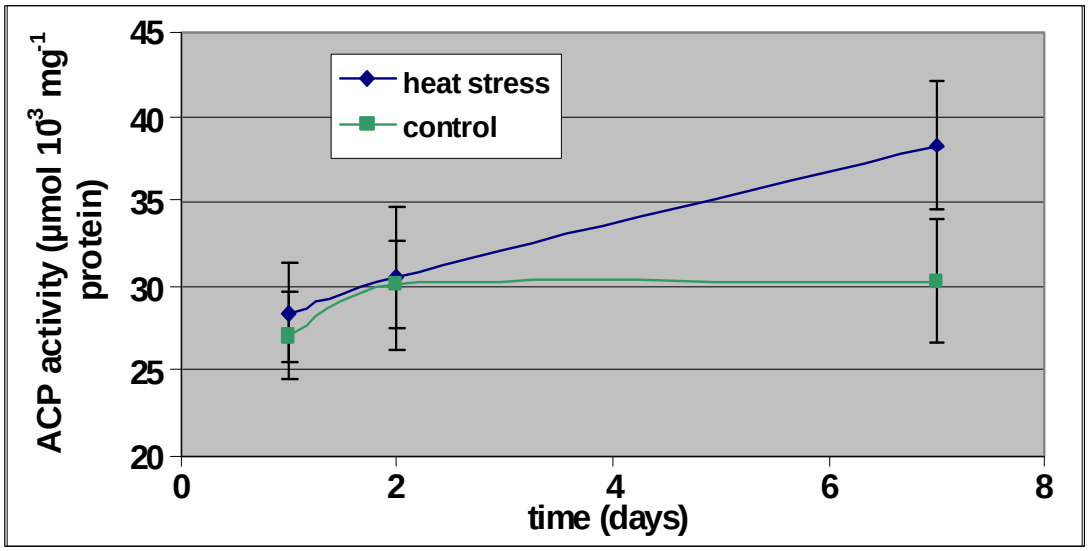



*Acid Phosphatase Activity*

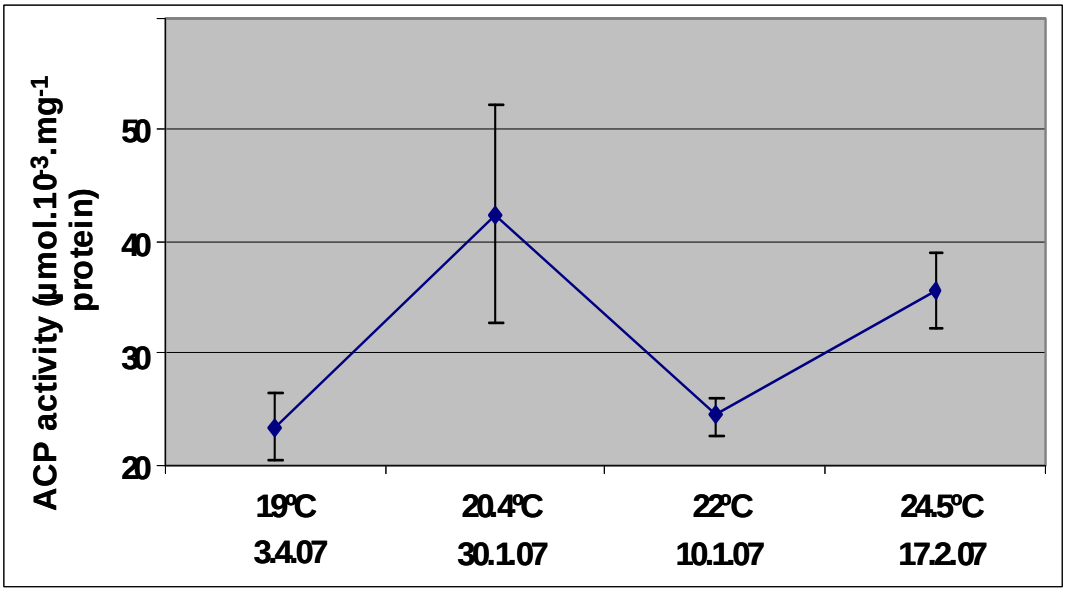
The laboratory experiments show an increased ACP activity after 7 days in the heat stressed abalone, but not between controls and treatments on days 1 & 2 of the experiment (Figure 9A). There was no evident trend in the on-farm data (Figure 9B)

**Figure 9: Acid phosphatase activity (mean +/- SE) in A) the laboratory heat stressed abalone held at 26°C for 1-7 days, with 16°C controls (n = 12); and B) in farm tanks on days with a range of water temperature conditions.**

A



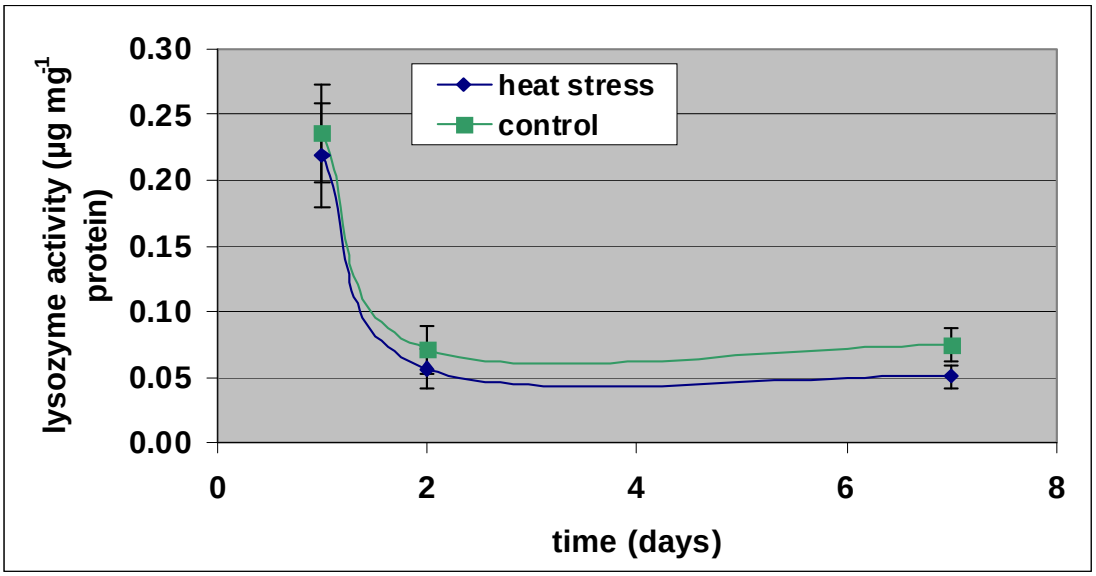

B




*Lysozyme Activity*

The results from the laboratory experiment show no clear difference between control and heat stressed abalone (Figure 10). As for several other assays, both change from day 1 to day 2. On day 7 the activity is slightly higher in controls than the heat stressed individuals. Lysozyme testing was not completed in the on-farm haemolymph samples.

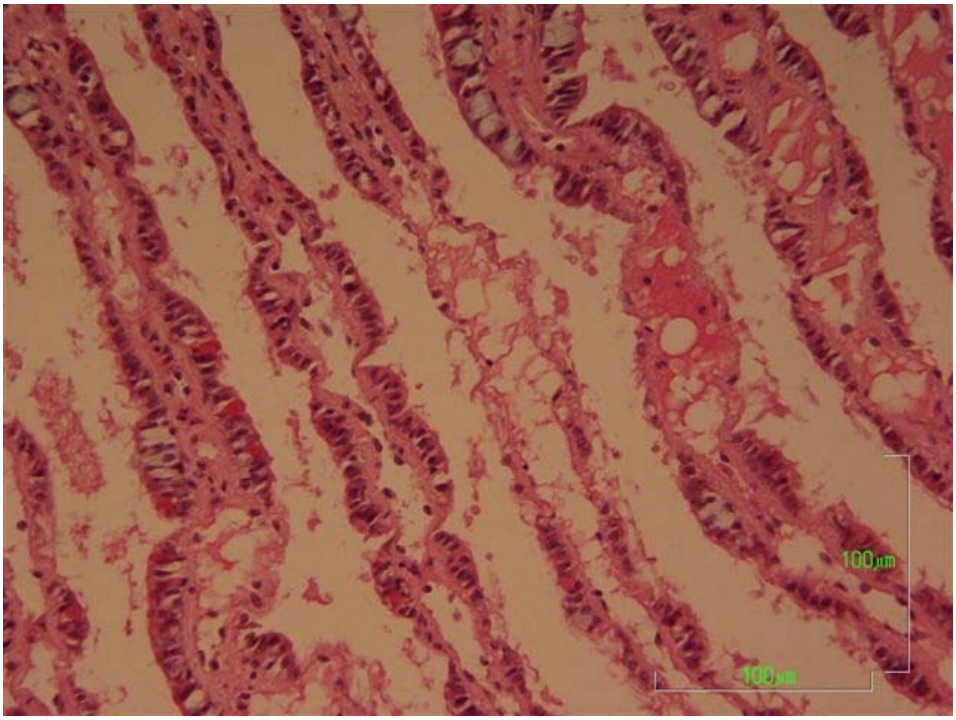
**Figure 10: Comparison of lysozyme activity (mean +/- SE µg.mg$^{-1}$ protein) in abalone kept at 16°C (control) and heat-treated individuals kept at 26°C for 1-7 days (n = 12).**




*Histopathology*

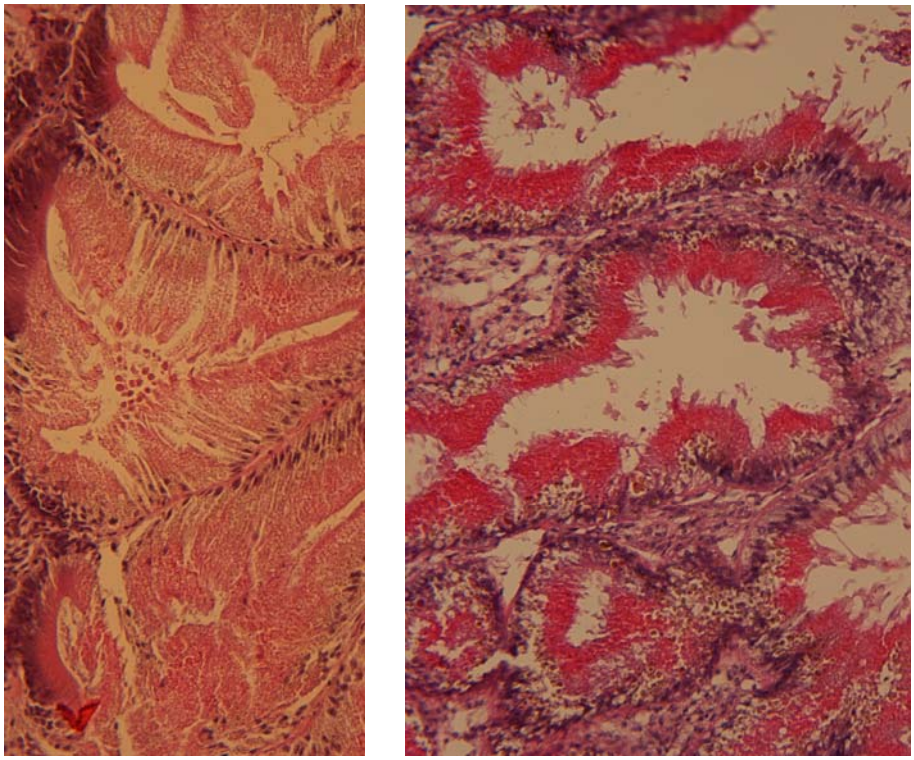
The gills of abalone in the laboratory experiment also had lesions after the severe heat stress, and these were most severe on day 1 but still present on day 2 and 7. The epithelium (outer layer of cells) had degenerated or sloughed off in a number of places, but this involved no more than 5% of the total gill area (Figure 11). This focus of damage would provide a portal of entry for bacteria in the water.

**Figure 11: Stained slide of gill filaments. The central gill filament has extensive necrosis and sloughing of the lining epithelial cells.**



At day 7 of the laboratory experiment, the digestive gland in some heat stressed abalone showed damage (see Figure 12) including edema and atrophy (the lobes of the gland decreased in size and their walls were thinner). In addition, there was a moderate infiltration of loose haemocytes and haemocytes formed into clusters (granulomas).

**Figure 12: Normal digestive gland (left photo) and after 7 days of severe heat stress (right photo) with edema, increased tubular lumen and haemocyte infiltration.**



## Heat Stress - Long term experiment.

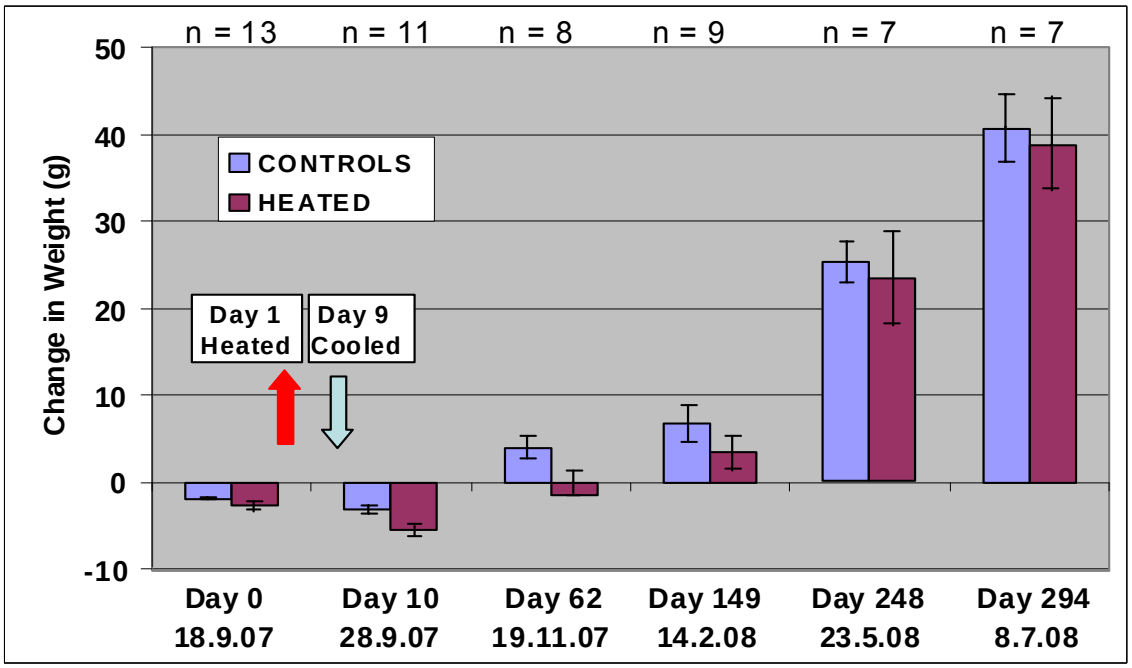
Nonlethal stress may not produce obvious production loss due to mortality, but may still result in reduced growth, and cause substantial reductions in overall farm productivity. Thus the longer term effects of severe but not lethal heat stress were the focus of subsequent work. A second experiment was carried out on the farm, to assess whether the abalone could recover and thrive in the months following severe heat stress.

In this long term experiment, 144 abalone were randomly selected from three stock tanks on the farm, tagged and placed into 12 smaller tanks on the farm used for brood stock work. The abalone were left in these 12 tanks for 5 days to acclimatise. Feeding ceased for treated and control abalone the day prior to the temperature being elevated. On day 1 the temperature was elevated in 6 of the tanks from the ambient 13º to 26ºC over a period of 24 hours, kept at 26ºC for 7 days, then slowly returned to 13ºC over another 24 hours. After the return to ambient temperature, the abalone were sampled, then moved into a single farm tank and were then subject to the ambient changes in water temperature and the routine feeding and cleaning regime used on all tanks throughout the farm. Two abalone from each of the 12 tanks were sampled the day before the temperature was elevated, the day after the temperature returned to 13ºC and 14-18 abalone were sampled every 2 - 3 months for the next 10 months. Ambient water temperatures were recorded.

*Weight*

Abalone weights were initially measured during tagging, 5 days prior to the start of the experiment, and the data are presented as changes from initial weights in Figure 13. Both control and heat-treated groups had lost some weight the day before the tanks were heated (18.9.07) and after the week of heat stress (28.9.07). The control group had recovered and had started to gain weight two months later, but the heat-treated group had recovered lost weight only after 4 months. Within 7 months, both heat-treated and control groups had similar weight gains, but the error bars were wider in the heat stressed group, reflecting a more variable weight gain. The loss of weight and slow weight gain in controls are probably due to the movement into the experimental tanks 5 days before, the cessation of feeding, and the movement back to the farm tank. The heated abalone were subject to these same effects.

**Figure 13. Change in weight (g) from initial tagging, of abalone monitored on the day before heat was applied (up arrow), the day after the water was cooled (down arrow), and every 2-3 months over 10 months. Sample sizes are shown.**

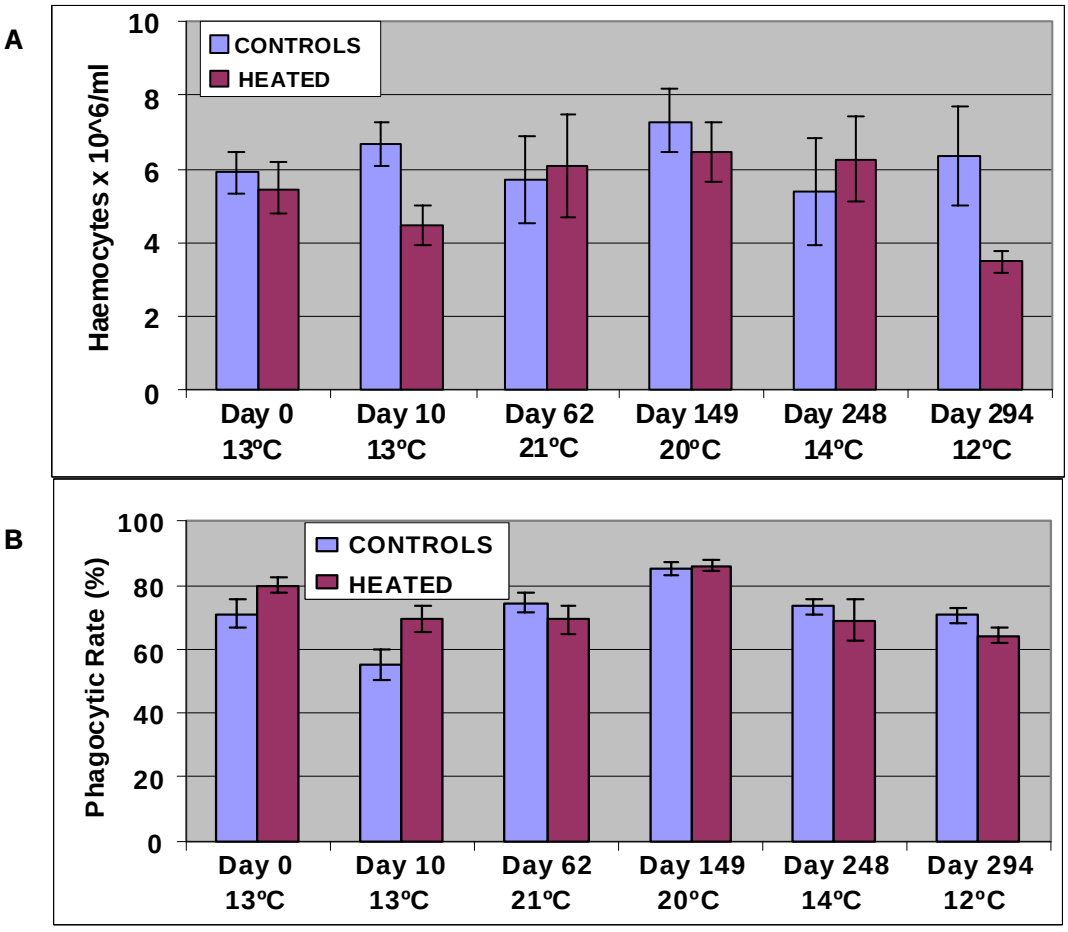



*Haemocyte counts and Phagocytic Rate*

As shown in Figure 14A, on the day after the severe heat stress had been applied for 7 days (28.9.07) the stressed abalone had lower cell counts, in contrast to the earlier laboratory heat stress experiment (Figure 3A). This could suggest depletion of haemocytes after the prolonged stress, but the difference is within the range of the controls over time, and may simply be due to interabalone variation. In any case haemocyte counts in the heated abalone had recovered by day 62. Haemocyte numbers were also lower in heat stressed abalone at the end of the experiment, after 9 months of recovery (8.7.08). We cannot explain this second, unexpected result, especially as at this time all abalone (controls and heat stressed) were kept in a single tank.

The phagocytic rate (Figure 14B) appeared to be higher in the treatment group just before the week of severe heat stress (18.9.07) and the day after the heat stress ceased (28.9.07), but neither of these differences were significant. There was no significant difference between control and heat-stressed abalone on any of the dates.

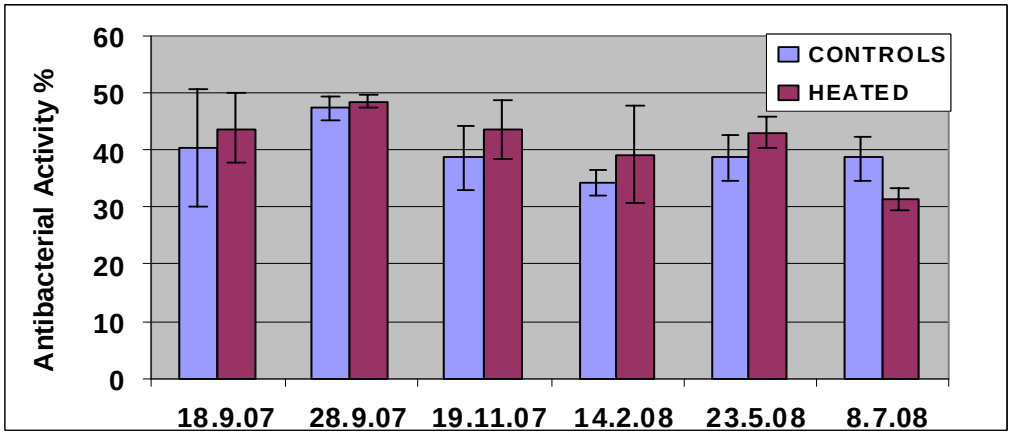
**Figure 14. A: Haemocyte counts, and B: Phagocytic rate (% cells phagocytosing zymosan) just before a severe heat stress (Day 0, 18.9.07), the day after a week of heat (Day 10, 28.9.07) and then every 2-3 months over 10 months. The temperatures on sampling days are shown.**




*Antibacterial Assay*

Due to a problem with the nutrient broth during initial analysis, reserve samples were analysed later (Figure 15). Both the control and heated abalone varied in antibacterial activity over time, but showed similar trends. We have discovered in subsequent work that the baseline antibacterial activity varies over time. There is no evidence of an effect of heat stress on antibacterial activity.

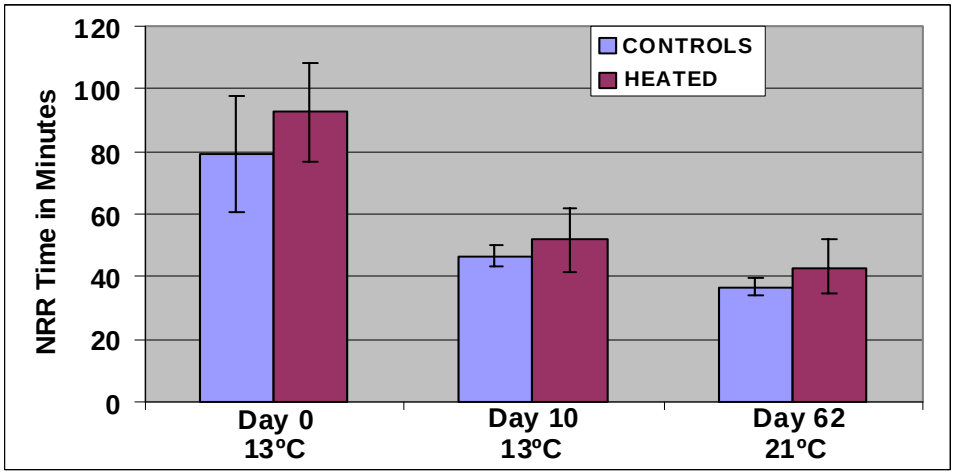
**Figure 15. Antibacterial activity just before heat stress (18.9.07), the day after a week of heat (28.9.07) and then every 2-3 months over 10 months.**



*Neutral Red Retention Assay*

The NRR assay was done on only the first 3 sampling days (Figure 16). There was no significant difference between the NRR times of the control and heat-treated abalone the day before the severe heat stress (Day 0), on the day just after the heat has ceased (Day 10) nor 2 months later (Day 62). Both control and heat-treated groups had reduced NRR times after the heating period.

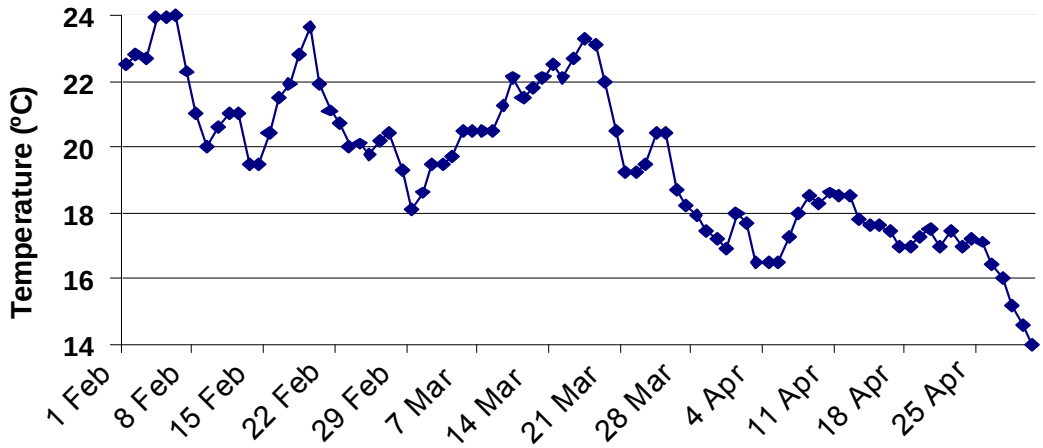
**Figure 16. NRR assay results just before a severe heat stress, the day after a week of severe heat and 2 months thereafter. Temperatures on the sampling days are shown.**




## Heat Stress – on-farm sampling, summer 2008

On-farm sampling was repeated in 2008, from early February until April. A different cohort of abalone to that sampled in 2007, stocked in a different shed, were used. The same five tanks were sampled during each farm visit in 2008 (3 abalone per tank). The temperature remained above $20^0$C for the first 7 sample times: i.e. from early February until mid March, when the temperature dropped and remained below $20^0$C. Thus temperature was unfortunately confounded with time, and the slowly increasing weight of abalone. The farm records of temperatures (Figure 17) show the temperature spikes during this seasonal decline over the sample period.

**Figure 17. GSW records of piped water temperatures, February to April 2008. Temperatures were measured twice daily and averaged. Dates at the start of each week are shown**



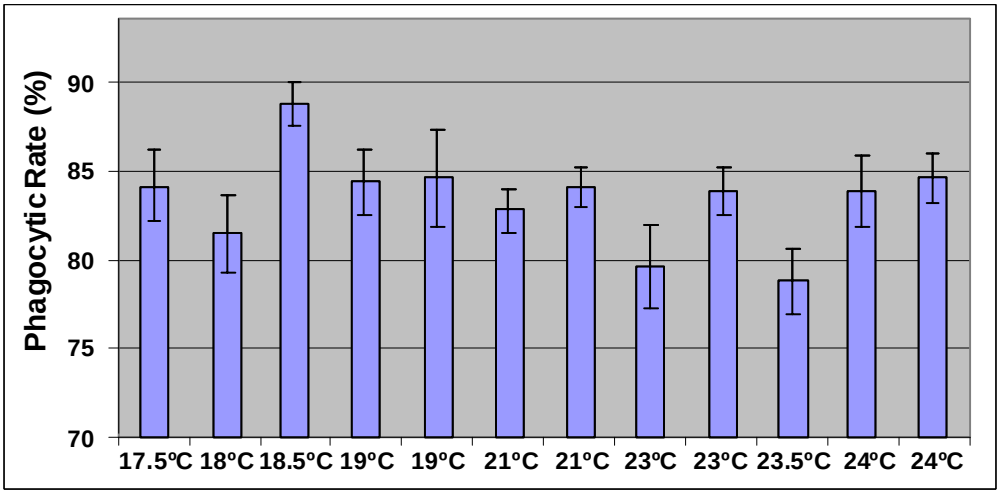
The biochemistry and complex enzyme assays done in 2007 were not repeated in 2008, as they had not proved useful. Table 7 below shows the results of the assays done. Weights gradually increased over the 2 months monitoring, as would be expected. The analysis of these results also show growth varied between tanks. Haemocyte counts varied between sampling days such that the highest and lowest values were significantly different, but they remained within the normal range seen on the farm, and there was no association with temperature variation (Table 7).

The phagocytic rate was fairly constant over the 2 months, with no obvious effect of temperature. This is consistent with the 2007 results. The rate declined as temperature rose in mid March, followed by a rise when there was a significant drop in temperature at the end of March (Figure 18). This trend was mimicked by the NRR assay (see below).

**Table 7: Temperatures (°C) on each sample date, and results (means) of long term monitoring: weights (g), haemocyte counts (x$10^6$ $ml^{-1}$), phagocytosis rates (% of cells), NRR times (mins), and MTS assay (% antibacterial activity).**

| Date | Water Temperature | Weight | Haemocyte count | Phagocytic rate (%) | Antibacterial % | NRR times |
|---|---|---|---|---|---|---|
| 5.2.08 | 24 | 61.03 | 6.02 | 83.83 | 41.42 | 58.27 |
| 18.2.08 | 23 | 59.25 | 5.93 | 83.87 | 43.94 | 37.60 |
| 19.2.08 | 24 | 62.45 | 5.20 | 84.63 | 45.26 | 43.00 |
| 27.2.08 | 21 | 69.35 | 4.91 | 84.07 | 43.77 | 35.13 |
| 3.3.08 | 21 | 69.85 | 3.71 | 82.77 | 35.87 | 34.13 |
| 14.3.08 | 23 | 75.40 | 4.90 | 79.63 | 42.39 | 36.87 |
| 17.3.08 | 23.5 | 72.25 | 4.31 | 78.80 | 40.24 | 47.13 |
| 28.3.08 | 18.5 | 76.88 | 6.61 | 88.77 | 35.15 | 72.93 |
| 3.4.08 | 17.5 | 73.03 | 6.42 | 84.13 | 44.94 | 60.80 |
| 7.4.08 | 19 | 74.56 | 5.10 | 84.43 | 40.79 | 66.47 |
| 9.4.08 | 19 | 74.51 | 5.73 | 84.63 | 38.07 | 76.93 |
| 21.4.08 | 18 | 79.97 | 4.68 | 81.47 | 43.60 | 65.93 |

**Figure 18. Phagocytic rate (%) related to the temperature when samples taken.**

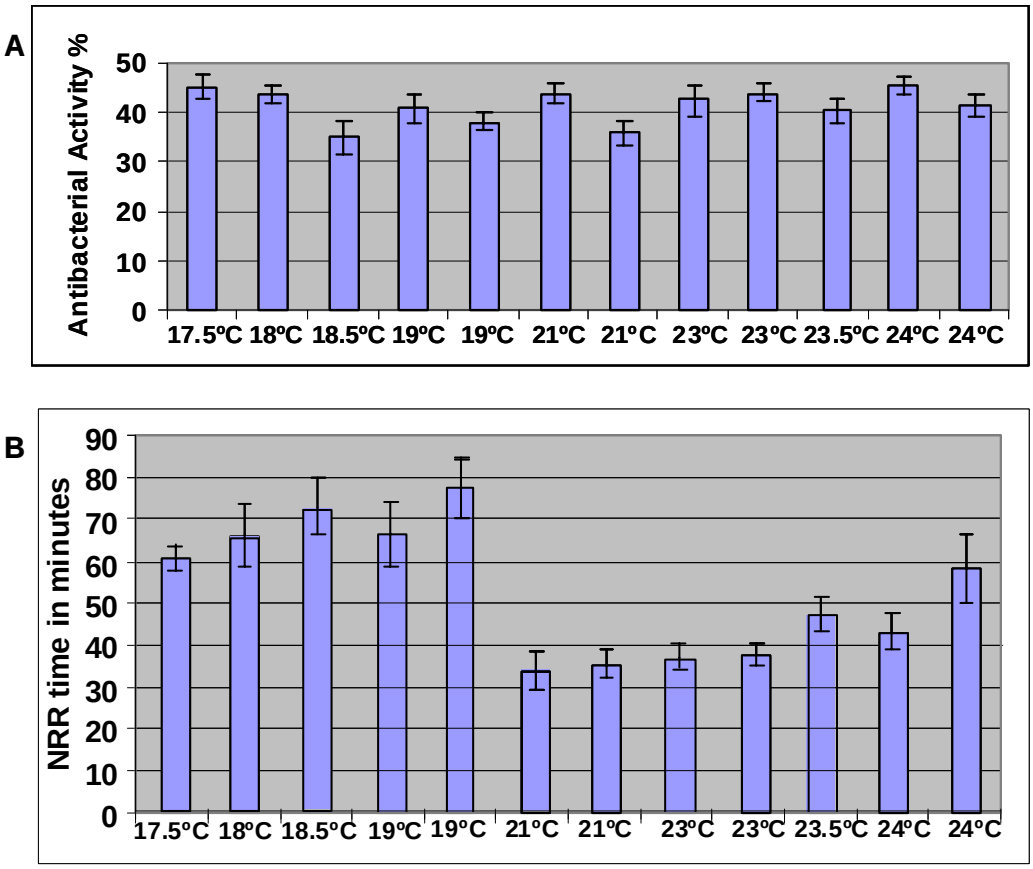



A problem with the broth peparation meant that analyses had to be repeated on frozen stored samples. There were significant variations in the antibacterial activity but again, this did not correspond with temperature variation (Figure 19A).

NRR times were lower during the warmer months, but not all the warm days were significantly lower than the cooler days (Figure 19B), and there is a trend to longer times

(denoting less stress) for the temperatures above 20 degrees. Note that NRR retention times increased at the end of March as the temperature dropped, in concert with the phagocytic rate (Table 7).

**Figure 19. A: Antibacterial activity, and B: Neutral Red Retention Time, each related to temperature on the sample day.**




## Discussion of heat stress experiments and monitoring.

Only one study of the critical thermal maximum temperature or upper lethal temperature in Australian abalone has been done and this found 50% of *H. rubra* and *H. laevigata* died at temperatures of 26.9ºC and 27.5ºC respectively (Gilroy et al. 1998). The preferred temperatures were 16.9ºC and 18.9ºC. Similar studies in hybrids of these two species, which are extensively used in Australian farms, have not been done. Hybrids of abalone can have different metabolic rates under stressful conditions (Ahmed et al. 2008). The farm has been involved in a genetic improvement program for some years, which included selection for reduced mortality.

Further, abalone are likely to acclimatise to some extent over hot periods. The study by Gilroy et al. (1998) was done in Tasmania, where the water remains colder. A temperature tolerance study done by Cheng *et al.* (2004c) on *H. diversicolor supertexta* reared at $20^0$, $25^0$ and $30^0$ C, showed the abalone reared at these temperatures survived temperatures from $3.5^0$C to $32.7^0$C, $5.3^0$C to $33.3^0$C and $10.6^0$C to $35.2^0$C respectively, when the water temperature was increased or decreased gradually. Part of acclimation to warmer temperatures is a reduction in mitochondrial density, leading to reduced oxygen needs at the cellular level (Portner 2002), and an expression of heat shock proteins to protect and repair essential proteins and other molecules in the cells.

In the short term severe heat stress experiment, a large number of assays were used to assess immune function and related biochemical responses in abalone under heat stress. As described in the Methods section, earlier published work (Cheng *et al*. 2004a-d) has validated some assays, showing that the assay corresponds to susceptibility to pathogenic bacteria. Some are also more convenient for on-farm use. However, some assays may be more sensitive indicators of immune function under heat stress, while others may be more useful for other forms of stress.

Haemocyte counts were significantly different between treatments within 3 hours after heat was applied in the short term severe heat stress experiment but the abalone had recovered by the following day. This suggests that this severe stress did have a marked impact in the acute stage, but the abalone were capable of adjusting themselves to the altered environmental conditions. The initial elevation in haemocyte count in the short term laboratory experiment may be an immediate adaptive response to the heat stress – the abalone appear to move haemocytes into the sinuses. Stress leukocytosis (the increased numbers of immune defence cells seen in the hemolymph) is seen in many species, and correlated with elevation in stress hormones such as catecholamines, corticotropin releasing hormone (CRH) and adrenocorticotropin (ACTH), all of which have been detected in abalone (Ottaviani & Franceschi 1997; Malham et al. 2003). The return to contol haemocyte densities in the sinuses by day 2 may simply be due to a return of many haemocytes from the hemolymph into the tissues.

On the farm the density of haemocytes in the sinuses appears to increase with temperature in the 2007 samples, following the trend seen with the weights, and the same pattern is evident to some extent in 2008. But all these changes are small – within the normal range for abalone, and thus do not indicate stress responses.

Typically, higher temperature causes increased oxygen requirements in ectothermic animals, as metabolism increases, requiring higher rates of respiration (Portner 2002). The microscopic damage to the gills in severely heat stressed abalone may relate to respiration stress. But haemocyanin (the copper carrying molecule that binds oxygen in the blood of molluscs) is in the plasma, not the haemocytes (Wilbur and Yonge 1966), so that respiration demands cannot explain an increase in circulating haemocytes. Moreover, copper concentrations did not increase during heat stress or on hotter days (Tables 5, 6). Molluscs can acclimatize to higher temperatures by reducing their metabolic rate and thus their oxygen demands, though this takes several days (Peck et al. 2002; Portner 2002), and it seems likely that abalone respond to low level or gradually developing heat stress in this way.

The atrophy and infiltration of haemocytes in the digestive gland may be the result of low oxygen in this metabolically active tissue, or simply the effect of increased cell turnover at a higher metabolic rate. The long term experiment shows they can recover on cessation of the heat stress, but the variable growth and histopathology show some at least cannot recover fully.

As haemocyte numbers were consistently higher in heat stressed abalone than in the controls in the short term experiment, the effect of heat on cell numbers may reduce the negative effects of heat stress on abalone immuno-competence. Haemocytes are the principal effector cells for both cell-mediated and humoral immunity in abalone (Hooper *et al.* 2007), and thus an elevated circulating haemocyte count could provide immune defence at any tissue location needed to rapidly combat infectious agents as they gain entry.

The results suggest that the mechanisms involved in phagocytosis are more resistant to heat stress than those that control antibacterial activity. In the laboratory experiment the only evidence of an effect of heat stress was on the first day, when heated abalone had higher phagocytic rates. In the second experiment there were no significant differences. This was unexpected because previous research showed a decline in phagocytic rate of heat-treated abalone (Cheng et al. 2004c). Perhaps the initial increase is simply increased metabolic activity at higher temperatures, and the abalone quickly adjusted, as discussed above. This assay and a number of others (NRR, LAP, Phenyloxidase, and Lysozyme activity) showed sharp changes in both controls and heated abalone between day 1 and 2 of the laboratory experiment, perhaps due to some unexplained temporary stress in this experiment, such as activity in the laboratory.

On the farm the phagocytic rate (PR) was significantly reduced on the warmest day, 17.2.07 compared to most of the other days in 2007. It mimicks the decline in NRR time on this day. The decline in PR suggests immunosuppression on this day, but it was not mirrored in the antibacterial levels. A mixed response in the PR was also reported by Cheng et al. (2004c), who found a decline in phagocytic rate when placed in moderately cooler water, then an increase in PR when placed in even cooler water, indicating that this assay is not predictable. It appears that phagocytosis is not a useful indicator of heat stress in abalone. It would be useful to compare this with similar work on other molluscs.

The MTS assay results show that during heat stress there is reduced antibacterial activity (as shown by the short term experiment), but the activity recovers rapidly after the stress ends (long term experiment). In the farm tanks however, there was no significant decline on the warmest days in antibacterial activity. Taking both years together, antibacterial activity was not clearly related to temperature, and remained robust at the higher temperatures.

NRR times decline with stress. In the short term experiment, the NRR times were consistently lower in the heat-treated group over each day tested, with no recovery over the 7 days of testing. The NRR results in the long term experiment remained low after the experimental period in both the heated and control groups, so reveal little about the effects of heat stress. Possibly these results reflect the effects of moving both groups in the experiment. While NRR may be more sensitive than the phagocytosis assay for detecting heat stress in abalone, the great variation in the control values, shown in both these experiments, may often mask the effects of heat stress.

The remaining results relate to assays done only in 2007. Most of the hemolymph electrolytes had no significant changes associated with heat stress. Abalone are osmoconformers (the overall concentration of ions in the body fluids is not different from the seawater around them), and this may apply not just to sodium and chloride, which largely determine overall salt concentration, but to some of the other electrolytes.

The laboratory experiment showed LAP activity increases with heat stress, but the on-farm data shows that although there was a significant decline in LAP activity on the warmest day compared to some but not all the cooler days, the decline in LAP was irregular, with the highest LAP activity recorded on one of the warmer days with 22ºC, then a large drop in LAP activity to $24.5^0$C (Figure 7B). The data collected on the day when the farm water temperature was $24.5^0$C may also be showing the effects of extremely warm air temperature ($38^0$C). The sampled abalone were exposed to this air temperature for a few minutes while haemolymph was extracted and collected into ependorff tubes. Perhaps this is sufficient to cause a destabilisation of some of the enzyme systems in the abalone. Enzymes are often highly susceptible to protein-denaturation by elevated temperature. Further work is needed to understand the response of LAP. It may increase with increased temperature, then collapse at a critical temperature.

Phenoloxidase activity appears to be reduced by heat stress in the laboratory experiment (Figure 8A), despite unexplained changes over time in the controls. This reduced phenoloxidase activity under temperature stress is similar to the findings of Cheng *et al.* (2004c) in *Haliotis diversicolor*. There is no evident trend in the on-farm data (Figure 8B), which may reflect acclimatisation in the farm stock to the moderate rise and fall of water temperatures in the summer months. Alternatively, phenoloxidase activity may not be a particularly sensitive indicator of heat stress in abalone. Shuhong *et al.* (2004) found that phenoloxidase activity was low and did not alter significantly in abalone injected with pathogenic bacteria compared to controls. Consequently, it may not be a reliable indicator for monitoring immune status in abalone.

The laboratory experiments show a long term increase in ACP activity in the heat stressed abalone compared to controls, after 7 days, but not on days 1 & 2 (Figure 9A). There was no evident trend in the on-farm data (Figure 9B), but this may be masked by the varying rates of change in temperature on the farm. Shuhong *et al*. (2004) found a marked elevation in ACP activity within 24 hours in abalone after injecting them with pathogenic bacteria. As a lyosomal enzyme, increased ACP activity can be expected during a bacterial infection as part of the cellular response. It is unclear why 7 days of severe heat stress were required for detection of a marked elevation in ACP during the in-lab experiment. ACP activity may respond only to some forms of stress, and sudden heat stress may not affect it.

The sloughed epithelium of the gills after severe heat stress in the experiments may provide a portal of entry to bacteria if they are present in high numbers in the water during hot water periods on farms. Possibly this is involved in bacterial infection on farms in times of acute heat stress. Gill damage is however, not a specific indicator of heat stress.

The digestive glands of many of the abalone from the heat stressed group on day 7 had significant lesions. This may have occurred because the digestive gland is more sensitive to the effects of heat on metabolism than other organs. The numerous mitochondria present in digestive gland cells (Bevelander 1988) suggest a high metabolic rate is present. As temperatures rise, less oxygen dissolves in water even if it is well aerated, so that the supply to the tissues by the haemolymph may be reduced. But oxygen consumption in tissues rises with increased temperature, so irreversible onset of anaerobic metabolism is likely to occur in cells with higher metabolic rates (Van Dijk et al. 1999). The elevated metabolism due to higher temperatures will also lead to elevated tissue catabolism and consumption of the abalone's energy stores. Since the digestive gland is a central metabolic organ (Wilbur and Young 1966; Bevelander 1988), it would be most affected by this process.

Damage to the digestive gland may well lead to poor growth and survival; and the damage seen in this organ raises the question of whether it is possible for abalone to recover from such a severe stress and resume normal growth.

The decline in weight in the laboratory experiment between day 1 and day 2 may be partly physiologic because there was some recovery by day 7. Weight loss could include loss of mucin and defaecation, but also some losses due to gill damage or changes in the digestive gland.

Both control and treated groups in the second experiment done on the farm had lost weight during the 5 days before the tanks were heated (by 18.9.09) and had lost further weight after the week of heat stress (28.9.07). This is probably an effect of movement of the experimental animals into the tanks from the farm, as well as cessation of feeding from the time of movement until after the heat stress had ceased. Both treated and control groups were then moved again, another stressful event. But the design of this experiment focuses

on recovery after the heat stress, by comparing the heated abalone with the controls. The abalone were moved to a single tank and were treated with the same husbandry as all the other farm stock, to see if recovery would occur when treated in the standard way used on this farm. The control group had recovered and had started to gain weight two months later, but the treated group had recovered the initial lost weight only after 2 months. The heat treated abalone had more variable growth rates over the following 9 months but the eventual mean weight gain was not significantly lower than the control groups. This indicates that some abalone had recovered from the very severe heat stress and made up growth losses, while others remained at a disadvantage in weight as a result of the stress.

The recovery in weight gain was mirrored in the histological findings. The day after the temperature had declined back to ambient levels, there were some abalone in the heat-treated group that had similar lesions in the digestive gland as described in the first shorter experiment, but not all the abalone had these lesions. That they occurred in the second experiment indicates that these lesions can be induced within a week of severe heat stress, but whether this occurs probably depends on the robustness of the individual abalone. The recovery of weight gain indicates that abalone can recover from the severe stress and may make up the lost growth, though others may be set back in terms of growth. The stress applied in both the experiments was severe but short lived. Whether the acuteness of the stress is a factor in being able to recover is not clear, but longer periods are unlikely on farms. Perhaps similar experiments should be done with longer term stresses such as overstocking or malnutrition.

Elevated temperature in summer is not the sole stress. It comes with other problems of decreased water quality and further research is needed to assess which factors in hot summers are responsible for increased mortality. Bacterial counts will rise in warmer weather due to a mixture of factors. Bacterial growth increases with increased temperature, and increased bacterial counts in the water would lead to increased infection even with a robust immune system, due to an elevated dose of bacteria. If a few abalone die, then these would become foci of dense bacterial growth leading to increased bacterial challenge doses to their cohorts. This is one possible explanation for the sudden rise in mortality that can be seen in individual tanks on farms in summer. The effect of water quality also needs to be investigated, for example the decline in dissolved oxygen and its association with elevated ammonia and water temperature in different farms. Careful monitoring of abalone from the same cohort in poorly performing tanks compared to tanks with low mortality may be useful to pinpoint the causes of elevated mortality that may be encountered on a farm during the summer months.

Some assays appeared to show marked effects in the laboratory experiment, but the data from hotter and colder days on the farm did not always correlate to this. It may be that the more gradual temperature changes on the farm do not produce the same effects, or that associated changes on-farm, such as increased bacteria in the warmer tank water, may be playing a role.

One reason why there was not more clear-cut depression in immune function may be that the abalone were still within the temperature range that they can tolerate. The warmest day recorded, 24.5ºC in 2007, resulted in significant differences in some but not all parameters. The phagocytic rate (PR) and Neutral Red Retention (NRR) times declined, the phenoloxidase elevated but there were no significant differences in antibacterial activity. This indicates a suite of assays is needed to examine immune function on-farm. In 2008 between the 7$^{th}$ and 8$^{th}$ visit (17.3.08 – 28.3.08) the temperature dropped greatly from 23.5ºC to 18.5ºC and this coincided with an elevation in each of the immune assays done. The haemocyte counts, PR, antibacterial activity and NRR times all rose.

The mixed results from the farm monitoring indicate that reduced immune function could well have been present in the very warm days of 2007, but this suite of assays cannot accurately predict on-farm the level of heat stress that will lead to immuno-suppression. While this is a negative result, it demonstrates it is important to study how well assays detect stresses in the farm setting, as we have done, to evaluate how useful assays are, rather than rely on laboratory based studies. Farmers have been looking for a simple on-farm test to indicate times when stress in their stock is elevated to the extent that the immune system may collapse, but none of these assays currently can predict accurately when this event will occur. The farm manager commented that prior to 2009 summer mortality (probably due to multiple stresses) remained under 1%, but the stock appeared more stressed and growth was lower over peak summer temperatures (>22ºC), so the farm initiated a range of measures to increase productivity, including a selective breeding program with mortality as one criterion, increased pump capacity, more intense husbandry in summer and a changed diet. Summer temperatures are now not seen as a production issue on this farm.

One problem for any assay detection program is that the mortality can vary between tanks, with a few tanks much worse than others. The stress problem may similarly be confined to a small subset of tanks, and if so, a random sample of tanks and abalone within them cannot easily detect the problem. Mortality may result only when abalone are subject to a combination of stresses with multiplicative effects (e.g. heat is combined with lower flow due to aggregations of abalone in the tank, or low dissolved oxygen).

## Stressors associated with stock movements.

Current farming methods for abalone include movement procedures to grade stock into similar sizes, change stocking density and harvest for sale. Increased mortality in the days following a procedure suggests these procedures are very stressful to abalone.

Stock movement methods typically involve anaesthesia applied to the entire tank followed by scooping up the abalone into large trays, which are then carried to other tanks for dispersal. The movement may include a grading step where the abalone are passed through holes of different sizes, or separated in other ways to enable abalone of a similar size to be stocked together. Such grading will invitably involve increased handling. Various anaesthetics have been used to relax the adductor muscle and simplify large-scale removal of abalone from a tank (Aquilina & Roberts 2000). Shortly after the anaesthetic is applied, the abalone extend their adductor muscle leading to elevation of the shell and they swivel the shell back and forth around the muscle axis for up to 5 minutes before becoming limp and gradually losing their grip, allowing for removal from the tank within 20-25 minutes. Due to the problem of anaesthetic residues in the meat, larger stock nearing the size for harvesting cannot be anaesthetised and are chipped (manually removed) from the tanks. Thus it was appropriate to consider the relative impacts of both movement methods commonly used by the industry.

The experiments described here followed a routine movement procedure at GSW, with sampling of abalone before and after the workers had anaesthetised and shifted stock. Movement procedures involve several potentially stressful components, but anaesthesia and handling are suspected to be paramount. To establish the relative effects of these two possible stresses, experiments on 2 year-old hybrid abalone were designed to determine which effects were most stressful to the abalone, to assist farmers in developing better movement methods. Manual removal and movement (referred to in the industry as "chipping") was compared to anaesthesia with subsequent movement from the tank (AM) and anaesthesia without movement (A). The parameters studied were haemocyte counts, phagocytosis rates, antibacterial levels in the cell free haemolymph and the neutral red

retention times (NRR assay). The control tanks were monitored for 2 weeks in order to investigate whether the baseline altered. The movement experiment was then repeated to see if any baseline changes occurred and altered the response to stress. Water temperatures for the first experiment varied from 13-14ºC and in the repeat experiment from 14.5-15.5ºC.

The first experiment started the day prior to commencement of a routine farm movement procedure. In three control tanks abalone were sampled each day of the experiment; in three tanks abalone were chipped; and in three tanks abalone were anaesthetised prior to movement of stock in the routine manner done on this farm. On day 1, two baseline samples were taken from each of the nine tanks. The treatments started on day 2. The water temperature on the day of anaesthesia was 13ºC (range 13-14ºC over the 5 days).

Manual chipping was done using a metal spatula to scoop up the abalone from the tank floor, taking care to avoid cutting the foot. These chipped abalone were tagged with plastic 10x3mm tags attached to the shell with superglue, then left out on a plastic tray for 45 minutes prior to being replaced into the same tanks. The tanks selected for anaesthesia on the day of movement had the in-flow seawater taps turned off, then were flooded with seawater to which benzocaine had been added (70 grams benzocaine in a litre of ethanol per 1000 litres of seawater). A fresh mixture was made up prior to anaesthesia of each tank. The abalone were moved once they had ceased adhering to the concrete floor of the tank (approximately 20-25 minutes). These abalone were swept together into small piles and then scooped up in handfuls and put into plastic trays by the farm workers. The trays were weighed and then the stock poured from the trays into new tanks or into the same tanks at two thirds the original stocking density. Thirty experimental abalone were randomly sampled from the trays, tagged and replaced into the same three tanks. The abalone were out of the water for 40 - 50 minutes. Thirty abalone were left behind untouched in the tanks, and these were tagged where they lay when the anaesthetised water was draining out, leaving the shells exposed to air. Fresh seawater, free of anaesthetic, was then pumped into the tanks.

Two abalone from each of the 3 tanks for each group were sampled within 4-6 hours of the treatments (N=6 per treatment or control group). Further abalone were sampled from control and treatment groups the following day (day 3) and two days later (day 5) to assess recovery. The anaesthetised abalone were not fed because this is routine practice on the farm. The farm has noted that abalone normally do not eat for several days after the movement procedure, so no feed is supplied. The chipped abalone were put back into the tanks they were removed from. These tanks were fed and cleaned daily because the experimental group was a tiny fraction of the total stock in this tank and the farm practice was to feed these tanks. It was not noted whether the chipped abalone ate any of the food supplied but anecdotal records from the farm suggests this is unlikely. Controls were from 3 tanks that were not otherwise handled, except for routine cleaning and feeding.

The experiment was repeated 2 weeks later, with some minor variations. Two abalone from 2 tanks (N=4) were assigned to each treatment group. The chipping and anaesthesia were done as described above. The baseline samples were taken from the same tanks as the treated groups and sampled immediately before treatment commenced. The tanks used for the chipping component were the same as those used in the first experiment. The chipped abalone were monitored on the day of treatment (2-3 hours after the treatment) and the day after only. The anaesthetised (A) and the anaesthesia + movement (AM) groups were sampled on the day of treatment only; the recovery from anaesthesia on the day after treatment was not examined. The water temperature on the day of anaesthesia was 15.5ºC (range 14.5-15.5ºC over the 3 days of experiments).

The control tanks were sampled two weeks after the first experiment to see if baseline values altered. Samples were taken from these tanks over 3 days, termed days 16, 17 and

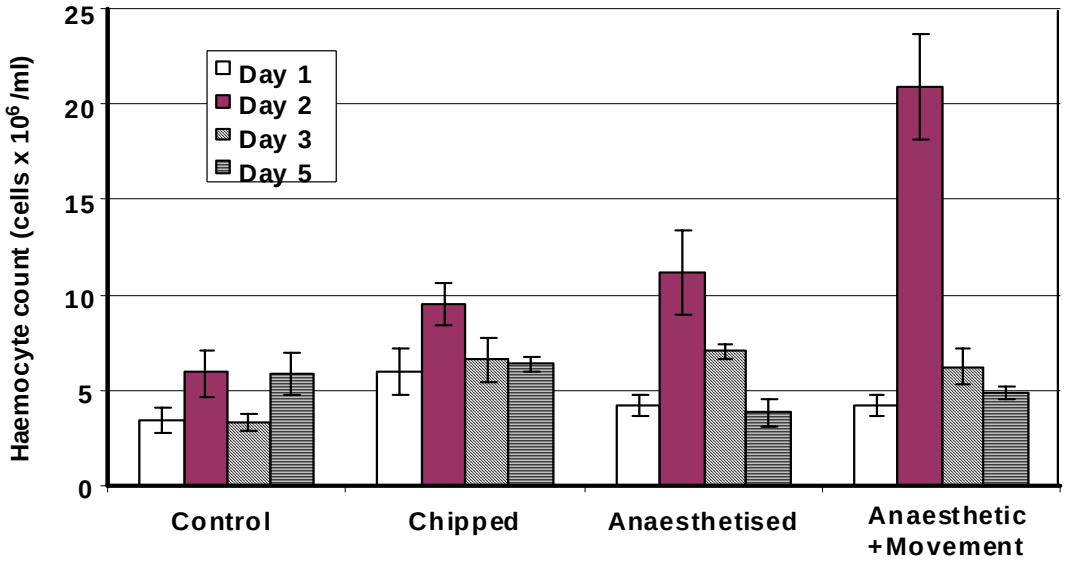
18 because this is the time from day 1 of the first experimental sampling sequence.

In the first experiment, partly nested two-way ANOVAs were used to compare the effects of tanks, day, treatments and their interactions. The treatments were applied to tanks, which were included in the general linear model nested within treatments. The tank effect was not significant so that the analysis focused on the day x treatment interaction. After the ANOVAs, planned comparisons (Day & Quinn 1989) were done to investigate whether the changes seen in the treatment tanks were significant when compared to changes seen in the control tanks over the same time period. These provide a powerful means of comparing treatments. A separate set of control tanks was not available for the repeated experiment. The treatment groups in the repeated experiment were compared to baseline samples taken from the same tanks just before treatment and Tukey's pairwise comparisons were done after the ANOVAs (day, treatment, tank effects) to compare the treatments. The NRR data were log transformed for analysis, but the graphs use raw data.

*Haemocyte Counts*

The haemocyte counts were elevated in all treatment groups, with recovery occurring within 1 day (Figure 20). For all these assays, Day 1 is before the treatment. The elevation and recovery were significant (Table 8) for the group subject to anaesthesia and movement (AM) as compared to the changes in the control tanks. The anaesthesia only (A) group and chipped group showed increased counts after treatment on day 2 and recovery on day 3, but the changes were not significant as compared to changes in the control tanks on the same days. The change in cell count between day 1 and 2 was significantly different between the AM group and the A group. Cell debris was noted in the haemolymph of both the anaesthesia treatment groups.

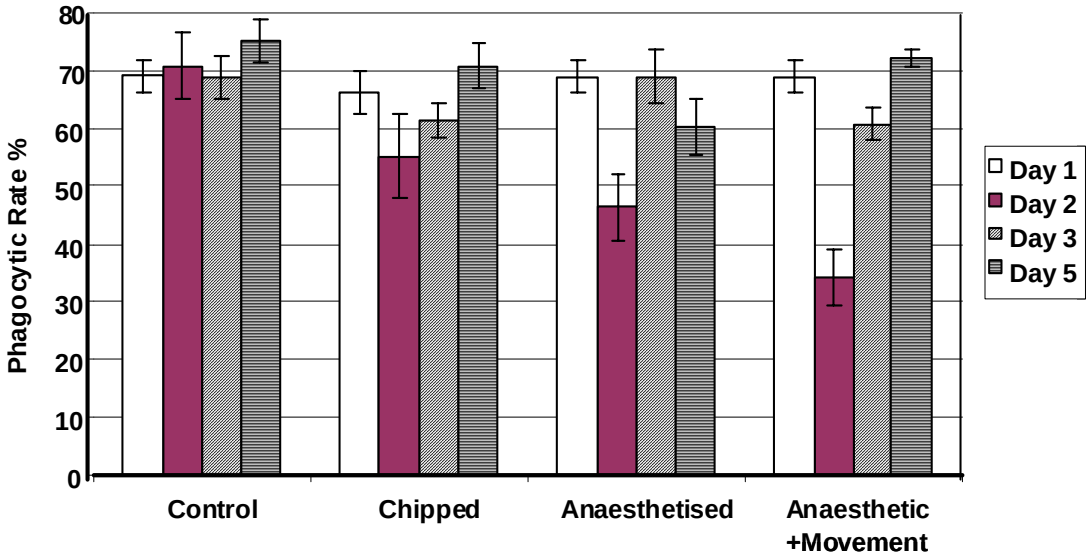
**Figure 20. Haemocyte counts (means +/-SE) over 5 days in controls, after chipping without anaesthesia, anaesthesia alone and anaesthesia+movement**




*Phagocytic Rate*

Phagocytic rate (PR) declined in all three treatment groups after movement on day 2 (Figure 21), with the AM group most severely affected. All 3 groups had largely recovered within 24 hours. The decline in PR is significant for the AM group as compared to the change in control tanks from day 1 to 2, and also compared to change in the A group (Table 8). The decline in the A group as compared to controls is close to significant. The recovery is significant for both A and AM. In both anaesthesia groups cell debris the same colour and appearance as haemocyte cytoplasm was seen in the stained smears.

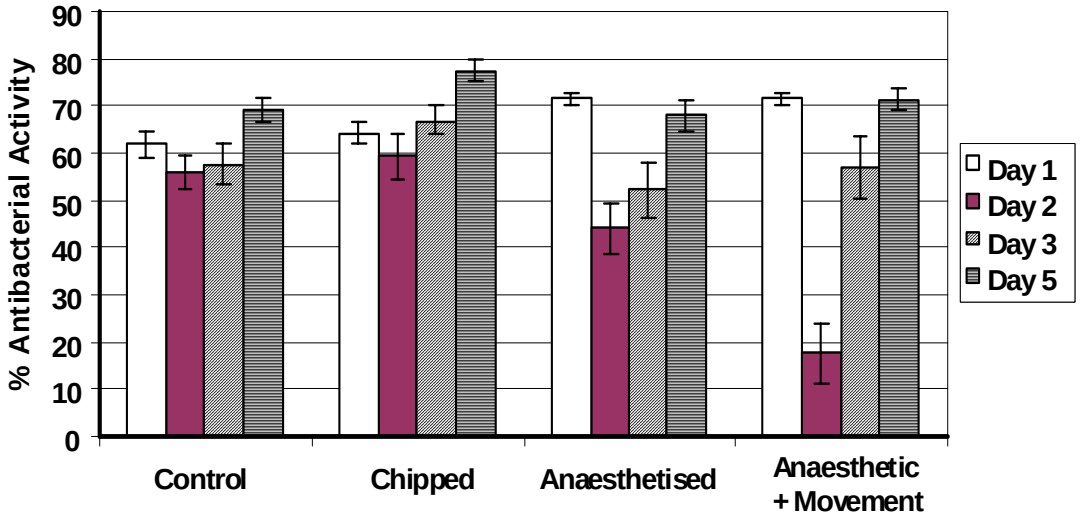
**Figure 21. Phagocytic rate (means +/-SE) over 5 days in controls, after chipping without anaesthesia, anaesthesia alone and anaesthesia+movement**




*Antibacterial Activity*

The decline in antibacterial activity (Figure 22) after treatment from day 1 to 2 is significant in both anaesthesia groups compared to the changes in the control tanks (Table 8) and the AM group is significantly more depressed than the A group after treatment. The recovery in the AM group between day 2 and 3 is significant, but the recovery for A between day 2 and 3 or 5 is not. The chipped abalone showed minimal decline in antibacterial activity on the first day of treatment and elevated antibacterial activity 3 days after movement, but neither change was significant.

**Figure 22. Antibacterial activity (means +/-SE) over 5 days in controls, after chipping without anaesthesia, anaesthesia alone and anaesthesia+movement**




*Neutral Red Retention Assay*

The control tanks as wells as all the treatment groups show a marked decline on the day of treatment with no evidence of recovery (Figure 23). The planned comparisons show there is no significant difference between the decline in the controls between day 1 and day 2 and the decline in the three different treatment groups (Table 8).

**Figure 23. Neutral Red Retention (means +/-SE) over 5 days in controls, after chipping without anaesthesia, anaesthesia alone and anaesthesia+movement**

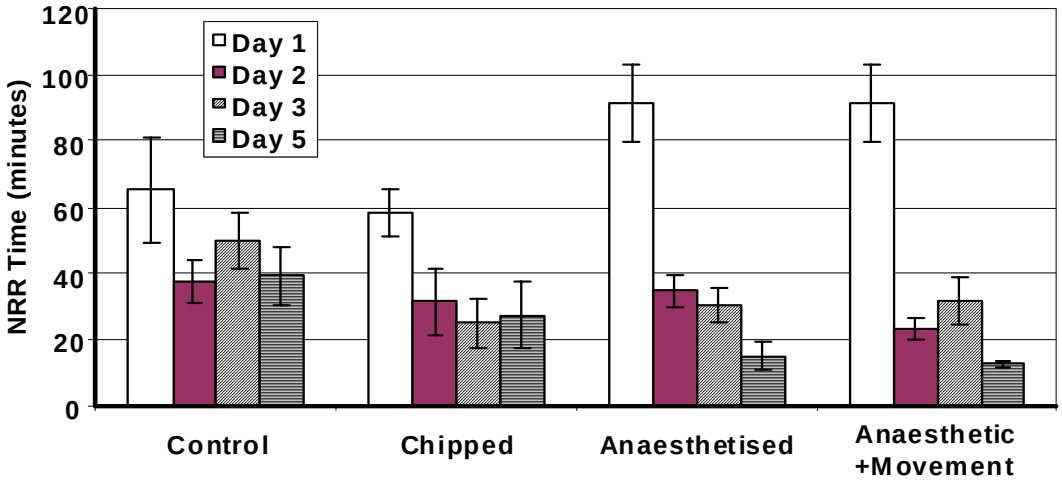



**Table 8: Comparison of immune assays in abalone from control and treatment tanks on day 1 (d1) and day 2 , and recovery on days 3 and 5 compared to day 2.**

| | df | Mean Squares | F ratio | p-value |
|---|---|---|---|---|
| **Haemocyte count** | | | | |
| Day x treatment | 9 | 63.7 | 7.1 | < 0.001 |
| Control d1-d2 vs A d1-d2 | 1 | 24.8 | 2.76 | 0.101 |
| Control d2-d3 vs A d2-d3 | 1 | 3.6 | 0.41 | 0.526 |
| Control d2-d5 vs A d2-d5 | 1 | 85.4 | 9.509 | 0.003 |
| Control d1-d2 vs AM d1-d2 | 1 | 246.5 | 27.4 | <0.001 |
| Control d2-d3 vs AM d2-d3 | 1 | 219.6 | 24.5 | <0.001 |
| A d1-d2 vs AM d1-d2 | 1 | 95.8 | 10.7 | 0.002 |
| Control d1-d2 vs Chip d1-d2 | 1 | 2.13 | 0.238 | 0.627 |
| Control d2-d3 vs Chip d2-d3 | 1 | 0.198 | 0.022 | 0.883 |
| Error | 74 | 8.98 | | |
| **Phagocytic Rate %** | | | | |
| Day x treatment | 9 | 398.2 | 3.6 | 0.001 |
| Control d1-d2 vs A d1-d2 | 1 | 418.1 | 3.8 | 0.054 |
| Control d2-d3 vs A d2-d3 | 1 | 906.5 | 8.3 | 0.005 |
| Control d1-d2 vs AM d1-d2 | 1 | 2133.6 | 19.52 | <0.001 |
| Control d2-d3 vs AM d2-d3 | 1 | 1232.7 | 11.28 | 0.001 |
| A d1-d2 vs AM d1-d2 | 1 | 552.3 | 5.05 | 0.028 |
| Control d1-d2 vs Chip d1-d2 | 1 | 237.5 | 2.235 | 0.139 |
| Control d2-d3 vs Chip d2-d3 | 1 | 100 | 0.92 | 0.342 |
| Error | 74 | 109.3 | | |
| **% Antibacterial Activity** | | | | |
| Day x treatment | 9 | 633.5 | 5.2 | < 0.001 |
| Control d1-d2 vs A d1-d2 | 1 | 492.7 | 4.08 | 0.047 |
| Control d2-d3 vs A d2-d3 | 1 | 277.7 | 2.3 | 0.134 |
| Control d2-d5 vs A d2-d5 | 1 | 168.6 | 1.4 | 0.241 |
| Control d1-d2 vs AM d1-d2 | 1 | 2910.7 | 24.08 | <0.001 |
| Control d2-d3 vs AM d2-d3 | 1 | 3012 | 24.92 | <0.001 |
| A d1-d2 vs AM d1-d2 | 1 | 840.3 | 6.95 | 0.01 |
| Control d1-d2 vs Chip d1-d2 | 1 | 0.6 | 0.005 | 0.944 |
| Error | 74 | 120.9 | | |
| **Log NRR** | | | | |
| Day x treatment | 9 | 0.63 | 2.8 | 0.007 |
| Control d1-d2 vs A d1-d2 | 1 | 0.38 | 1.708 | 0.195 |
| Control d1-d2 vs AM d1-d2 | 1 | 0.37 | 1.623 | 0.207 |
| Control d1-d2 vs Chip d1-d2 | 1 | 0.11 | 0.481 | 0.49 |
| Error | 74 | 0.23 | | |

*Histopathology*

Movement processes led to erosion and ulceration or laceration of the foot epithelium, with later scarring, both for chipping and anaesthesia. There was also an immediate enlargement of sinuses in the foot muscle of abalone, filled with haemocytes. This second result correlates directly to the increased haemocyte counts in the haemocytometer, and suggests that abalone respond to anaesthesia or chipping by immediately moving haemocytes into the sinuses, and as a result both the volume of the sinuses and the density of cells in the haemolymph increase. Perhaps this allows the haemocytes to circulate rapidly to any site of erosion or laceration of the foot, so as to respond to bacterial invasion at those sites.

*Repeated Experiment*

The results were similar to those in the first experiment (Table 9). The haemocyte counts and phagocytic rates were significantly different between the chipped and control groups in the tanks on the day of chipping. The phagocytic rate was also significantly different between the chipped abalone on the treatment day and those sampled on the day after chipping. The antibacterial activity baseline was higher in the tanks used for chipping than in the initial experiment, and there was no significant decline after treatment. There were significant differences between baseline and treatment groups in all anaesthetised abalone, with the Anaesthesia plus Movement group sometimes more severely affected than the Anaesthesia only group, and sometimes less affected (Table 9). The NRR results declined significantly in anaesthetised abalone as compared to the baseline abalone sampled in the same tank prior to anaesthesia.

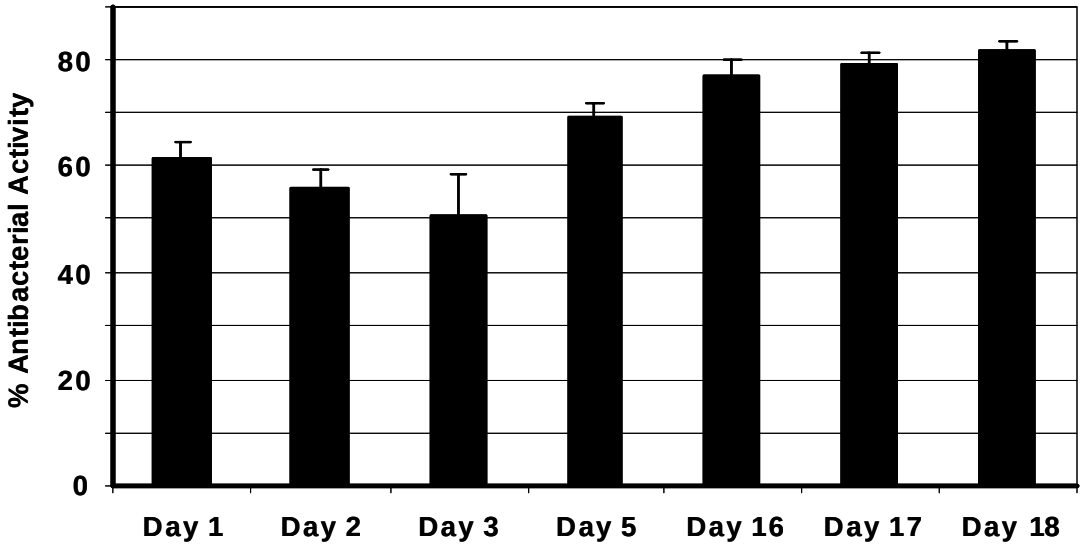
**Table 9. Results from the repeat experiment. N=4. Means +/- SE for Haemocyte counts (HC), phagocytic rate (PR), antibacterial activity (AA) and the Neutral Red Retention assay times (NRR); and below these, the *p* values for pairwise comparisons using Tukey's tests after ANOVA.**

| Samples /Comparisons | HC (x $10^6$/ml) | PR (%) | AA (%) | NRR (mins) |
|---|---|---|---|---|
| Chip baseline | 5 +/- 1 | 72 +/-4 | 80 +/- 2 | 39 +/- 9 |
| Chip same day | 10 +/- 1 | 49 +/- 3 | 79 +/- 3 | 18 +/- 5 |
| Chip next day | 10 +/- 0.5 | 80 +/- 2 | 81 +/- 3 | 32 +/- 10 |
| Chip same day vs next day | p=0.881 | p<0.001 | p=0.892 | p=0.492 |
| Chip same day vs baseline | p=0.011 | p<0.001 | p=0.930 | p=0.246 |
| Chip next day vs baseline | p=0.023 | 0.214 | p=0.994 | p=0.849 |
| Anaesthesia baseline | 5 +/- 0.5 | 62 +/- 7 | 82 +/- 2 | 57 +/- 13 |
| A same day as baseline | 13 +/- 1 | 28 +/- 6 | 69 +/- 2 | 10 +/- 0.0 |
| AM same day as baseline | 20 +/- 2 | 32 +/-5 | 65 +/- 2 | 12 +/- 3 |
| A vs AM | p=0.033 | 0.882 | 0.354 | 0.908 |
| A vs baseline | p=0.012 | p=0.017 | p=0.006 | p<0.001 |
| AM vs baseline | p<0.001 | p=0.029 | p=0.002 | p<0.001 |

*Long term monitoring of control tanks*

The control tanks were sampled repeatedly over 18 days to monitor the baseline levels (Figure 24). Antibacterial activity did not vary significantly in the first 5 days, although there was an initial decline, but levels on day 16, 17 and 18 were significantly elevated compared to day 1 ($p < 0.05$). The abalone in the control tanks had no significant differences in the haemocyte counts, phagocytic rate and NRR times during this period.

**Figure 24. The % antibacterial activity in the control tanks over time.**




## Discussion of stock movement stress effects

The results show the value of controlled experiments done on-farm to compare different management strategies and show that abalone are sensitive to the current industry practices of stock movement. The assays used consistently showed the effect of each stress. Clearly these stresses have more effect on immune function than the heat stress studied above and explain the anecdotal evidence from farmers that movement in warm weather is more detrimental to subsequent stock health. The greatest changes were seen in abalone that were subjected to both anaesthesia and handling. Abalone were less affected by anaesthesia without handling and were least affected by manual chipping without anaesthesia. These differences between treatments were apparent in all the parameters examined. The repeated study had similar responses, even though the baseline levels of some parameters had changed. The parameters studied were a mix of physiologic and immune parameters (haemocyte counts, NRR assay), plasma immune properties (antibacterial levels in the cell free haemolymph) and cellular immunity (phagocytosis rates). Although the assays measured different parameters, the concordance of the results shows that the assays reflect the effects of stress on the abalone in a similar way, i.e. there is a predictable response to the stressors examined.

Both anaesthesia treatment groups had more severely depressed immune assay results than the chipped abalone, indicating that anaesthesia is a more stressful way to carry out stock movement. Several types of anaesthesia have been trialled in abalone and compared in terms of their effect on metabolic rate, recovery and subsequent growth rate (Edwards et al. 2000; Chacón et al. 2003) but not on the immune response. The consistent results show the methods used here provide an alternate means of comparing anaesthetic techniques that directly examines their effect on immunity.

The total haemocyte counts in this experiment showed a marked elevation that subsequently subsided to baseline levels by the following day. The literature shows a variable response in haemocyte counts depending on the stressor applied to abalone and the time lag that follows before sampling (Malham et al. 2003; Travers et al. 2008). The cause of the increased density of haemocytes in the sinuses and the source of the haemocytes in abalone is not yet known. The increased numbers in circulation could be triggered by an early transient increase in biogenic amines (Malham et al. 2003), and the walls of the digestive

tract are one likely source of haemocyte reserves, although further work is needed.

There was a significant impact of anaesthesia, with or without movement, on phagocytic rate (PR). This decline in phagocytic rate, which reflects reduced cellular immunity, has been reported with many other stressors and is a common response in abalone to significant stress (Cheng et al. 2004a; Chen et al. 2005; Travers et al. 2008). Cell debris was seen microscopically after anaesthesia with or without movement, suggesting that haemocytes may die or be damaged as a result of this stress. The cell debris was evident in the haemocytometer when counting haemocytes and in the stained smears used to assess phagocytic rate. Under light microscopy, it had an appearance consistent with cytoplasmic fragments, and the most obvious source is fragments of haemocytes, but it might possibly be from cells in any tissue as they all connect to the open circulatory system of abalone. In this study the PR recovered within 24 hours. If the fragments are from haemocytes, this recovery suggests that there were sufficient haemocytes in reserve for any damaged cells to be replaced, as cell fragmentation or loss of cytoplasm is likely to be lethal. The reduced phagocytic rate occurs at the same time as the elevated haemocyte counts, and suggests that moving haemocytes into circulation may be a favourable evolutionary adaptation to counteract the reduction in phagocytic capacity of the haemocytes under stress.

The control tanks were monitored over 18 days to see if baseline values change. On days 16, 17 and 18 the antibacterial activity had significantly increased in the control tanks compared to day 1 but haemocyte counts, phagocytic rate, and NRR times had not significantly altered. The repeated chipping done two weeks after the first experiment also showed a higher baseline level of antibacterial activity, but the increase was not significant. These results suggest that improved antibacterial activity may have been induced by some factor during this period. Previous work has shown that antibacterial activity in haemolymph is inducible by pre-immunising abalone with killed bacteria (Cushing et al. 1971), but there are no reports that environmental alterations can have this effect. Over the two weeks of this study there were increased light, noise and anaesthetic fumes in the shed due to the stock movement work in many tanks. Possibly one of these, or a combination, induced the increased antibacterial activity. Oxygen consumption and ammonia excretion rates are higher under light conditions than in the dark in some abalone species (Ahmed et al. 2008), indicating that increased light affects the physiology of abalone, but further work is needed to investigate whether it affects the immune system.

Why the antibacterial properties of haemolymph but not the phagocytic rate of the haemocytes would be stimulated is not clear. Both are important in dealing with pathogenic bacterial infections, but measure different aspects of an abalone's immune defences. This indicates that studies using single assays such as phagocytic rate or antibacterial activity, taken at a single time, could not reasonably assess the state of an abalone's immunity. It is important to establish a reliable set of complimentary assays to interpret immune status, and where possible use treatment groups together with controls.

On farms, stock movement procedures are applied to whole tanks of abalone, not individuals, and conditions may vary between tanks. In this study the abalone in the different tanks used were found to have very similar responses, so that the treatment effects were consistent across tanks. This may be a result of the very uniform construction and management of these tanks by the farm, and the very similar origin and size of the abalone in these tanks. However, a lack of differences between tanks cannot be assumed in future on-farm studies; and experimental study designs should accommodate for this potential influence on results.

The farm records show similar mortality rates when stock is moved using anaesthesia or manually chipping. The clear differences seen in this experiment may reflect greater care

taken when chipping the relatively low numbers of abalone required for this experiment than is possible when commercial numbers must be moved, so that the chipping stress in normal operations may be much more severe. This suggests that greater care or training to surprise the abalone during manual chipping might be effective in reducing the stress on the stock. But it would be most useful to find a less stress-inducing anaesthetic.

## BENEFITS AND ADOPTION

All Australian abalone growers now have available the opportunity to use a set of tested and well described assays that are convenient to use on-farm, to investigate the immune status in their stocks during periods of increased stress. This is most likely to be done in association with a research facility. Some have already sent staff to training sessions on how to take haemolymph samples for microbiology and samples for histopathology processing, to be part of the surveillance program.

Further, our on-farm experiments have provided information to farmers on the relative importance of various types of stressors. With the information on the severe stress that benzocaine anaesthesia produces, farmers should be able to reduce mortality and poor subsequent growth by ensuring this stress is imposed only as often as needed, and not when other stressors such as heat are present. In the long term they can investigate alternative stock movement methods using these assays.

Our results for the heat stress work suggest that abalone can cope with high temperatures and indicate the need for more work into the mix of factors that can lead to summer mortality. In particular we recommend further work into examining the synergistic effects of elevated heat combined with reduced water quality and increased bacterial counts. The assay methods, together with the designs of experiments and methods for data analysis we used, which test focused and relevant hypothesis in such experiments, provide a pathway to further useful information for farmers in the future. Our experiments were designed to work on farms, so that they relate to the issues on farms, rather than a laboratory simulation of the stress. This approach will produce useful results, including new questions for future research that may be better conducted in the laboratory. Further, we have demonstrated powerful analyses, such as planned comparisons of treatments, which can detect effects better than the commonly used methods of ANOVA followed by multiple comparisons. Growers can now support their staff, or students, to use these methods to evaluate new innovations in farm management. This provides a pathway to more cost-effective production on farms over time.

We have also distributed to farmers, via reports and presentations at growers workshops etc., a review of what is known, a glossary of technical terms, and reports of what we have learned about how abalone defend themselves from disease, and how stress affects them. This should facilitate the innovative capacity of growers, who can potentially use their increased understanding to optimise the management of farms. Further, our review of what is known and the results of our experiments are published, or now in preparation for publication. This should stimulate work elsewhere on abalone aquaculture that will add to the information farmers can use to guide cost-effective abalone husbandry. Some work in France has already built on our work.

Thus the benefits of our project to growers have encompassed all those benefits described in our project application. The response of farmers at Abalone Growers Association workshops where the work was presented has been very favourable.

## FURTHER DEVELOPMENT

The stock movement experiments in particular, indicate the future potential of the suite of assays that we have developed for on-farm use. These can now be used to compare the impact on the abalone immune status of various husbandry methods that are developed by farmers in the future, or are now used on different farms. This should facilitate the development of management strategies that impose less stress during stock movement, by testing alternative anaesthetics to find one that imposes less stress, and perhaps by revised handling procedures. The assays also provide a means to determine the importance of other factors, such as low water flows or high tank densities, and to evaluate ways to mitigate these stressors. Most reported studies have been done in the laboratory situation and may not reflect the complexity of farm situations.

Much of the work to exploit the new information we have provided will be most cost-effectively done by growers, using their innovative capacity and trials to see what works on their farms. For example monitoring dissolved oxygen at the tail end of tanks, with flow adjustments and observations of how tanks differ, should reveal when this factor is responsible for raised mortality. Similar trials of methods to reduce bacterial counts in the water should prove useful.

We suggest that growers may want to provide support to research students to undertake small scale research projects on problems afflicting particular farms. This should be cost-effective and successful, as we have now developed and defined the assays, most of which do not require complex laboratory equipment. Haemolymph for the MTS assay could be sent to a laboratory with the required facilities on a contract basis. Further, the experimental designs and analyses in this report (such as the planned comparison method) provide a model to study the stress associated with other husbandry methods, and the success of various mitigation methods to reduce environmental stresses. With well defined methods and models to follow, many of the problems particular farms face due to stress effects on abalone should be well within the scope of honours and masters students.

This is not to suggest that further work by experts in the field would not reap important benefits for growers. While we have provided simple techniques for on-farm work, we have not advanced very far in the understanding of abalone diseases or how abalone protect themselves from them – how the immune system of abalone works. We would expect great benefits from further study of abalone diseases and how they infect abalone, and from more detailed research on how the abalone immune system functions.

As our review (see Appendix 4) points out, the immense knowledge available about immune function in vertebrates can only provide a rough guide to what mollusc immune systems *may* be like, and very little work on molluscs has been done, especially on abalone or their close relatives. In fact work on prawns or even oysters (which have been cultured and thus studied for longer) also only provides a guide. Based on evolutionary distance, our immune systems may well be more similar to those of sea urchins than the immune systems of prawns are to the system in abalone. Oysters and abalone are both molluscs, but the ancestors of these animals separated about twice as long ago as the ancestors of birds and mammals separated from fish. Thus there is a huge field of knowledge to be uncovered about how abalone immunity works, and that knowledge will provide the means to solve disease problems in abalone aquaculture and enhance the security and profitability of this industry.

## PLANNED OUTCOMES

The planned outcome was to improve understanding of methods to assess abalone health, leading to increased productivity and profitability. We proposed the following outputs:

1. Establishment of normal variation in abalone immune parameters will provide a standard for use in future studies and any routine health monitoring program.
2. Providing an assessment of the effect of particular stressors on immune function, possibly leading to specific recommendations for minimizing stress and maximizing health under stressful conditions on abalone farms.
3. Providing an assessment of the relative value of adding immunological parameters to the basic histological assessment used in the South African abalone health monitoring program.

We subsequently replaced the first output, once we realised that the 'normal' ranges of abalone vary from farm to farm, and from time to time, so that controls must be used to monitor stresses. In fact there were also no obvious standard methods applicable to on-farm work, so that we proposed the new objective of providing a description of the best methods to measure abalone immune function on abalone farms.

The outputs of the project have been:

1. A review of what is known about both stress effects and immune function in abalone, and the various assays of immune function that have been measured in abalone.
2. A detailed description of many immune assay methods, including details of how suitable assays can be applied to investigate stress effects and immune status on abalone farms.
3. Descriptions of the results of experiments on the effects of heat stress, and various components of a stock movement process (anaesthesia, chipping, and anaesthesia plus movement), which show the relative effects of these stresses on abalone immune functions and provide recommendations for how stress may be reduced, together with the values of immune parameters in control abalone and how these can vary.
4. Descriptions of the effects of farm stresses on the growth and histology of farm abalone, and the relation of these effects to the haemolymph immune parameters measured in experiments.
5. Model designs for on-farm experiments and powerful statistical analyses (especially planned comparisons of treatments) that can be used in future work.

Most of these outputs have been communicated to farmers by the distribution of reports, and presentations at Australian Abalone Growers annual meetings by Celia Hooper. They largely provide the overall outcome planned, but we also have applied these methods and thus given farmers a better understanding of the nature and degree of summer heat stress, the importance of stress during stock movements, and especially the stress associated with anaesthesia. These methods and the model designs of on-farm experiments and analysis also establish the way in which monitoring on farms and trials of stress reduction strategies can be done in future. Thus we have provided a pathway for many more outcomes to come.

An unintended outcome was the early detection and description of the disease Abalone Viral Ganglioneuritis. As part of this project Celia Hooper was carrying out assays on abalone haemolymph at Southern Ocean Mariculture, when she was contacted by the nearby Coastal Sea Farms to investigate sick abalone there. When she recognised that this was a new disease, she suggested the involvement of the veterinary aquaculture specialists Paul Hardy-Smith and Judith Handlinger. The samples were then sent to the government laboratories at Attwood.

## CONCLUSIONS

Our experiments and monitoring show that the combination of several methods: haemocyte counts, phagocytic rate, the NRR assay, the MTS antibacterial assay, and histopathology, are extremely useful in detecting stress effects on the immune system and the physiology of abalone. The four assays using hemolymph cover various components of the immune system, and the first three can be done on-farm. All four indicate the stress of the stock movement process, but the response to severe heat stress was mixed. Thus these assays should be used in combination. We are confident that these methods provide the means to investigate how to improve management processes to reduce stress and thus increase productive efficiency.

The Neutral Red Retention Assay (NRR Assay), which measures lysosomal membrane stability, is reported to be the earliest indicator of stress in molluscs (Moore et al. 1979; Harding et al. 2004; Song et al. 2007), and it does not require complex equipment. The assay appears to be sensitive in picking up stress responses when standardised against control data taken on the same day. We found however that the variation between samples is high, so that error bars are large. It also is hard to standardise between observers, and requires practice for consiistent results. Thus it is not useful as a health monitoring tool or as a ”stress test” but does have value in the research setting.

The longer term monitoring in our experiments shows that baseline conditions for some assays do change, and thus these tests cannot be used for occasional health monitoring but are useful as a research tool to ask a specific management question, for example which type of anaesthetic procedure causes the least immunosuppression in your stock. It is also important to ensure that the effects on these assays are consistent; and that the main effects identified are repeatable in experimental studies. In particular the variation in antibacterial activity in the control tanks in the movement experiment was not expected, and highlights the need for more research to be done on commercial abalone farms and not just in controlled laboratory situations. This should generate relevant research questions that more accurately reflect important issues in abalone farms.

Our stock movement experiments have shown that the stresses associated with stock movement appear to be very important. Anaesthesia with benzocaine is a very serious stress for abalone, far worse than careful chipping. This indicates that more work needs to be done with assays like these to accurately compare different movement and anaesthetic procedures.

The short term heat stress experiments show that some assays detect heat stress more effectively than others. But it appears likely that abalone are well adapted to short term heat stress. Heat appears to be a stress that abalone have mechanisms to cope with, provided the temperatures remain within a range and duration tolerated by the stock, depending on their genetics and acclimatization. This work indicates other areas of research to be pursued, to further identify the problems leading to summer mortality.

This last conclusion illustrates that even where the assays produce a negative result – showing that the heat stress alone is not likely to produce extensive mortality – the result can point to ways to improve husbandry and thus increase farm production.

While the conclusions set out above about the stressors associated with stock movements are the most definitive and important in themselves, we consider that the most important contribution of our work is to facilitate future work. We have assessed and defined a series of appropriate methods to monitor stress effects on immune function in abalone on farms. We have also provided model designs and statistical analyses to allow on-farm - and thus

very relevant - experiments to study how stress can be reduced in abalone mariculture to increase farm profits. In the field of ecology, the recognition that experiments need to be done in the field and not the laboratory, using designs for field experiments, is now hailed as a very important breakthrough. Conditions do not act in isolation, so that apparently extraneous laboratory conditions can change the outcome of experiments. We look forward to the use of the methods we have developed and tested for experiments and monitoring carried out on farms, to provide further benefits to abalone farms on a continuing basis.

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# APPENDICES

## APPENDIX 1: INTELLECTUAL PROPERTY

While there is no commercially valuable IP arising from this research, there is considerable valuable information to Australian farmers. Further, the methods could be used by commercial veterinary services to provide a service to abalone farmers.

The work is either published, or is currently being prepared for publication. While the farming methods used overseas for abalone are not the same, so that the results described will not assist abalone farms elsewhere directly, we expect these papers to stimulate and speed further work overseas, so that more information will become available that is of some assistance to farmers here. This has already been the case with our early review of what was known of abalone stress and immunity: work in France has built on the information in our paper, and a French student has spent a period in our laboratory, contributing to the work in this report.

## APPENDIX 2: STAFF

No salaried staff were required for this project but students were employed on a casual basis, in particular Athol Whitten, who provided many days of field assistance. Most of the work was carried out by Celia Hooper, who had 50% employment at Gribbles Veterinary services, and worked 50% time on this project.
Many students volunteered time to assist us, and GSW staff provided extensive support, such as feeeding and cleaning of experimental tanks, assistance in the setup of experiments, or chipping animals for laboratory experiments.

## APPENDIX 3: A GLOSSARY OF TECHNICAL TERMS IN THIS REPORT

| | |
|---|---|
| Actin-myosin filaments | Muscle filaments made of actin and myosin; proteins which work together to contract and move cells. |
| Adhesins | Surface antigens involved in forming adhesions between cells. |
| Adrenocorticotropin | A hormone that stimulates the adrenal cortex in mammals |
| Agglutination | Sticking together of a large number of cells |
| Amines | Organic molecules with an N-$H_2$ component |
| Amoebocyte | Amoeba-like cells that actively seek and phagocytose foreign particles. Sometimes used as a synonym for haemocyte. |
| Amoeboblasts | Cells in molluscs that appear to be precursors to amoebocytes |
| Antigen | A molecule recognized by the immune system. |
| Apoptosis | Programmed cell death |
| Atrophy | Process where tissues become smaller |
| Basophilic | Blue colour seen microscopically due to certain acidic compounds when stained with haematoxylin dyes. |
| Blast like cells | Primitive cells that have a small amount of cytoplasm surrounding the nucleus, and are typically progenitors to specialized cells |
| Cambrian era | A geological period from 600 to 500 million years ago, when many animal groups appeared |
| Caseous deposit | Necrotic (dead) material with a crumbly cheesy consistency |
| Chemotaxis | Movement along a concentration gradient, e.g. towards the source of a chemical stimulus |
| Class | The taxonomic category below Phylum. Abalone are in the class Gastropoda of the phylum Mollusca |
| Collagen | A group of structural proteins forming flexible, strong fibers important for the strength in tissues. |
| Colorimetric assays | Chemical assays carried out using a change of colour as an indicator of biological (metabolic or enzymatic) activity |
| Complement cascade | A series of blood proteins involved in the mediation of coagulation |
| Cortisol | A steroid hormone produced by the adrenal cortex in response to a stressor, which has various metabolic and anti-inflammatory effects in the body. |
| Cytokines | Chemicals that carry messages between cells |
| Cytoplasm | The jelly-like matter in a cell outside the nucleus, in which organelles are embedded |
| Cytoskeleton | The structures maintaining the shape of the cell, mainly microtubules |
| Cytosol | The fluid of the cytoplasm of cells |
| Cytotoxicity | Ability of a toxin to kill cells |
| Diapedesis | Migration of cells across a membrane or through tissues |
| Differentiation | A process whereby a cell becomes specialized due to the expression of particular genes |

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|---|---|
| Effector cells | Cells that act to produce an effect; in this case destruction of pathogens |
| Efferent | Carrying messages (in the case of nerves) outwards from the center to the periphery of an animal |
| Endemic | With a local origin, not introduced from elsewhere (opposite to epidemic). An infection is said to be "*endemic*" when that infection is maintained in the population without the need for external inputs |
| Endocrine system | The system of circulating hormones in animals that controls tissue functions |
| Endogenous | Arising from within the organism (opposite of exogenous) |
| Endothelium | A layer of specialised cells lining the inner surface of blood vessels. |
| Epithelium | A specialized sheet of cells lining the surface of an organ, eg the lining of the gastrointestinal tract, urinary tract, or the skin as well as the cells that make up glandular organs. |
| Epizootics | Disease epidemics among animal populations. |
| Exocytosis | The extrusion by a cell of material that was previously in a vacuole (capsule) in the cell |
| Exogenous | From a source outside the organism |
| Fibrinogen | A blood protein that causes clotting |
| Fibronectin | A structural protein found in the fibrous tissues of animals, involved in adhesion |
| Ganglia | A collection of nerve cells forming a local processing centre with particular functions |
| Glucocorticoids | A group of steroid hormones produced by the adrenal cortex, which have metabolic and anti-inflammatory effects. |
| Glycogen | A carbohydrate used as an energy store (stored in the foot of abalone) |
| Glycoprotein | Complex molecule formed by a protein linked with carbohydrate molecules. |
| Gram negative bacteria | Bacteria with chemical characteristics to their cell walls which causes them to stain red on gram staining |
| Gram positive bacteria | Bacteria with chemical characteristics to their cell walls which causes them to stain blue on gram staining |
| Haematopoiesis | The formation of blood cells |
| Haemocytes | Cells that are found in the blood of molluscs |
| Haemocytopenia | A lowered concentration of haemocytes in the haemolymph |
| Haemolymph | The circulatory fluid in molluscs, arthropods and other organisms where the fluid is not confined to blood vessels |
| Haemolymph plasma | The fluid without the cells |
| Haplosporidian parasite | A parasitic protistan from the phylum Apicomplexa (sporozoans, which includes *Perkinsus* and *Plasmodium*, the malaria parasite) |
| Heparin | An anticoagulant that prevents clots forming in the blood of mammals |
| Histamine | A protein involved in inflammation, commonly associated with hypersensitivity reactions |
| Histological examination | Sections of tissue are placed on glass slides, stained and studied under a microscope |

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|---|---|
| Homeostasis | The maintenance of a steady state in an organism's internal environment |
| Homology | Similarity in structure or function |
| Humoral factors | Non-cellular components in the body fluids |
| Hypercortisolemia | Elevated levels of cortisol in the blood / body fluids |
| Hyperplasia | Enlargement of tissues, the opposite of atrophy. |
| Ill-thrift | A loss of condition and reduction of normal growth and activity |
| Integrins | A family of adhesion molecules expressed on cell surfaces. |
| Inter-endothelial junctions | Junctions between the cells forming the endothelium |
| Intermediate hosts | A host used by a parasite as a means to get to the final host, where it becomes mature and reproduces sexually. |
| Invaginations | Pockets formed in a membrane where there is an opening to the outside |
| *In vitro* | Studies conducted outside the animal, often using blood fluid or cells suspended in culture media (as opposed to within the animal – *in vivo*) |
| Isoelectric point | Point to which proteins migrate in a gel when placed in an electric field as they move to the point where their attraction to each electric pole is balanced. Gel electrophoresis is a common method used to separate proteins |
| Lamellipod | The frontal `fan'-like part of a migrating cell - a directional thin protrusion of the cytoskeleton that is followed by the remainder of the cell body. |
| Lectin | Any protein that binds to specific carbohydrate groups on proteins or on cell membranes |
| Lesions | Areas of damage in tissues e.g. due to infection or trauma |
| Leukocytes | White blood cells, which form part of the immune system in vertebrates |
| Lysosome | A vacuole in the cell that contains reactive enzymes or other chemicals |
| Lyse | To split open a cell, releasing its contents |
| Macrophages | Large white blood cells in vertebrates that phagocytose pathogens |
| Mast cell | Cells with basophilic granules which when released from the cell promote inflammation and vascular leakage |
| Metaplasia | The transformation of one differentiated cell type into another, typically in response to chronic injury |
| Microtubules | Tiny tubes found in the cytoplasm of cells that have a variety of functions, especially secretion of cell products |
| Mitotic rate | Rate of cell division |
| Monosaccharides | Sugars with a single chemical ring, e.g. glucose |
| Morphology | Part of the visible structure of an animal |
| Multimeric | A structure composed of several identical or different subunits held together by weak bonds e.g. a protein with several peptide chains that aggregate to produce a complex tertiary structur |
| Necrotic | Dead tissue |
| Neuromuscular | Involving messages carried by nerves to muscles |
| Neuro-endocrine | Involving messages carried by nerves that result in the production of hormones or other chemicals |

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|---|---|
| Neutrophils | One of the types of phagocytic white blood cells of vertebrates, which forms part of the effector cell component of the immune system in vertebrates |
| Oedema | Accumulation of fluid (serum or hemolymph) in tissues |
| Opsonin | A molecule in the blood that coats foreign particles and mediates attachment and phagocytosis by immune system cells. |
| Organelle | A structure within a cell that carries out a particular function: e.g. a flagellum |
| Osmoconformer | The overall concentration of ions in the body fluids is not different from the seawater around the animal |
| Pathogenicity | Damaging effects to the organism |
| Peptide | A chain composed of a small number of amino acids and having less tertiary structure than proteins |
| Phagocytosis | Engulfing of an object by a cell so that it is enclosed in a vacuole |
| Phagosome | The vacuole in a cell enclosing an engulfed pathogen or other foreign material |
| Phenol | A chemical with an aromatic carbon ring structure and a hydroxyl group |
| Phospholipase | An enzyme that breaks up phospholipids |
| Phylum | A major category of animals. The Kingdom Animalia is divided into Phyla, such as the Mollusca |
| Plasma membrane | The membrane bounding the cell |
| Pore cells | Fixed cells that have a phagocytic function and sieve-like structures |
| Prophenoloxidase system | A protein cascade in the invertebrate immune system leading to the production of phenoloxidase (a protein that attaches nonspecifically to various surfaces), cytotoxins and melanin |
| Protein kinase | An enzyme that cuts proteins into sections |
| Protease | An enzyme that digests proteins |
| Pseudopodia | Lobe-like extensions of an amoeba-like cell |
| Pulmonates | A group of Gastropods (snails) that breathe air, and thus do not have gills |
| Quinone | A class of yellow aromatic compounds containing a double ketone and often having important biological activity as coenzymes, vitamins or cytotoxins |
| Renopericardium | The part of the space around the heart in some molluscs that acts as a kidney |
| Respiratory burst | Production of superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) and other oxygen radicals by stimulated inflammatory cells after they phagocytose pathogens, in order to kill them. |
| Selectins | A family of transmembrane molecules, expressed on the surface of leukocytes and activated endothelial cells. They are responsible for the initial attachment of leukocytes during inflammation, and cause a slow downstream movement of leukocytes along the endothelium via transient, reversible, adhesive interactions. |
| Senescent | Aging. Senescent cells gradually lose their capacity to function. |
| Sequestration | Process of removal and storage of material or cells to isolate them from tissues or the circulation |

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|---|---|
| Schistosome | Trematode worms of the genus *Schistosoma,* many of which are parasitic in the blood of humans and other mammals. Also called *bilharzia*, *blood fluke*. |
| Sporocysts | A larval stage of trematodes (flatworms) that is formed in snails and which multiplies asexually |
| Stem cell | Primitive cell that has the potential to form many different specialized cell types as it proliferates |
| Superorder | The taxonomic category below Class |
| Terminal invader/pathogen | Organism that invades when the animal is already dying of other causes, but which is not typically capable of causing disease alone |
| Titres | A quantity of chemical activity measured by titration against a standard chemical |
| Trematodes | A class of flatworms that are parasites, such as liver flukes and schistosomes |
| Trophozoite | One of the life stages of a protist, in which it feeds and grows |
| Vascular | Of the vessels (usually blood vessels) |
| Ventrocaudal surface | The underside, towards the tail |

## APPENDIX 4: A REVIEW OF STRESS AND IMMUNITY IN ABALONE

This review was carried out at the satrt of the project, to establish what was known prior to our work. We found first, that many measures of how the blood fluid and blood cells of animals respond to stress could be, and had been used in various animals. These blood tests (called *in vitro* because they are tests on the extracted blood fluid) are convenient ways to measure the physiology of an animal because blood samples are easily taken and stored. Many of these tests however, might not provide useful information about how well abalone could resist infection (immune function). Second, we found that there was confusion in the published literature about the relationships between abalone and other invertebrate animals; and thus which of them should be used to inform work on abalone stress and immunity. Thus we focused our review on these issues. The version of the review below was written for, and distributed to abalone farmers as one of the outputs and extension activities of the project. The references can be found in the reference list above, and as in all of this report, technical terms have been minimized, and are explained in Appendix 3.

A revised, more technical version of this review was published in the journal 'Fish and Shellfish Immunology': Hooper C, Day R, Slocombe R, Handlinger J. & Benkendorff K. (2007) Stress and immune responses in abalone: limitations in current knowledge and investigative methods based on other models. *Fish and Shellfish Immunol*ogy 22:363-379.


### ***Summary***

We do not fully understand how stress affects the abalone immune response and how *in vitro* studies on immune responses relate to suppressed immunity in production systems. Mild stressors can cause an "apparent" immune stimulation in *in vitro* studies but concurrently there is increased susceptibility to infectious disease, which is the only genuine measure of immune capacity. An important aim of future research should be identifying those *in vitro* tests of immune function which can be reliably measured and which are relevant in assessing on-farm immune functional capacity. This review details current knowledge on the immune response; including permeability of blood vessels, chemotaxis (the movement of cells towards a chemical stimulus), non-self recognition, phagocytosis, intracellular and extracellular killing mechanisms, the stress response and integration of the immune response with the stress response. Where work has been done in abalone, this is discussed. To fill the considerable gaps in the knowledge of stress responses and the immune system in abalone, references to other invertebrate taxa have been made, concentrating on those closest to abalone. Because invertebrate taxa have diverged over long evolutionary periods, the immune and stress response systems in other classes and phyla of invertebrates should not be assumed to be the same, as has happened in some previous studies.


### ***Introduction***

In abalone, the stress response and the immune response both appear to be based on the haemocyte. Haemocytes produce mediators of both the stress response and the main immune responses (Ottaviani & Franceschi 1996, Ottaviani et al. 1997, Ottaviani & Franceschi 1997). Some uncertainty exists in abalone as to the types of immunocytes that may be involved, as both a circulating haemocyte and a fixed tissue granulocyte of uncertain relationship are present. (Bevelander 1988). The types of immune parameters that could be used to monitor immune health have been discussed by Benkendorff (Benkendorff 2003), but further information is required to establish which parameters are likely to be reliable indicators of immunosuppression in abalone subject to stress.

Stress has a complex relationship with disease. It has been implicated in disease outbreaks in many animals including abalone. A link has been established in abalone between increased

stress and decreased immune functional capacity leading to increased rates of bacterial infections and increased mortality (Martello & Tjeerdema 2001, Malham et al. 2003, Cheng et al. 2004a, b, Cheng et al. 2004c, Cheng et al. 2004d, Cheng et al. 2004e). This link is based on immune function tests after applying stressors such as altered salinity, shaking, low dissolved oxygen, increased concentrations of ammonia and nitrate and increased temperature.

The term "stress" has diverse meanings in current usage. It has been used to describe both the stressor and the stress response (Barton 1997) . The term "stressor" refers to the cause; and "stress" or "stress response" are the terms used for the animal's response to the stressor. Different stressors may produce a different specific response, e.g. the specific response to bacterial invasion is increased phagocytic activity and the specific response to the presence of a predator includes the neuromuscular reactions involved in flight. In addition to the specific response caused by the stressor however, there is also the generalized stress response. The generalized stress response is a nonspecific pattern of neuroendocrine reactions to a situation that threatens homeostasis. Some define the stress response as a physiologic term meaning any internal or environmental variable that causes a rise in adrenocorticotropin (Cooper 1996) and others will apply the term to any cause inducing a demand on the body (Cooper 1996, Ottaviani & Franceschi 1996).

Notably however, there has been a tendency to fill the gaps in our knowledge about immunity or stress responses of one invertebrate species by using another invertebrate species that is very distant in the evolutionary scale. Recently, molecular evidence has revolutionised our understanding of the relationships between phyla, and between groups of molluscs. For this reason, the taxonomic relationships of abalone to other commonly studied invertebrates are outlined here (Figure 1). Abalone belong to the Phylum Mollusca, in the superorder Vetigastropoda of the large and diverse class Gastropoda. Vetigastropods retain many of the ancestral characters of the Gastropoda, which appear to have been benthic herbivores (Moore 2001). Recent extensive revisions of molluscan systematics are described in Beesley *et al.* (Beesley et al. 1998). More derived lines in the superorder Caenogastropoda may also be herbivorous, such as the periwinkle *Littorina*, or suspension feeders such as the freshwater *Viviparus*, or have become adapted to carnivory, such as the whelks and cones. In the superorder Heterobranchia, some have reduced or lost the shell ( 'Sea-slugs' such as the sea hare *Aplysia*) or adapted to freshwater and terrestrial habitats (the pulmonate snails). Immunity has been more intensively studied in some freshwater pulmonates (e.g. *Biomphalaria, Lymnaea*), because they are intermediate hosts for parasites of humans and domestic stock. Other well-known classes are the Cephalopods (e.g. squid and octopuses) and the Bivalvia (e.g. oysters and clams). All the major classes of molluscs appeared 500 million years ago, so there may be considerable evolutionary divergence between them.

Recent work suggests the phylum Mollusca is most closely related to the Annelids (segmented worms). The Arthropoda (Insects, spiders, lobsters etc.), associated with the Nematodes in the Ecdysozoa group, are very distantly related (Pechenik 2005). Vertebrate animals form a distinct lineage (the Deuterostomes) with the Urochordata (sea-squirts) and Echinoderms (sea urchins, starfish, etc). Thus the immune and stress response systems of molluscs and lobsters are more distantly related and perhaps more different than those of mammals and sea-urchins. Generalisations about 'immune systems in invertebrates' should be avoided.

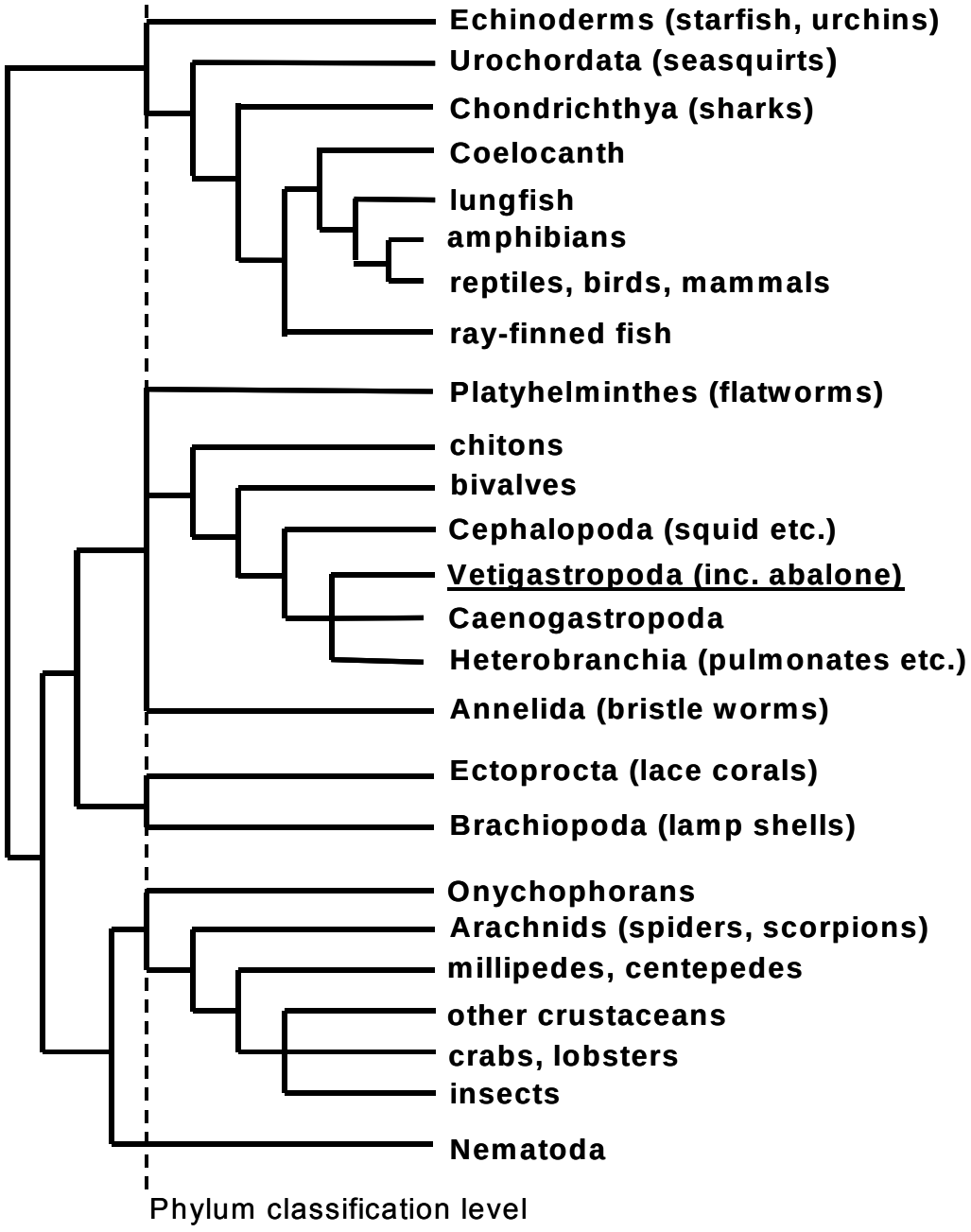


**Figure 1. Modern classification of common animal taxa, based on molecular and morphological evidence, emphasizing relations of molluscan taxa. Modified from Lecointre and Guyader (2001) and Beesley *et al.* 1998.**

**Infectious Disease in Abalone**

It is important to understand the immune system and the effects of stress on abalone because the development of abalone mariculture has meant that abalone are kept in high density and traded live. These are ideal conditions for disease spread, if appropriate measures are not taken. Such measures can be informed both by an understanding of the disease agents and by an understanding of the abalone immune system and how it is affected by stress. Management of farmed abalone uses systems that can become stressful to the stock and this can lead to an increase in endemic infectious disease problems or an outbreak of new infectious disease.

Little is known internationally of abalone diseases. Table 1 shows the most frequently reported diseases.

**Appendix Table 1: Frequently reported infectious diseases in abalone.**

| *DISEASES* | REFERENCE |
|---|---|
| *VIRAL* | |
| Amyotrophia | Nakatsugawa *et al.* 1999 |
| Abalone Viral Mortality | Shi and Handlinger 2005 |
| *BACTERIAL/FUNGAL* | |
| *Vibrio harveyi* | Reuter and McOrist 1999, Handlinger *et al.* 2005 |
| *Vibrio fluvialis* | Li *et al.* 1998 |
| *Vibrio carchariae* | Nicholas *et al.* 2002 |
| *Vibrio splendidus* | Handlinger *et al.* 2005 |
| Rickettsia (Withering Foot Syndrome) | Friedman *et al.* 2000, 2002 |
| Shell Mycosis | Friedman *et al.* 1997, Grindley 1998 |
| Tubercle Mycosis (*Atkinsiella awabi*) | Hatai 1982, Kitancharoen *et al.* 1994 |
| *PARASITIC DISEASES* | |
| *Perkinsus olseni* | Goggin and Lester 1995, Handlinger *et al.* 2003 |
| *Haplosporidia* | Diggles *et al.* 2002 |
| *Labyrinthuloides haliotidis* | Bower 2000 |
| Shell boring organisms (*Cliona* spp, *Polydora* spp, Boccardia knoxi) | Bower 2000, Lleonart 2001, McDiarmid *et al.* 2005 |
| Sabellid shell worm | Mouton 2003 |
| Metazoan tissue invaders (*Echinocephalus pseudouncinatus,* Trematode Metacercaria) | Milleman 1951, Harrison and Grant 1971 |

A national survey of abalone diseases is currently underway in Australia (Handlinger et al. 2003) and it appears that infection rates are quite low in this region. Nevertheless, the only internationally notifiable abalone disease (*Perkinsus*) has been recorded in Australia

(Goggin & Lester 1995, Handlinger et al. 2003), along with incidents of Vibriosis and endemic mudworm shell infestations (Handlinger et al 2003). The most concerning recent international outbreaks involve viral diseases that have decimated the abalone industry in China, but have not yet spread into Australia waters. Bacterial diseases are most commonly opportunistic infections especially by *Vibrio* species (Vandepeer 2003). Numerous parasite infections have been reported, but these are of questionable significance apart from *Perkinsus* (Goggin & Lester 1995, Handlinger et al. 2003) and mud worm infestations (Lleonart 2001).

**The Immune Response - Haemocytes**

Molluscan haemocytes are involved in many physiological and pathological functions including nutrient transport and digestion, shell repair, exogenous and endogenous material excretion and immune defense (Sahaphong et al. 2001). The haemocyte is the main defense cell of molluscs (Adema et al. 1991, Bachere 2000)). They are capable of recognition, chemotaxis, attachment followed by agglutination and phagocytosis, and exocytosis of antimicrobial factors. Depending on the size of the invading foreign organism or foreign body, it is either phagocytosed by a haemocyte or encapsulated by a number of haemocytes.

There are two main cell types in abalone haemolymph: the granulocyte and hyalinocyte or agranulocyte (Sahaphong et al. 2001). Sahaphong et al. reported 11.43% granulocytes and 88.57% hyalinocytes in their study on *Haliotis asinina*. The granulocyte cytoplasm has a peripheral zone filled with dense granules of various types. Most of the granules are polyhedral and elongated with a few being spherical or oval. The average size of the granules is 0.34 um. Some of the granules fuse or protrude from the plasma membrane. Sahaphong et al. (2001) speculated that this fusion probably reflects the process of releasing the internal products of the granule into the haemolymph. It is not clear however, how stimulated these haemocytes were: after extraction and pooling, the haemolymph was immediately fixed, which would suggest the cells were only in the initial stages of stimulation. The granulocyte has fewer organelles than the hyalinocyte.

Abalone hyalinocytes contain prominent aggregates of glycogen, which appear as clear zones in the cytoplasm under light microscopy. They have similar organelles to granulocytes but have more of them. While abalone show predominantly hyalinocytes in the circulation (Bevelander 1988, Sahaphong et al. 2001), abalone also have abundant fixed granulocytes in connective tissues, which may be related cells (Bevelander 1988).

Minimal investigation into the functional differences of abalone haemocytes has been carried out, but the relationship between hyalinocytes and granulocytes of other gastropod molluscs has been more extensively studied. It is possible that they are the same cell type at different developmental stages: the granulocyte being the fully developed cell. Morphologic and lysosomal functional differences between hyalinocytes and granulocytes in the pulmonate landsnail *Lymnaea stagnalis* were investigated and determined to differ quantitatively only (Sminia & Barendsen 1980, Van der Knaap et al. 1993). Subpopulations of *L. stagnalis* haemocytes were identified based on size and lysosomal enzyme contents but the subgroups did not separate out into completely distinct groups, leading the authors to surmise the presence of one heterogenous haemocyte, which probably varies due to age and differentiation.

Other invertebrates have been more studied than abalone, and it is tempting to try to directly surmise abalone haemocyte organelle function from what has been established in

other invertebrate species. As might be expected however, there is substantial variation among other invertebrate classes. More surprisingly, variation has been reported within species, for example, granulocytes of genotypically different strains of oysters from different sites may show different lectin binding patterns and react differently to pathogens (Cheng et al. 1995). Bivalve granulocytes phagocytose bacteria, yeasts and protozoa, and phagocytosis is usually but not always accompanied by generation of reactive oxygen species. The agranular haemocytes may be non-phagocytic or less phagocytic, although oyster hyalinocytes phagocytose some haplosporidian parasites (Hine & Wesney 1994). Some bivalves have haemocytes with the general appearance of granulocytes but lacking granules or having only a few granules. These may be senescent granulocytes. Degranulation of granulocytes will occur following phagocytosis, and this also leads to morphologic ambiguity (Hine 1999). In crustaceans the terminology for haemocytes appears similar but studies have shown a different function for the cell types. In crayfish, the hyaline cells are chiefly involved in phagocytosis, semigranular cells are active in encapsulation and granular cells are involved in storage and release of the prophenoloxidase system and cytotoxicity (Johansson et al. 2000).

Apart from circulating haemocytes, gastropods have a population of fixed phagocytes, the reticulum cells (Sminia 1980, Adema et al. 1991, Hine 1999). The reticulum cells studied in *L. stagnalis* have intimate connections with collagenous connective tissue fibrils and are actively phagocytic (Sminia 1980). In molluscs that have been parasitised, the fixed phagocytes are filled with pigment and have been called brown cells (Adema et al. 1991). In bivalves, the brown cells aggregate in lesions and around parasites; and they may be involved in parasite phagocytosis (Hine 1999). These brown cells appear to have limited ability to degrade phagocytosed material, but may remove such matter by diapedesis (Hine 1999). Senescent haemocytes, like other degenerate tissues, are probably removed by phagocytosis by other haemocytes.

Pore cells have been identified in molluscs (Sminia 1980, Haszprunar 1996). They have a great but selective phagocytic capacity. Their morphology differs from the free moving haemocytes of the haemolymph, especially in the presence of the cytoplasmic invaginations forming sieve-like structures involved in phagocytosis (Sminia 1980). They will selectively phagocytose only materials of the size that fits into the cytoplasmic sieve and they select based on the nature of the material. They will phagocytose foreign material, but the digestion rate is very low; possibly indicating that this is not their primary phagocytic function. Pore cells may be more involved in phagocytosis of haemolymph proteins (Sminia 1980, Haszprunar 1996). Whether pore cells and circulating haemocytes have a common stem cell or arise from very different cell types is not clear. Considering their phagocytic capacity, they possibly arise from a similar stem cell line.

**Haematopoiesis – the formation of haemocytes**

The site of haematopoiesis has not been established in abalone. Stem cells and mitotic figures (indicating cell division, thus new cell formation) have been reported from the amoebocyte producing organ in the renopericardial region of the pulmonate gastropods *Biomphalaria glabrata* and *Lymnaea truncatula* (Joky & Matricon-Gondran 1985, Adema et al. 1991). Blast like cells called amoeboblasts were identified in the amoebocyte producing organ of *B. glabrata*. These cells had a high nuclear to cytoplasmic ratio, a narrow rim of basophilic cytoplasm and a paucity of organelles, suggesting these cells are immature. These were identified as the cells from which haemocytes develop because of the hyperplasia (enlargement of the tissue) and increased mitotic rate seen in this site

alone after infection by trematodes (Joky & Matricon-Gondran 1985). In *L. truncatula* and *L. stagnalis*, haemocytes divide in the haemolymph, in connective tissue and at the site of wound repair (Sminia 1974, Van der Knaap et al. 1993). *L. stagnalis* continue to grow for their entire lives and their haemocyte numbers also increase continuously by replication (Van der Knaap et al. 1993).

**Haemacytopenia – loss of haemocytes**

Stressed abalone can show a transient drop in haemocyte counts (haemacytopenia) with mild stressors (Malham et al. 2003, Cheng et al. 2004a, Cheng et al. 2004e). The cause of the decreased count is not clear, as none of the possible mechanisms have been investigated in abalone. One possible explanation is that the haemocytes migrate from the haemolymph towards areas prone to injury or pathogen attack (Malham et al. 2003), but this would not explain the transient haemacytopenia seen after a mild stressor. Transient haemacytopenia would be seen if the cells become more closely associated with the endothelial cells of the vessel wall, thus avoiding being sucked out during haemolymph extraction; this would probably be mediated through intercellular adhesion molecules, an area which is poorly understood in molluscs. Haemocytes may undergo apoptosis or lyse if the stressor is severe or prolonged. Haemacytopenia will also occur if the haemocytes leave the abalone via diapedesis across epithelial tissues, as seen in oysters and clams. Yeast cells, bacterial spores and carbon particles injected into *Crassostrea virginica* were taken up by haemocytes and removed from the tissues as the cells migrated through epithelial tissues to the exterior (Tripp 1960). Carbon particles injected into the clam *Tridacna maxima* were phagocytosed by circulating haemocytes, transported across the epithelial surface of the digestive organs into the lumen and then eliminated from the organism (Reade & Reade 1972).

**Haemocyte Involvement in the Immune Response**

Few studies have been done on abalone to characterize the action of the haemocyte on invading pathogens. Those studies done on abalone immune responses to infection have concentrated on *in vitro* assays for haemocyte functions such as phagocytosis, migration assays and respiratory burst (Malham et al. 2003, Cheng et al. 2004a, b, Cheng et al. 2004c, Cheng et al. 2004d, Cheng et al. 2004e). Numerous studies have been done on other gastropods however, such as the pulmonate gastropod *Biomphalaria glabrata,* the intermediate host of the parasite causing schistosomiasis in humans, *Schistosoma mansoni*.

Studies on the immune interactions of *B. glabrata* and the parasite have indicated that haemocytes are the dominant effector cells in killing *Schistosoma* sporocysts (Bayne et al. 2001). *B. glabrata* have two types of haemocytes, hyalinocytes and granulocytes. Electron microscopy indicates the granulocytes are the effector cells. When sporocysts and haemolymph interact *in vivo* or *in vitro*, the granulocytes are the cells in contact with the parasites. The parasites are killed by *B. glabrata* haemocytes in the absence of haemolymph plasma, indicating that haemocytes can kill in the absence of humoral factors (Bayne et al. 2001). There is however, a significant decrease in phagocytosis of haemocytes in medium containing no plasma proteins as compared to the rates of phagocytosis in plasma (Yakovleva et al. 2001), indicating that phagocytosis in gastropods is opsonin dependent to some extent. Injection of cell free plasma from resistant strains decreased infection frequencies in normally susceptible *B. glabrata*, which implies the presence of humoral immunity (Bayne et al. 2001). Killing requires only sufficient cells to adhere to less than half the sporocysts surface. This indicates that killing is an active process, because complete envelopment of the parasite (encapsulation) and suffocation or starvation are not needed.

**Vascular Permeability**

The aorta and larger blood vessels are lined by endothelium. Smaller blood vessels and the sinuses are not lined by endothelium (Bevelander 1988). Where the connective tissue is loose, it can be difficult to distinguish the circulatory system from tissue, hence the circulation system is referred to as open. Haemocytes are able to gain access to the extravascular connective tissue and return to the circulation without the need for the adhesion molecules required to cross interendothelial junctions. Lectin-like receptors have been identified on the endothelial cells with which they trap microorganisms prior to phagocytosis by the haemocytes (Van der Knaap et al. 1981, Van der Knaap et al. 1993), but whether these lectin receptors participate in endothelial-haemocyte binding prior to emigration from endothelial lined vascular beds is not known.

Oedema associated with acute inflammation is due to both changes in hydrostatic pressure (congestion in the affected site) and alterations in vascular permeability due to biochemical mediators of inflammation. Histamine, which plays such a prominent role in vascular permeability in vertebrates, is not known to have a role in molluscs. In molluscs, it is present in the neurons of the central nervous system (Karhunen & Panula 1991) where it is considered to have an important role in efferent, sensory and integrative functions (Hegedus et al. 2004), but has not been reported in the cytoplasmic granules of haemocytes. Cells with mast-cell like features containing both histamine and heparin have been reported in the subepithelial connective tissue and several mucosal organs of the mollusc *Anomalocardia brasiliana* (Santos et al. 2002), but the effect of histamine in these areas is not clear.

**Adhesion Molecules**

Phagocytes crawl through tissue; they do not swim. This requires reversible adhesiveness to endothelial cells and extracellular matrix (ECM). Abalone have endothelium only lining their larger vessels and haemocytes could pass into the connective tissue through smaller vessels with no endothelial lining. Adhesion molecules are needed for intercellular communication between haemacytes and endothelial cells if they are to navigate the intercellular junctions between endothelial cells, and between haemocytes and ECM for chemotaxis in connective tissue. In vertebrate species, numerous adhesion molecules have been described for leukocytes and endothelial cells. In pulmonate gastropods, adhesins including integrins and selectins have been identified but not characterized (Duclermortier et al. 1991, Davids & Yoshino 1998). Davids and Yoshino (1998) reported differences in the integrin expression between strains of *B. glabrata* that were resistant or sensitive to parasitism by *S. mansoni*. In the resistant strains of *B. glabrata*, cell spreading and adhesion were less affected by integrin inhibitor molecules. Adhesins are better characterized in crustaceans, eg. peroxinectin, which enhances phagocytosis and degranulation (Holmblad & Soderhall 1999).

**Chemotaxis**

Chemotaxis involves directional migration in response to a chemoattractant gradient. Orientation of the haemocyte towards the chemotaxin occurs prior to movement. Orientation is due to the microtubules and movement is due to the actin-myosin filaments (Slauson & Cooper 2002). The vertebrate chemotaxis model is well-characterized and may provide a useful model for studies in abalone. After the chemoattractant binds the cell surface receptor, there is a release of $Ca^{++}$ from intracytoplasmic stores, causing an increase in cytosolic $Ca^{++}$. This triggers depolarisation of the membrane, followed by repolarisation. This is linked with the actin-myosin network that is responsible for movement. The $Ca^{++}$ is then sequestered from the cytosol prior to the next wave of

activation and movement. Different integrins bind different ECM proteins. The cell attaches, the actin-myosin filaments contract and the cell is pulled forward. The adhesion molecules are then down-regulated and the ECM is released. The adhesion molecule receptors can be recycled and reused (Slauson & Cooper 2002). Actin microfilaments and the ECM protein fibronectin have been identified and investigated in mussels, with the haemocytes being identified as the producers of fibronectin (Panara et al. 1996).

Evidence for the existence in abalone of signal transduction pathways, similar to those well identified in vertebrates, comes from work on the induction of heat shock protein 70 (hsp 70) in *Haliotis tuberculata* after incubation with noradrenaline. The production of hsp 70 was prevented by a phospholipase C inhibitor, a protein kinase C inhibitor, a $Ca^{++}$ - dependent protein kinase inhibitor and a phosphatidylinositol 3-kinase inhibitor (Lacoste et al. 2001). This indicates the potential usefulness of the vertebrate model.

Chemotaxins are chemical mediators of chemotaxis. Chemotaxins that have been identified in molluscs include Interleukin-8 (IL-8), Lipopolysaccharide (LPS) and Formyl-methionyl-leucyl-phenylalanine (fMLP) (Scneeweiss & Renwrantz 1993, Fawcett & M.R.Tripp 1994, Ottaviani et al. 2000). IL-8 is a chemotaxin of the chemokine family identified in mammalian neutrophils and macrophages (Slauson & Cooper 2002). IL-8 has been identified in the haemocytes of the mussel *Mytilus galloprovincialis* and found to affect conformational change in haemocytes via reorganization of the actin microfilaments. It has been found to induce chemotaxis and increase bacterial phagocytic activity (Ottaviani et al. 2000). IL-8 induces these changes via the protein kinase A and C pathways. fMLP is a chemoattractant peptide produced by bacteria that has been well-characterized in mammalian neutrophil chemotaxis.

**Recognition of non-self and opsonisation**

Discrimination of self from non-self is achieved by molecules in the haemocyte membrane recognizing conserved motifs in pathogens called pathogen associated molecular patterns (PAMPS) or pattern recognition proteins (PRPs) (Roitt et al. 2001). Molecules recognised in pathogens include lipopolysaccarides (LPS) in gram negative bacteria, peptidoglycans of gram positive bacterial cell walls, B-1, 3-glucans of fungal cell walls and the double stranded RNA of viruses.

Attachment of foreign particles to the haemocyte membrane may occur directly or be mediated via opsonins or agglutinins (Roitt et al. 2001). When an opsonin is involved, the antigen-opsonin complex is bound to a haemocyte membrane receptor specific for the opsonin. A large subgroup of opsonins are lectins. Lectins are multimeric glycoprotein complexes that carry several sites for binding targets and are able to recognise and bind to "non-self". Lectins contain identical subunits of specific monosaccharides. After the lectin binds the target cell or particle, a conformational change occurs, which makes available binding sites on the lectin. The haemocyte membrane receptors then can interact with the revealed binding site on the lectin.

The haemolymph from an abalone species, *Haliotis asinina*, is reported to agglutinate chicken and mouse erythrocytes but not guinea pig, rat or rabbit erythrocytes (Yakovleva et al. 2001). The agglutinins were not further characterized. Specific agglutinin receptors have been identified on haemocytes of the pulmonate *Helix pomatia*. The carbohydrate portions of these receptors include galactose, fucose, mannose or glucose or both and N-acetylneuraminic acid (Renwrantz & Cheng 1977). The humoral factors involved in opsonising and agglutinating foreign material in the pulmonate *Lymnaea stagnalis* are produced within the haemocyte and incorporated into the plasma membrane (Sminia

1980). An agglutinin was essential to the chemotactic response of the caenogastropod *Viviparus malleatus* haemocytes to killed *Staphylococcus aureus* cells (Schmid 1975). A member of the Ig superfamily identified in *Lymnaea stagnalis* and called molluscan defense molecule (MDM) is produced by granulocytes and is possibly an opsonin involved as a mediator in non-self recognition (Hoek et al. 1996).

The fresh water pulmonate snail *Biomphalaria glabrata* contains lectins that bind to yeasts, thus targeting them for phagocytosis (Fryer et al. 1989). Eight sugars thought to occur on the surface of *S. mansoni* sporocysts were tested for their ability to stimulate the respiratory burst in *B. glabrata* haemocytes (Hahn et al. 2000, Bayne et al. 2001). *B. glabrata* haemocytes recognised galactose, mannose and fucose; and responded by producing reactive oxygen species. These results were similar in haemocytes from resistant and susceptible strains of *B. glabrata* and suggest that the cells do not differ with respect to their lectin-type surface receptors. These results imply that haemocytes from both strains are equally capable of producing reactive oxygen species; that receptor activation requires a patterned presentation of multiple ligands; and that the difference between susceptible and resistant strains of *B. glabrata* is due to something other than different recognition capabilities. Fibrinogen related peptides (FREPs) in *B. glabrata* are another family of haemolymph proteins that have lectin like properties, allowing them to precipitate soluble antigens derived from trematode parasites. FREPs contain immunoglobulin domains and are produced in increased abundance after infection with *S. mansoni*. The immunoglobulin domain of the FREP3 subfamily is very diverse, implying diversification of non-self recognition molecules functioning in immune defense (Zhang et al. 2004).

Plasma of some bivalve species contain at least two lectins (Stein 1979, Yakovleva et al. 2001) composed of subunits differing in mass or isoelectric point. Differing subunits are capable of binding several monosaccarides with different affinity, resulting in a wider spectrum of targets. Bivalve lectins composed of identical subunits have been found to react with different monosaccharides and other bivalve lectins have been identified with narrow specificity. Unlike bivalve lectins, gastropod lectins often consist of subunits of the same type and are strongly specific to a certain monosaccharide. Achatinin, a lectin from the giant african pulmonate snail, *Achatina fulica,* binds specifically to 9-O-acetylsialic acid (Yakovleva et al. 2001). Bivalve species have higher agglutination titres than gastropod species, with some gastropods such as *Littorina littorea*, a marine Caenogastropod, having no detectable agglutination ability. The more potent agglutination capacity of Bivalvia is possibly due to a higher concentration of plasma agglutinins and polyreactivity (Yakovleva et al. 2001). Since the different classes of invertebrates efficiently remove foreign matter, plasma agglutinating activity must not be the only measure of the efficiency of an immune system.

Complement receptor 3-like activity was detected in the central and peripheral nervous system of *Lymnaea stagnalis* but there are no reports of its activity as an opsonin (Hatakeyama et al. 2000). Sea urchins however, contain a complement cascade comparable to the vertebrate alternative complement pathway. Two components have been identified with significant homology to vertebrate complement factors, C3 and factor B, called SpC3, which is inducible and SpBf, which is constitutive. SpC3 functions as an opsonin in sea urchins, causing augmented phagocytosis. Sea urchins are members of the phylum Echinodermata of the deuterostome lineage, and not close to the molluscs. This is an interesting case of an invertebrate species, the sea urchin, having immunologic abilities related more to vertebrate species than other invertebrates, and illustrates the need to pay attention to taxonomic affinities.

**Phagocytosis**

Phagocytosis in gastropods proceeds through well defined stages including recognition, chemotaxis, attachment, ingestion and destruction of foreign particles (Bayne 1990, Adema et al. 1991, Van der Knaap et al. 1993, Adema et al. 1994, Hahn et al. 2000, Bayne et al. 2001, Yakovleva et al. 2001). After internalisation, the foreign particles are enclosed in a primary phagosome, which then fuses with lysosomes to form a phagolysosome. The activation of lysosomes is $Ca^{++}$/phospholipase A dependent (Burlando et al. 2002).

The purpose of enclosing the foreign agent within the phagolysosome is to eliminate it. Two main systems will do this: the oxygen dependent systems and the oxygen independent systems. The oxygen independent systems include the array of cytotoxic lysosomal enzymes. The oxygen dependent systems include the production of reactive oxygen species (ROS) and the hydrogen peroxide-myeloperoxidase-halide system (Slauson & Cooper 2002).

The products of particle digestion (e.g. glycogen), can contribute to the nutrient supply of the animal. Undigestible particles, after phagocytosis, can be removed to the external environment by diapedesis across epithelial surfaces in oysters and clams, but this has not been demonstrated in abalone (Tripp 1960, Reade & Reade 1972). Alternately, sequestration may occur where the phagocyte becomes metabolically quiescent (Bayne 1990). Objects too big to be phagocytosed are encapsulated by multiple layers of haemocytes, then phagocytosed bit by bit (Van der Knaap & Loker 1990). An ultrastructural study on *Perkinsus* infection of the clam showed encapsulation leading to nodule formation. Encapsulation of the *Perkinsus* trophozoite was followed by haemocyte lysis and degeneration with some of the released granules coalescing to form megagranules. Trophozoites were within the interior of these nodules (Sagrista et al. 1995). This appears to be a granuloma-type response to an agent that cannot be properly eliminated.

***In vitro* assays of phagocytosis**

Phagocytic activity can be recorded by measuring the proportion of ingested particles or the proportion of cells that have ingested labeled particles (Chang et al. 2000, Malham et al. 2003). Research methods vary in whether adhered cells or suspended cells are examined. Haemocytes are easily manipulated when adhered to a glass or plastic surface. As the cell adheres, it spreads and its morphology changes from roughly spherical with short or unapparent pseudopodia to a flattened cell with long pseudopodia extended over the surface. Integral membrane proteins are likely to redistribute during the spreading process, as this is the scenario in chemotaxis and adherence in mammalian phagocytes (Bayne 1990, Slauson & Cooper 2002). This redistribution would also include receptors for foreign agents, decreasing their availability on the cell surface away from the solid surface and hence interfering with studies on immune reactions (Bayne 1990). Other assays have been developed to examine haemocytes in suspension, but there are different problems with these. Haemocytes removed with haemolymph from the animal will start to clump in seconds. Various anticoagulants have been trialed to stop this, including EDTA and modified Alsever's solution. These anticoagulants interfere biochemically with the haemocyte functioning to stop clumping. They chelate calcium ions and calcium ions are involved in triggering cell adhesion processes in mammals (Slauson & Cooper 2002). Hence, the anticoagulants will interfere with experiments examining the different components of haemocyte phagocytosis and production of reactive oxygen species, especially quantitative studies of calcium-dependent processes.

This has been shown in the bivalves *Mytilus galloprovincialis* (Torreilles et al. 1999) and *Crassostrea gigas* (Lambert et al. 2003). Use of a citrate-EDTA anticoagulant resulted in inactivation of approximately 50% of the prophenoloxidase activity in *in vitro* studies in the cockroach *Blaberus craniifer* (Leonard et al. 1985).

**Respiratory Burst**

During phagocytosis, there is an increase in oxygen consumption and an increase in production of superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$); the so-called respiratory burst that occurs within the phagolysosome. Neither superoxide nor hydrogen peroxide are hugely potent microbicidal molecules themselves, but will be involved in further reactions to form more potent reactive oxygen species (ROS). Superoxide anion contributes to formation of the very toxic hydroxyl radical ($^{\cdot}OH$) which produces oxidant damage and peroxidation of membrane lipids (Slauson & Cooper 2002).

The pathway of production of reactive oxygen species commences with activation of the NADPH oxidase complex in the phagocyte lysosomal plasma membrane (Slauson & Cooper 2002). This complex catalyses the production of superoxide. Superoxide can spontaneously change into hydrogen peroxide, a reaction that is enhanced by the enzyme superoxide dismutase. The hydrogen peroxide can combine with the enzyme myeloperoxidase and chloride ion to form hypochlorous acid (HOCl), which is a powerful oxidant. Hydrogen peroxide can also react with iron in the Fenton reaction to produce hydroxyl radical, another potent oxidant. The enzyme nitric oxide synthase combines oxygen and L-arginine to produce nitric oxide (NO). Nitric oxide has cytotoxic activity but can also combine with superoxide to form the more potent oxidant peroxynitrite ($ONOO^-$). Each oxidant varies in its reactive properties (DNA strand breaks, lipid peroxidation, enzyme inactivation) and the relative cytotoxic roles of specific oxidants are dependent on the bacteria or fungi involved (Bayne et al. 2001, Slauson & Cooper 2002). $H_2O_2$ and NO are the ROS mainly responsible for killing the sporocysts of *S. mansoni* by resistant species of *B. glabrata*. Scavengers for $O_2^-$, HOCl, $^{\cdot}OH$ and $^1O_2$ did not protect sporocysts from being killed (Bayne et al. 2001), suggesting to the authors that these ROS did not play a significant role in killing the phagocytosed and encapsulated parasites. Mussel haemocytes have been shown to produce peroxynitrite (Torreilles & Guerin 1999).

These oxidants react in processes associated with photon-generating oxidation-reduction reactions that can be measured using *in vitro* assays. The light emitted can be detected by chemiluminescence (Trush et al. 1978). This method has been used in abalone to test for reactive oxygen species. Malham et al. (2003), used chemiluminescence to test for respiratory burst as well as directly measuring superoxide anion via the reduction of nitroblue tetrazolium. Cheng et al (2004 a,b,c,d,e) tested directly for superoxide production. Black abalone (*H. cracherodii*) produced more chemiluminescence than red abalone (*H. rufescens*) after applying a salinity stressor combined with the environmental pollutant pentachlorophenol (Martello & Tjeerdema 2001).

Shuhong identified superoxide dismutase in *H. diversicolor* (Shuhong et al. 2004). Clams and mussels have been found to contain antioxidants (superoxide dismutase, catalase and glutathione peroxidase) that will protect against oxidative stress (Wenning & DiGiulio 1988). Varying concentrations of scavengers responsible for neutralising or packaging reactive oxygen species may exist between species of abalone and account for the varying rate of ROS production seen between different abalone species exposed to similar stressors (Martello & Tjeerdema 2001). When the stressor is a toxin, it is not known whether the increase in ROS is due to augmented production or suppression of

inhibitory antioxidant enzymes. Inhibiting antioxidant enzymes may reflect some level of cellular damage or by prolonguing exposure to ROS may aid in the immune response.

**Non-oxygen dependent killing mechanisms: Antimicrobial factors**

The granules of abalone haemocytes have not been thoroughly investigated as to their content, but they are probably lysosomes. Lysosomes contain a variety of cytotoxic and microbicidal enzymes, but there is evidence that their contents vary between molluscan species. Martello and Tjeerdema (2001) specifically checked for lysozyme in two species of abalone (*H. cracherodii* and *H. rufescens*) but found no evidence for its existence. Shuhong identified lysozyme in the haemolymph of *H. diversicolor* but reported it was present only at low levels (Shuhong et al. 2004) and there was no significant change in haemolymph levels of lysozyme between groups of *H. diversicolor* infected with *Vibrio parahaemolyticus* versus the control groups. One possible explanation for this is that lysozyme is not as important in abalone defense responses as other lysosomal enzymes. Shuhong et al (2004) also identified acid phosphatase and alkaline phosphatase in abalone haemolymph and the levels did significantly rise in groups infected with *V. parahaemolyticus*. Lysosomal enzymes have been studied in other molluscan species. Lysozyme, acid phosphatase, arylsulphate, peroxidase, non-specific esterase, elastase, cathepsin B and G and B-glucuronidase have been identified in haemocytes of various species of molluscs (Cheng et al. 1975, Yoshino & Cheng 1976, Moore & Lowe 1977, Sminia & Barendsen 1980, Pipe 1990, Van der Knaap et al. 1993, Lobo-da-Cunha 2002).

Studies on abalone haemolymph have demonstrated the presence of both antibacterial (Li 1960, Grant 2001) and antiviral activities (Li et al. 1962). Li (1960) demonstrated that the antibacterial factor is most likely a protein, but no further characterisation has been undertaken. Cushing et al. (1971) demonstrated inducible antibacterial activity in the haemolymph of three species of abalone. The bactericidal activity was induced by preimmunizing the animals with killed bacteria. A rapid rise in haemolymph bactericidal activity occurred, with the peak reached within one to two days, before subsiding after five to eight days. These bactericidal factors have not been identified.

Antibacterial and antifungal peptides isolated from *Mytilus edulis* have been partially characterised. The molecules are classified into four groups: mytilins, defensins, myticins and mytimycin; and are produced in haemacytes and stored in haemocyte granules (Charlet et al. 1996, Mitta et al. 2000). They have a role in killing within phagolysosomes and are also released into haemolymph. The defensins in mussels have similarities with the defensin family of molecules in arthropods. Defensins have broad spectrum antimicrobial properties, including toxicity to gram positive and gram negative bacteria, fungi and some enveloped viruses, and are widespread among insects and scorpions. The mytilin and myticin group have potent bactericidal activity, but different isoforms have different bactericidal capacity. Mytimycin has antifungal properties.

**Prophenoloxidase system**

The Prophenoloxidase (proPO) system is directly involved in non-self recognition and defense. It is well characterised in arthropods (Johansson & Soderhall 1989, Soderhall et al. 1996), but has also been investigated in other invertebrate groups, including the bivalves *Venus antiqua* (Mercado et al. 2002), *Mytilus edulis* (Coles & Pipe 1994) and *Perna viridis* (Asokan et al. 1997). It has not been characterized in abalone but several workers have included phenoloxidase measurement in their investigations into the effects of environmental and pathogen stressors on the abalone immune response (Cheng et al. 2004 a,b,c,d,e). Evidence for the importance of phenoloxidase in molluscs stems from a study by Peters and Raftos (Peters & Raftos 2003), who found that phenoloxidase was

suppressed in QX disease outbreaks among Sydney rock oysters (*Saccostrea glomerata*). Rock oysters selected for resistance to QX disease had significantly higher levels of phenoloxidase activity than unselected wild type oysters and the resistant oysters were shown to possess a novel isoform of the enzyme phenoloxidase (Newton et al. 2004). This implies a genetic basis for the enhanced level of phenoloxidase activity in oysters. Studies on the Pacific oyster (*Crassostrea gigas*) have also shown significant differences in the levels of phenoloxidase activity between family lines (Manning 2004). Thus selective breeding in molluscan aquaculture could provide improved resistance to disease via enhanced immune functions, such as phenoloxidase activity.

In both insects and crustaceans, phenoloxidase is a "sticky" protein that attaches nonspecifically to various surfaces (eg. fungal hyphae) (Johansson & Soderhall 1989). It produces intermediate cytotoxic *o*-quinones and indole derivatives, ultimately leading to the polymer melanin, a pigment integral to the wound healing process. Phenoloxidase (PO) is present in arthropod haemolymph as an inactive proenzyme, prophenoloxidase (proPo). Prophenoloxidase is activated in a stepwise process, generating a protein cascade, with the oxidation of L-DOPA to melanin as the final common pathway. Prophenoloxidase is activated by a serine protease (ppA), which is itself activated by β-1,3-glucans and LPS in crustaceans. Crustacean ppA can be activated *in vitro* in a haemocyte lysate, suggesting that their haemocytes contain a β-1,3-glycan binding protein. In crustaceans, proPo and ppA are stored in secretory granules in semigranular and granular haemocytes from where it is released by exocytosis (Johansson & Soderhall 1989). In the bivalve *Perna viridis*, *in vitro* activation by exogenous proteases has been described, including trypsin, α-chymotrypsin and the detergent sodium dodecyl sulphate .

A glycoprotein named 76 kDa cell-adhesion factor is also found in crustacean haemocytes and is involved in the proPo system (Johansson & Soderhall 1989). It is activated simultaneously with the proPo system. This protein mediates haemocyte attachment and spreading and promotes encapsulation. It may stimulate phagocytosis, whereas phenoloxidase does not stimulate phagocytosis or encapsulation in crustaceans. When in solution, the 76 kDa cell adhesion factor triggers degranulation and aggregation of crustacean haemocytes. It also causes rapid decrease in RNA and protein synthesis in the haemocytes, which likely leads eventually to death of the host cell. The 76kDa protein is stored in granules in the semigranular and granular cells and exocytosis is stimulated by B-1,3-glucans or LPS. The 76kDa protein and proPo systems are activated spontaneously in the presence of low calcium ion concentration. Johanssen (1989) proposes the following model for reacting to foreign matter in crustaceans. The semigranular cells degranulate, releasing proPo and 76kDa protein into the plasma. The activated proteins then attach to foreign substances and fungistatic substances are produced. The 76kDa protein mediates the attachment and spreading of granular and semigranular haemocytes onto the foreign substances. To control this system, there are two protease inhibitors in crayfish plasma which inhibit the proPo system (Johansson & Soderhall 1989).

Colorimetric enzyme assays can be used for the determination of phenoloxidase activity *in vitro*. In previous research on bivalve molluscs, L-3,4-dihydroxyphenylalanine (L-DOPA) has been used as the primary substrate for the catalytic reaction (Coles & Pipe 1994, Asokan et al. 1997). However, phenoloxidase catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). Consequently, Espin et al. (1997) has developed a method for the determination of monophenolase activity. Monophenolase activity should distinguish phenoloxidase from other phenol-oxidizing enzymes, such as laccase (oxidizes only diphenols) and peroxidase (oxidizes catechols to quinones)

(Maramas et al. 1996). A recent study on Pacific oysters has raised concern however, over the possibility that both assays are measuring the auto-oxidation of phenoloxidase-like substances, stock substrate solutions and various other phenol or quinone products that are present within the haemolymph (Manning 2004). This highlights the importance of including positive and negative controls, as well as the need for further optimization of the assays.

**Links between the Neuroendocrine and Immune Systems**

Vertebrate species rely on three systems for cell to cell communication: nervous, endocrine and immune systems, with these systems being considerably integrated (Ottaviani & Franceschi 1997). A relationship does exist between these three systems in abalone but it is still poorly characterised. The stress response originates in the endocrine system: corticotropin releasing hormone (CRH) stimulating the release of adrenocorticotrophic hormone (ACTH) leading to the release of biogenic amines, which then mediate secondary effects. These are characterised by metabolic changes in other organs as the animal responds to the stressor and attempts to maintain homeostasis. There is considerable overlap between the extracellular signals utilised by the immune and nervous systems in mollusks, and the molecules involved in both systems are present in the haemocytes, as opposed to being spread through multiple organs as seen in vertebrate species (Ottaviani & Franceschi 1997).

The molecules of the molluscan stress response are comparable to the mammalian stress response. Corticotropin-releasing hormone (CRH), adrenocorticotropin hormone (ACTH), cytokines, biogenic amines (noradrenaline, adrenaline and dopamine) and cortisol-like molecules have been found in the haemocytes of various molluscan species (Ottaviani & Franceschi 1997). The nervous system, haemocytes and cell-free hemolymph contain the neuroactive factors dopamine and noradrenaline. Haemocytes of the Caenogastropod *Viviparus ater* contain neuroactive peptides, including substance P, somatostatin and neurotensin (Ottaviani & Franceschi 1996).

The CRH - ACTH - biogenic amine cascade controls the stress response in vertebrates and those invertebrates which have been studied (Ottaviani et al. 1997). The process has not been studied in abalone, but has been studied in other gastropods. The hemolymph of *P. corneus* and *V. ater*, when incubated with CRH and ACTH showed a rapid and marked release of biogenic amines, especially noradrenaline and dopamine (Ottaviani & Franceschi 1996). When CRH was added to the hemolymph it provoked a release of ACTH from the haemocytes. CRH and anti-ACTH antibodies did not induce an elevation in biogenic amines, indicating that the ACTH, not the CRH, was responsible for the release of the biogenic amines; and confirming the CRH-ACTH-biogenic amine cascade (Ottaviani & Franceschi 1996). Platelet derived growth factor (PDGF) inhibited the release of adrenaline and noradrenaline, whereas transforming growth factor (TGF) stimulated their release. Neither growth factor had a significant effect on dopamine release. The growth factors influenced each other's effects. When the hemolymph was preincubated with PDGF, the depressant effect was not overcome by TGF. Preincubation with TGF caused elevation in noradrenaline that was not countered by the subsequent addition of PDGF, though the adrenaline levels were depressed. CRH and ACTH both caused increased release of noradrenaline and adrenaline after addition to hemolymph. Both CRH and ACTH were associated with decreased adrenaline and noradrenaline levels if added after the growth factors (Ottaviani et al. 1997). These results show an integration of the molecules involved in the stress response.

Haemolymph levels of noradrenaline and dopamine increase in abalone when they are stressed (Malham et al. 2003), but the other neuroactive peptides have not yet been characterised in abalone. Noradrenaline will induce the expression of heat shock protein 70 in *Haliotis tuberculata* (Lacoste et al. 2001). Heat shock proteins such as hsp 70 have several functions in mammals, including as chaperones and in stimulating macrophages via toll-like receptors to produce inflammatory cytokines (Akira 2003).

Cytokines are soluble mediators involved in the effector phase of immune responses. ACTH, β-endorphin, the interleukins IL-1α, IL-1β, IL-2, IL-6 and tumor necrosis factor (TNF)α have been demonstrated in haemocytes in the pulmonate gastropod *Planorbarius corneus* and in the Caenogastropod *Viviparus ater* (Ottaviani & Franceschi 1997). Il-1, IL-6, INF-γ and TNF have been identified in the pulmonate gastropod *Lymnaea stagnalis* (Van der Knaap et al. 1993). In molluscs, IL-2 will increase the hemolymph levels of biogenic amines, reflecting the link between the immune and neuroendocrine systems. IL-2 elicited the release of adrenaline and noradrenaline, but this effect was inhibited in the presence of CRH. Similar inhibition was noted with IL-1a, IL-1b, TNFα and TNFβ (Ottaviani & Franceschi 1996). The purpose of this competition is not clear, but shows a relationship between immune and endocrine functions.

Peptide fragments of ACTH 1-24, β-endorphin 1-31 and some cytokines can influence the migration, chemotaxis and phagocytosis in vitro of different invertebrate hemocytes (LeRoith et al. 1982, Stefano et al. 1989, Ottaviani & Franceschi 1997, Salzet 2000) Ottaviani et al (1995) demonstrated that IL-1α and TNFα increased the motility of *P. corneus*, but not *V. ater* haemocytes. IL-1α, IL-2 and TNFα increased the phagocytic activity and provoked the induction of nitric oxide synthase (NOS) in the haemacytes of both *P. corneus* and *V. ater*. The similar responses of increased phagocytosis and NOS induction but different responses to motility suggests that some cytokine effects are species specific (Ottaviani et al. 1995). The ACTH fragments ACTH 1-24, ACTH 1-4, ACTH 4-9, ACTH 1-13, ACTH 1-17 and ACTH 11-24 increase the migratory activity of the haemocytes of *P. corneus* and *V. ater* with some differences between the species. The whole sequence ACTH 1-39 and the fragment ACTH 4-11 were inhibitory for haemocyte migration (Genedani et al. 1994). ACTH can induce modifications of the cytoskeleton of the molluscan haemocyte, such as rearrangement of microfilament bundles under the plasma membrane, concentration of actin in the protruded lamellipods and an increase in microtubules. ACTH also alters cell-adhesive properties and localisation patterns of extracellular fibronectin, which is likely to be involved in the activation and mobilisation of haemocytes during the immune response (Ottaviani & Franceschi 1997).

Studies on *Aplysia californica* (a gastropod in the superorder Heterobranchia) have also shown a relation between the nervous system and haemocytes (Clatworthy 1996). One group of mechanosensory neurons on the ventrocaudal surface of the pleural ganglion play an important role in triggering defensive withdrawal reflexes following noxious stimulation. These neurons show long term changes in their electrophysiologic properties following axonal injury or a foreign body inflammatory response in close proximity to the axons. Both these types of lesions resulted in the accumulation of numerous haemocytes close to the axons allowing for potential neural-immune interactions to occur. In vertebrates, interleukin-1 (IL-1) and tumor necrosis factor (TNF) can influence neuronal signaling properties, neuronal survival during development and regeneration of injured neurons (Plata-Salaman 1991). IL-1 and TNF have been identified in several molluscan species (Ottaviani & Franceschi 1997) including *Aplysia*, though the neural effects of these cytokines have not been widely studied in most molluscan species. Studies in *Aplysia* have shown IL-1 and TNF modulating ionic channels in the soma of neurons

(Sawada et al. 1991) and IL-1 has been shown to enhance the firing from injured sensory nerves (Clatworthy et al. 1994).

**Stress and Immunosuppression in Abalone**

Several recent studies have greatly advanced our knowledge of how stress affects the immune function of abalone (Malham et al. 2003, Cheng et al. 2004a, b, Cheng et al. 2004c, Cheng et al. 2004d, Cheng et al. 2004e, Shuhong et al. 2004). Malham et al. (2003) demonstrated that the haemolymph levels of noradrenaline and dopamine in *Haliotis tuberculata* rise after application of a controlled stressor (mechanical shaking), concomitant with the decreased immune response. The stress hormones decreased back to basal levels 15- 60 minutes after the stressor ceased. Consistent with these findings, Ottaviani et al. (1996) incubated hemolymph with CRH and ACTH and found that the release of biogenic amines reached their peak after 15 minutes and lasted approximately 45 minutes in the gastropods *V. ater* and *P. corneus* (Ottaviani & Franceschi 1996). Malham et al. (2003) reported an initial depression in haemocyte counts, migratory activity, phagocytosis and superoxide production, which was followed by an increase in these immune responses lasting for between 2 hours (migratory activity, superoxide production) and 4 hours (haemocyte counts and phagocytic ability). This was then followed by decreased values in all immune responses (Malham et al. 2003). The suggested explanation for this was a period of transient immune stimulation followed by depression. Likewise, the series of experiments done on the abalone *Haliotis diversicolor supertexta* by Cheng et al. (2004 a,b,c,d,e) showed a similar period of apparent immune stimulation with milder stressors. Haemocyte counts increased for 72 hours with the lower elevations of the stressor nitrite followed by decreased counts (Cheng et al. 2004b) and there was a transient rise in haemocyte counts for 24 hours with the lower increased ammonia levels, followed by significant decreases (Cheng et al. 2004a). The higher concentrations of ammonia and nitrite both caused decreased haemocyte counts. This indicates mild stressors have a different effect to severe stressors on the immune response though the overall effect is immunosuppression.

The experiments by Cheng et al. (2004a,b,c,d,e) showed increased susceptibility to infection by *Vibrio parahaemolyticus* and increased mortality in each case a stressor was applied, including with mild stressors. This is evidence that stress leads to immunosuppression, which will be exploited by opportunistic pathogens. It also indicates that the apparent immune stimulation seen transiently does not correspond to increased immune functional capacity, because there was increased susceptibility to infection and increased mortality. For example, abalone exposed to the stressor of increased nitrite concentrations showed elevated phenoloxidase activity and superoxide production, but the mortality of the abalone after infection with *V. parahaemolyticus* increased in direct parallel to the nitrite concentration (Cheng et al. 2004b). Thus, increased susceptibility to *Vibrio parahaemolyticus* is occuring irrespective of elevated phenoloxidase activity and superoxide production with some stressors, supporting the notion that stressed abalone are immunosuppressed.

Cheng et al.'s (2004a,b,c,d,e) work on stress and immunosuppression studied the effects of elevated temperature, nitrite, ammonia, reduced dissolved oxygen, and increased/decreased salinity outside the normal ranges of the species he was studying, *H. diversicolor*. Phagocytosis and clearance efficiency assays were all depressed when the abalone were subjected to these conditions, which can be experienced on farms. The haemocyte counts, phenoloxidase measurements and superoxide production varied, with both increased responses and decreased responses seen in different circumstances. The cause of this is not certain and until understood better, these assays are not reliable

indicators of immunosuppression in abalone. By comparison, the phagocytic ability of abalone haemocytes does appear to decrease consistently after the application of stressors (Malham et al. 2003) and this correlates to increased susceptibility and mortality due to *Vibrio parahaemolyticus* infection (Cheng et al. 2004 a,b,c,d,e). Phagocytosis assays are probably a more reliable immune parameter for future studies on stress-related immune function.

Recent work on the cell-free haemolymph of *H. diversicolor* (Shuhong et al. 2004), showed increased levels of the lysosomal enzymes, acid phosphatase and alkaline phosphatase, in abalone infected with the pathogen *Vibrio parahaemolyticus* but not with the nonpathogenic bacterium *Escherichia coli*. This indicates that *V. parahaemolyticus* activated the immune defense response but *E.coli* did not. It remains unclear how the abalone distinguish pathogenic from nonpathogenic bacteria. Shuhong et al (2004) found levels of phenoloxidase to be low and there were no significant differences between infected abalone and control groups. The activity of superoxide dismutase (SOD) was significantly lower in the *V. parahaemolyticus* exposed group. The reducing of SOD activities allows reactive oxygen species to persist longer and may aid in the response to bacterial infection (Shuhong et al. 2004).

**Effect of Stress on Physiology**

Immunity like all other physiologic functions requires adequate energy to sustain optimal function. Energy is not a limitless resource. Finite energy sources must be allocated to all biological functions in the animal and these functions may compete. At times of stress, energy needs to be re-allocated from less critical needs such as growth to those more important for immediate survival (Demas 2004). One question that needs defining is how costly is the stress response in terms of energy?

Several biochemical parameters have been explored as indicators of physiological stress in abalone. Glucose levels in the haemolymph of *H. laevigata* have been shown to rise after exercise stress and in response to increasing temperature (Haldane 2002). This is consistent with findings on the heterobranch mollusc *Aplysia dactylomela* (Carefoot 1991, 1994). Although Haldane (2002) found no concurrent drop in the glycogen levels during exercise stress, suggesting that this activity is supported by aerobic metabolism, this is contradictory to the findings of Baldwin et al. (1992), who reported reduced glycogen levels after exercise in *H. iris.* Anaerobic glycolysis results in the production of lactate and a concomitant fall in the pH. Exercise stress was found to produce a significant drop in pH for both *H. laevigata* (Haldane, 2002) and *H. iris* (Baldwin et al. 1992), suggesting anaerobic metabolism is occurring in these animals when stressed. A drop in pH levels was also recorded after applying temperature stress in *H. laevigata* (Haldane, 2002) and air exposure in *H. iris* (Ryder et al. 1994). Baldwin et al. (1992) also reported a concomitant increase in D-lactate and the production of tauropine, another end product of anaerobic glycolysis. Other biochemical parameters, such as adenylate energy charge, IMP-load and haemolymph ions were not found to be useful indicators of stress in *H. laevigata* (Haldane, 2002).

The longer-term effects of chronic stress may be better represented by the growth and survivorship of the animals. Growth decreases with increased stocking density of farmed abalone (Capinpin et al. 1998, Huchette et al. 2003b, a, Day et al. 2005), which could reflect increased stress due to density dependent competition for space or food. Abalone tend to stack at high densities due to a lack of primary attachment space (Huchette et al. 2003a). Stacking reduces the movement associated with feeding, hence food limitation occurs at high density even if there is enough food. Abalone are erratic feeders, sensitive

to a number of environmental and physiologic influences (Greenier & Takekawa 1992). Growth is also inhibited by increased metabolic wastes, especially ammonia, which becomes a problem in higher stocking densities and which has been shown to cause stress in abalone (Huchette et al. 2003b, Cheng et al. 2004a).

Abalone as well as other molluscs possess growth hormone-like molecules (Taylor et al. 1996). Two growth hormone like substances have been purified from *Haliotis discus hannai* (Moriyama et al. 1989). A growth hormone isolated from the neurosecretory cells in the cerebral ganglia of *Lymnaea stagnalis* stimulated shell growth and influenced metabolic pathways (Dogterom 1980, Dogterom & Jentjens 1980). The effect of stress on the function of these molecules has not been examined. Since there is a neuroendocrine link between stress and immune function, there may also be a link between stress and the neuroendocrine system directly affecting growth hormones. This link is seen in mammals, e.g. stress induces hypercortisolemia, which inhibits growth through several mechanisms, including blocking the secretion of growth hormone and promoting protein breakdown (Sherwood 1997).

**Conclusions**

The stress response in those molluscs that have been closely studied has the same key molecules and cascade as seen in vertebrates; i.e. CRH stimulating release of ACTH stimulating release of biogenic amines. In molluscs, the molecules are all produced by the haemocyte. The haemocyte is also the central cell of the immune response, participating in migration, phagocytosis, superoxide production, etc. Further work needs to be done to clarify the interrelationship between the endocrine and immune systems. There is competition among the hormones and cytokines in influencing the immune system and the benefits and costs to the animals ability to survive cannot be ascertained until more is known about how these molecules inter-relate. The role of glucocorticoids needs to be investigated further, considering how important these molecules are in mediating secondary effects on all body systems, including the immune system, in vertebrates.

How growth hormones interact with the other hormones and the immune system is another area to be investigated. After a disease outbreak, the dead abalone can be disposed of and the cost measured, but we do not understand the mechanisms involved in stunted growth in the survivors, which will often be of greater economic importance to the farmer. How much stress will cause a brief stunting of growth and how much will lead to permanent ill-thrift is not known.

We do not yet know the source of stem cells; which haemocytes are involved in which immune responses; and whether granulocytes and hyalinocytes are related to each other in terms of haematopoiesis. The location of haemocytes involved in the transient drop in haemocyte counts seen at the start of some of the stress responses has not been determined.

Understanding the immune response in abalone requires further research. The work done so far indicates immune suppression and increased susceptibility to infection due to stress, but the immune responses (e.g. haemocyte counts, phenoloxidase activity, superoxide production) have not yet been fully characterised. We do not yet know which changes are good indicators of immune response because we do not understand how the physiologic response relates to immune defense. There appears to be a period of apparent immunostimulation, followed by return to basal levels after a mild, controlled stress is applied, but overall susceptibility to infection by pathogens is increased, indicating immunosuppression has occurred.

In a series of recent papers, abalone were subjected to a variety of stressors, including temperature stress, shaking, decreased dissolved oxygen, increased ammonia concentrations, altered salinity and increased nitrite concentrations (Malham et al. 2003, Cheng et al. 2004 a,b,c,d,e). These stressors had similar depressant effects on phagocytosis and clearance efficiency, but haemocyte counts, phenoloxidase activity and respiratory burst responses varied. In some cases they increased and in other cases they were diminished; and the significance of this needs further investigation. Does increased phenoloxidase activity after stressing the abalone by decreased dissolved oxygen reflect immunostimulation? This is unlikely because the alterations in the immune response after exposure to low dissolved oxygen were concomitant with increased rates of infection and mortality (Cheng et al. 2004e). Increased susceptibility to infection is the most valid indicator of immunosuppression. These results show why further work on the use of apparent indicators of immune responses is needed.