Reduction in Pacific Oyster Mortality by Improving Farming and Processing Technologies in South Australia

FRDC Final Report (Project No. 2003/208)

Xiaoxu Li

January 2008

SARDI Publication No: F2006/000334-1
SARDI Research Report Series No: 186

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SARDI Publication No: F2006/000334-1
SARDI Research Report Series No: 186
ISBN No: 0 7308 5357 8

Printed in January 2008 by:
PIRSA
Corporate Services
101 Grenfell St
Adelaide
SA 5001

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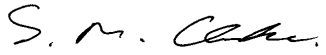
Approved by: Steven Clarke



Signed:

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2. NON-TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE

The research on stress assays conducted in this project has provided a tool to determine best practice techniques in oyster farming. Once adopted, this should result in an improvement in the performance of oyster farms. A 1% reduction in oyster mortalities would save approximately $200,000 per annum for the South Australian oyster industry. In addition, this project has contributed to a better understanding of the frequency, location and possible causes of Pacific oyster mortalities in South Australia and on disease agents that occur in oysters farmed in South Australian waters. This information will be used to help reduce and manage future mortalities. Finally, the survey of oyster processors has provided valuable information regarding the quality of South Australian oysters and the most important traits with respect to their marketing. This has highlighted to the industry where improvements need to be made to increase the saleability of oysters and to expend into new markets.

Ever since mortalities in Pacific oysters (*Crassostrea gigas*) were first recorded in South Australia (SA) about 10 years ago, oyster farmers have continually refined farming methods in an attempt to increase oyster survival, although limited improvements have been made. In some regions unusual mortalities occurred nearly every year prior to 2002, especially during the summer periods when ambient temperatures have remained high for several days. The purpose of this project was to investigate oyster mortalities and to minimise their occurrence by developing low stress management strategies for the oyster industry.

In April 2005 it was decided at a Project Steering Committee meeting (comprising SARDI and SAORC representatives, but subsequently involving FRDC) that the project should be terminated due to:

- no unusual oyster mortalities having occurred in South Australia during the two years since the project's commencement in June 2003;
- further refinement of the stress assays would be required prior to their application; and
- oyster industry representatives pursuing a best practice husbandy manual for the farming of Pacific oysters in SA waters through other means (industry bench mark workshops).

At that time objectives 1, 3, 5 and 8 had been achieved:

1. Design and conduct an industry survey to assess the location, time and prevalence of oyster mortality.
3. Design and conduct an oyster health survey to investigate the prevalence of oyster pathogens in stock

in SA coastal waters.

5. Identify quantitatively the stress levels created by different equipment and different farming and processing activities.
8. Communicate advice to farmers.

Objectives 2, 4, 6, 7 and 9 were not achieved and were subsequently cancelled:

2. Design and conduct on farm experiments to investigate the correlation between oyster mortalities and environmental and biological factors.
4. Reduce the number of possible causative factors by analysing the data from the industry survey, on farm experiments and oyster health survey.
6. Identify quantitatively and qualitatively the likely pathogens responsible for oyster mortality.
7. Develop best practice advice for farmers.
9. Develop a best practice husbandry manual for the farming of Pacific oysters in SA water.

The 1st project objective was to design and conduct an industry survey to assess the location, time and prevalence of oyster mortalities during the last five years from 1998 to 2003. Two industry surveys were conducted to gather information relating to mortality, health and quality of oysters. The farm-based survey found that farmers from Coffin Bay and Cowell had not experienced any unusual mortality events whilst some Denial and Smoky Bay farmers did report oyster mortalities. Some farmers reported higher mortalities in family lines compared to standard commercial stock. Many farmers thought that mortality occurred at the spat stage. The percentage of spat farmers expected to reach saleable size ranged from 50 to 95%. Most farmers thought that low mortality occurred in adult stock with estimated losses at each grade ranging between 1-2%. From the survey it was apparent that most farmers attributed mortalities to stock management and stress related issues, including grading, air exposure, high air and water temperatures and the reproductive cycle of the oysters. The survey also revealed that most farmers did not keep detailed records that allowed determination of when and where oyster mortalities were occurring.

The main issues raised from the processor/wholesaler survey were spawny oysters during summer, mudworm infestation and non-uniformity in oyster size and condition. Five out of eight processors/wholesalers stated that meat to shell ratio was the most important quality trait. The rest ranked uniform shell size as the most important quality trait. Four processors/wholesalers reported having observed abnormalities in oysters they had received during summer months. Two processors/wholesalers observed oysters with a dark or black spot in the white of the meat and an unusual smell.

The 3rd objective of the project was to design and conduct an oyster health survey to investigate the prevalence of oyster pathogens in stock in SA coastal waters. A shellfish health survey was conducted by the

PIRSA Aquaculture group. A total of 2,238 Pacific oysters from 16 sites in SA were collected for assessment. The pathological findings of the survey were:

- the detection of microcell-like cells in vesicular connective tissue;
- the abnormal hypertrophied nuclei with marginated chromatin in vesicular connective tissue cells;
- atrophy of digestive tubules;
- a viral gametocytic hypertrophy-like lesion in the gonad;
- metaplastic changes of the digestive tubule epithelium (a lesion distinct from tubule atrophy);
- diapedesis through the gut epithelium;
- parasites and symbionts; and
- rickettsia-like organisms.

The health survey has helped to produce a pathology-based overview of the disease agents that currently occur in SA. This information will further help the SA oyster industry to determine the relationship between oyster mortalities and pathogens.

The 5th project objective was to quantitatively measure the stress levels created by different oyster farming equipment and different farming and processing activities. Three techniques were developed and evaluated to measure stress as part of six student projects (five Masters projects from Dalian Fisheries University, China, and one Honours project from Flinders University, South Australia). The three techniques evaluated were: 1) measurement of catecholamine levels in oyster blood, 2) measurement of phenoloxidase activity in the blood and 3) assessment of lysosomal membrane integrity using neutral red staining. Results from laboratory and field experiments showed, based on the methodology used, that the phenoloxidase assay was not a reliable method for detecting stress responses in Pacific oysters. Measurement of catecholamine activity was suitable for single stressors while the neutral red retention assay could be applied to both single and multiple stressor analysis.

In this study the major factors identified in the industry survey as contributing to oyster mortalities or poor performance were assessed using either the neutral red retention (NRR) assay, catecholamine analysis or both. Significant differences were found when animals were subjected to:

- slow and rapid temperature changes;
- exposure to different air temperatures for different durations;
- recovery after different air exposure treatments;
- starvation or food deprivation;
- gonad development;

- spawning and post spawning recovery;
- grading for different durations;
- grading with different types of graders; and
- recovery after grading.

In addition, significant differences were also found between animals of different sizes in response to some of the above-mentioned treatments using the NRR assay.

It is therefore reasonable to assume that these assays could be used as a tool to evaluate the stresses induced by different farming practices and thus identify those which reslt in the least stress, leading to an improvement in oyster performance on farms.

The 8th project objective was to communicate advice to farmers. Results that could be used to improve the oyster farming practices were immediately circulated to the Project Steering Committee and the South Australian Oyster Research Council (SAORC). The oyster growers were directly informed through four articles in the "SAORC Newsletters" and two presentations at SAORC annual meetings. Five articles in scientific journals have also been published at this time.

3. ACKNOWLEDGEMENTS

The author wishes to acknowledge the important contribution made to this project by five Masters' students, Ms Yi Qu, Ms Yi Yu, Mr Zehu Zhang, Ms Ting Wang and Mr Liang Song of the Dalian Fisheries University, China and one Honours student, Mr Andrew Manning of the Flinders University of South Australia. Parts of this report are based on the results from their studies.

The author would also like to acknowledge the contribution from industry representatives who participated in the "Pacific Oyster Industry Survey of South Australia" and the "Pacific Oyster Processor/Wholesaler Survey": Gary Zippel, Ashley Zippel, Jeff Holmes, Geoff Hammat, Gorden Gardner, John Mitchell, John Pedler, Gary Seidl, Theo Eleftheriou, Craig Lowe, Mike Whillas, Bill Stevenson, Brendan Guidera, Mark Jarvis, David Pepper, Barry Guidera, Simon Turner, Richie Baker and Tony Schultz. The author is grateful to Mr Gary Zippel of Zippel's Enterprises Pty Ltd for making available the oysters and one of the graders and to Mr Jeff Holmes and Mr Geoff Hammat for providing the other two graders used in this project. I am grateful to Mr Kriston Bott for help in preparing this report, Ms Annette Doonan for preparing the maps and to Dr Meegan Vandepeer and Mr Steven Clarke, all from South Australian Research and Development Institute (SARDI) for editing the report. I would also like to thank the managerial and academic staff at the Aquatic Sciences Centre of SARDI for their support and valuable discussions.

4. BACKGROUND

The South Australian Pacific oyster industry is recognised as one of the fastest development aquaculture sectors and a major economic contributor of seafood to the state. During the last five years the production value of Pacific oysters in South Australia (SA) has increased by 82.8%, from $11.6 million in the 2000/01 financial year to 21.2 million dollars in the 2004/05 financial year. Currently the industry employs approximately 320 people in the rural areas of SA (Econsearch, 2006).

The Pacific or Japanese oyster is the most widely cultivated oyster species in the world. It has been transplanted from its native range in East Asia and Southeast Asia and is now cultured in large quantities in Australia, North America, and Europe. Currently, the global production of this species exceeds one million metric tons live weight from more than 11 countries (FAO, 2003).

The oysters currently farmed in SA are the descendants of the Pacific oysters from Japan. Although this species has been farmed in SA waters for more than two decades and every summer the animals spawn on the leases, only small numbers of feral Pacific oysters have been found in select bays along the SA coast. This suggests the range in some environmental variables required for oyster larval development and/or metamorphosis are exceeded in SA coastal waters. For example, the maximum air temperature and salinity of the water in SA during summer are approximately 40°C and 40ppt respectively, whereas in Japan the maximum air temperature and salinity are approximately 35°C and 35ppt. The high air temperatures and salinities in SA might not affect the ability of the oysters to grow, however, exposure to these kinds of environmental conditions for long periods of time might make them more sensitive to other environmental stressors. This is because they would need to invest more energy in maintaining homeostasis than oysters living in an area where environmental conditions are optimal. The consequence of this is that less energy is available for other body functions such as the immune responses.

Oysters are permanently exposed to various microbes and their defence system is continuously solicited to prevent accumulation of invading and pathogenic organisms. Therefore, impairment of the animal's defence system usually results in mass mortalities in cultured oyster stocks or increased bacterial loads in food products intended for human consumption. Published studies indicate that stress decreases the efficiency of the defence system in molluscs and therefore may be associated with high bacterial loads, disease outbreaks and subsequent mortalities (Lacoste et al. 2001; Berthelin et al., 2000).

Ever since unusual oyster mortalities were first recorded in SA about 10 years ago, oyster farmers have continually refined farming methods in an attempt to increase oyster survival. It has been suggested that limited improvements to oyster survival have been made, if any, via altering farming methods. This suggests that predisposing factors causing oyster mortality are more complicated than were initially thought. *Vibrio* sp.,

pathogens or flatworm proliferation, grading activities, physiological changes after spawning, long periods of exposure to sublethal temperatures, high stocking densities, food availability and their combinations are all thought to be predisposing factors to oyster mortality. The oyster industry now believes a systematic, scientific, research based approach is essential to addressing the problem.

Summer mortality is one of the major issues facing oyster aquaculture industries in temperate climates (Lipøvsky and Chew, 1972; Beattie et al., 1980; Perdue et al., 1981; Berthelin et al., 2000). In France, losses of up to 80% in some areas have been recorded (Berthelin et al., 2000). The production of a few molluscan species from a number of large Asian producing countries has been decimated (Yang, personal communication). In some countries the main predisposing factors for the mortality have not been resolved since the crash of the industry. This is mainly due to the lack of historical environmental information, non-standardized experiments on individual farms, the complexity of the factors investigated, the physiological changes of the animal itself and changes to the environment caused by the animals.

The aim of this project is to investigate the correlation between environmental factors and oyster mortality in SA and reduce mortalities by developing low stress management practices for the oyster industry. In addition, the success of the project could also contribute to the development of an oyster aquaculture monitoring and management program in South Australia.

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5. NEED

The SA oyster industry is recognised as one of the fastest development aquaculture sectors and a major economic contributor to seafood production. The value of the Pacific oyster production in SA has increased by 82.8% during the last 5 years, from $11.6 million in the 2000/01 financial year to $21.2 million in the 2004/05 financial year. If the mortality of oysters was not controlled and remained at the level as estimated by the SAORC in 2002 (5 to 20% losses each year of marketable stocks), the industry would experience $1.1 to 4.2 million losses in the 2004/05 financial year. A less optimistic forecast is that epidemiological factors could result in a much greater and possibly catastrophic rate of increase in losses in the future.

The industry views the need to understand and control the reasons for these losses as a matter of urgency. However, farmers' ad-hoc approaches have failed to identify any clear direction to address the mortality issue. Due to this, the South Australian Oyster Research Council spent considerable time discussing the matter with various researchers and decided to support a specific research project to address this issue systematically.

If this project can achieve a 50% reduction in mortality one year after its completion, the savings to growers will be at least $0.6-2.1 million per annum based on the 2004/05 production figure.

In addition, minimising the impact of mortality on the Pacific oyster aquaculture industry in SA will also strengthen the confidence of new growers and investors in this industry thereby encouraging its further expansion.

6. OBJECTIVES

The original project objectives were to:

1. Design and conduct an industry survey to assess the location, time and prevalence of oyster mortality.
2. Design and conduct on farm experiments to investigate the correlation between oyster mortalities and environmental and biological factors.
3. Design and conduct an oyster health survey to investigate the prevalence of oyster pathogens in stock in SA coastal waters.
4. Reduce the number of possible causative factors by analysing the data from the industry survey, on farm experiments and oyster health survey.
5. Identify quantitatively the stress levels created by different equipment and different farming and processing activities.
6. Identify quantitatively and qualitatively the likely pathogens responsible for oyster mortality.
7. Develop best practice advice for farmers.

8. Communicate advice to farmers.
9. Develop a best practice husbandry manual for the farming of Pacific oysters in SA water.

Those finally agreed and reported against were:

1. Design and conduct an industry survey to assess the location, time and prevalence of oyster mortality.
2. Design and conduct an oyster health survey to investigate the prevalence of oyster pathogens in stock in SA coastal waters.
3. Identify quantitatively the stress levels created by different equipment and different farming and processing activities
4. Communicate advice to farmers.

7. TECHNICAL SUMMARY

Research conducted in this project was focused in two areas. The first addressed the information gaps between the Pacific oyster mortality previously reported by the South Australian (SA) oyster industry and the actual prevalence of these events and opinions as to their potential causative factors. The second developed techniques that could be used to differentiate the stress levels imposed by different farming practices on Pacific oysters. It was anticipated that the results from this research would lead to the development of best husbandry practice for the SA Pacific oyster aquaculture industry through the implementation of low stress techniques at different production stages.

7.1. Surveys

Three surveys were carried out to achieve the following agreed project objectives:

- design and conduct an industry survey to assess the location, time and prevalence of oyster mortality (1);
- design and conduct an oyster health survey to investigate the prevalence of oyster pathogens in stock in SA coastal waters (2); and
- communicate advice to farmers (4).

7.1.1 Methods

The methods used in these surveys are detailed in the Appendix 3: Pacific oyster farm survey; Appendix 4: Pacific oyster processor/wholesaler survey; and Appendix 5: Oyster health survey.

7.1.2. Major results and discussions

A mortality survey of 24 oyster farms in SA (Appendix 3) found that:

- farmers from Coffin Bay and Cowell had not experienced any unusual oyster mortality events whilst some farmers from Denial and Smoky Bay had;
- some farmers reported observing higher mortalities in family lines compared with standard commercial stock;
- many farmers thought that mortalities mainly occurred at the spat stage;
- the percentage of spat farmers expected to reach saleable size ranged from 50 to 95%; and
- most farmers thought that low mortality occurred in adult stock with losses at each grading event estimated to be between 1-2%.

From the survey it was apparent that most farmers attributed mortalities to stock management and stress related issues, including grading, air exposure, high air and water temperatures, and the reproductive cycle of the oysters.

The main issues raised from the Pacific oyster processor/wholesaler survey (Appendix 4) were:

- spawny oysters during summer;
- mudworm infestation; and
- variability in size and condition throughout the year.

The most important quality traits for Pacific oyster marketing ranked by processors/wholesalers were:

1. meat to shell ratio (five out of eight processors/wholesalers); and
2. uniform shell size (five out of eight processors/wholesalers).

A total of 2,238 Pacific oysters from 16 sites in South Australia (SA) were collected and assessed for aquatic animal health issues (Appendix 5). The pathological findings of the survey were:

1. the detection of microcell-like cells in vesicular connective tissue;
2. the abnormal hypertrophied nuclei with marginated chromatin in vesicular connective tissue cells;
3. atrophy of digestive tubules;
4. a viral gametocytic hypertrophy-like lesion in the gonad;
5. metaplastic changes of the digestive tubule epithelium (a lesion distinct from tubule atrophy);
6. diapedesis through the gut epithelia;
7. parasites and symbionts; and
8. rickettsia-like organisms.

The Pacific oyster health survey has helped to produce a pathology-based overview of the disease agents that currently occur in South Australia.

7.2. Stress assessments

Three stress measurement techniques were evaluated and/or developed. These are the neutral red retention assay, the catecholamine assay and the phenoloxidase assay. The experiments conducted were to achieve the following two project objectives:

- identify quantitatively the stress levels created by different equipment and different farming and processing activities (3); and
- communicate advice to farmers (4).

7.2.1. Neutral red retention (NRR) assay

Neutral red is a lipophilic dye that diffuses through cell membrane passively (Lowe et al., 1992). In unstressed cells their lysosomes can retain this dye for a long period. However, once the lysosomal membrane is destabilized, the dye will leak into the cytosol more quickly. The NRR assay is to assess the lysosomal membrane integrity by directly measuring lysosomes' ability to retain neutral red dye. Because NRR assay is simpler, more convenient and more cost efficient, the method has been widely applied to evaluate the effects of environmental and physiological changes and mechanical stresses on molluscs (Lowe and Pipe, 1994; Lowe et al., 1995a, b; Fernley et al., 2000; Grøsvik et al., 2000; Da Ros et al., 2002; Castro et al., 2004; Harding et al., 2004a, b). In addition, the published data have also showed that lysosome is the earliest site of detection for the response of bivalves to various stressors and the relative value of the NRR assay can be correlated to the overall stress of the animal (Harding et al., 2004a, b). The lower the NRR time the higher the stress level.

7.2.1.1. Methods

Details of the experimental procedures and NRR assay are reported in Appendix 6: Effects of water temperature and air exposure on the lysosomal membrane stability of hemocytes in Pacific oysters, *Crassostrea gigas* (Thunberg); Appendix 7: Differences in the response of two size classes of Pacific oysters, *Crassostrea gigas* (Thunberg) to changes in water temperature and air exposure; Appendix 8: Application of the neutral red retention assay to evaluate differences in stress response to sexual maturation and spawning between different sized Pacific oysters, *Crassostrea gigas* (Thunberg); Appendix 9: Evaluation of the effects of grading and starvation on the lysosomal membrane stability in Pacific oysters, *Crassostrea gigas* (Thunberg); and Appendix 11: Preliminary study on the effects of different grading methods on lysosomal membrane stability in Pacific oysters, *Crassostrea gigas* (Thunberg), using the neutral red retention assay.

The NRR assay was used to evaluate the effects on oysters from:

a. rapid and gradual water temperature changes (Appendix 6);
b. exposure to different air temperatures (Appendix 6);
c. recovery in water after air exposure (Appendix 6);
d. sexual maturation (Appendix 8);
e. spawning (Appendix 8);
f. post spawning recovery (Appendix 8);
g. simulated rotational grading (Appendix 9);
h. starvation (Appendix 9);
i. combination of starvation and rotational grading (Appendix 9);

j. different types of graders commonly used in SA – the Inside/Out, Rotary and Flat Bed graders (Appendix 11); and
k. size and/or age classes (Appendixes 7 and 8).

7.2.1.2. Major results and discussions

The results from this project show that:

- the NRR times of oysters held in 5, 15 and 25°C seawater were about 60, 145, and 66 min, respectively (Appendix 6);
- when water temperatures were changed between 5°C and 15°C, and 15°C and 25°C, the NRR time of oysters at the final temperature of 5, 15 or 25°C was not influenced by the speed, 2°C per day or directly between them, at which the temperature was changed to these levels (Appendix 6);
- the time for lysosomal membrane integrity recovery was longer than its destabilization in Pacific oysters (Appendix 6);
- the water temperature between 13 and 17°C was the optimal range for maintaining lysosomal membrane integrity in this species (Appendix 6);
- when exposed to air the lysosomal membrane integrity within the first 1.5 hr was mainly affected by air temperature; from 3 hr onward both air exposure duration and temperature affected the integrity; after 12 hr higher air temperatures resulted in shorter NRR times than lower temperatures (Appendix 6);
- during recovery in 15°C water after 72 hr air exposure, significantly shorter time periods were required for NRR times to recover in oysters exposed to 5°C than those exposed to 15 and 25°C, indicating that exposure to lower air temperatures caused less damage to lysosomal membranes in Pacific oysters (Appendix 6);
- when oysters were transferred directly between 5°C and 15°C, 15°C and 25°C, and 10°C and 20°C water, only the NRR times in oysters transferred between 10°C and 20°C initially decreased significantly, and then gradually increased to the levels corresponding with the new temperatures, indicating that different ranges of water temperature change can affect the lysosomal membrane integrity differently (Appendix 7);
- when oysters were moved directly between 5°C and 15°C, 15°C and 25°C, and 10°C and 20°C in water, differences in the NRR times between large and small oysters were not significant when they were transferred from 15°C to 5°C and 25°C, and from 10°C to 20°C, but were significant when they were transferred from 5°C and 25°C to 15°C, and from 20°C to 10°C (Appendix 7);
- after exposure to an air temperature of 5°C, 15°C or 25°C the lysosomal membrane integrity of the large oysters recovered at a slower rate than that of the small oysters in 15°C water (Appendix 7);

- prior to spawning the decrease in lysosomal membrane stability in the oysters fed with microalgae at approximately 2×10^6 cells mL^{-1} at a rate of 1.0 L per oyster per day was negatively correlated with the dry meat weight, condition index and maturation index, indicating that any stress experienced by the oysters during this period was mainly related to gametogenesis and the related metabolic alterations it caused (Appendix 8);
- spawning further impaired lysosomal membrane stability ($P < 0.05$); after spawning the NRR times reached the lowest levels recorded before recovering to levels corresponding with the water temperature in which the animals were maintained (Appendix 8);
- the smaller oysters recovered from spawning stress much faster than the larger animals ($P < 0.05$) (Appendix 8);
- prior to spawning the dry meat weights of both large and small oysters increased by approximately 85% in the first 42 days, whereas after spawning the dry meat weights only increased by 20% during the same time period, suggesting that available energy was used to recover from the stress created by spawning (Appendix 8);
- both simulated grading duration and subsequent recovery in 15°C water had a significant effect on lysosomal stability. In general, the oysters that underwent longer durations of grading had lower NRR times. The NRR times in the oysters graded for shorter periods recovered more quickly than those graded for longer periods during the post-grading recovery in 15°C water (Appendix 9);
- after 42 days starvation, the NRR times in starved oysters were significantly lower than those in fed and control animals (Appendix 9);
- after 3 min grading the ability to retain neutral red in lysosomes in starved oysters was significantly lower than that in the fed animals (Appendix 9); and
- the Inside/Out grader had a significant effect on NRR time ($P = 0.013$), whilst the rotational grader had a close to significant effect ($P = 0.052$), suggesting that both these graders elicited a stress response in oysters under the existing operational settings (Appendix 11).

7.2.2. Catecholamine assay

When animals are exposed to a stressor, their physiological stress response is initiated by the recognition of a real or perceived threat by the nervous system, which then stimulates the release of catecholamines. Catecholamines include adrenaline, noradrenaline and dopamine. One of the well-characterized effects of catecholamine is the stimulation of glycogenolysis, the conversion of stored glycogen to glucose, in response to increased metabolic demands during a stress response (Randall and Perry 1992). In oysters, recent studies show that the release of catecholamine is rapid and the circulating levels can change immediately with stress (Lacoste et al., 2001b; Lacoste et al., 2002). Furthermore, the physiological changes imposed by stress have

been observed to increase the vulnerability of oysters to *Vibrio splendidus* (Lacoste et al., 2001a). The higher the catecholamine level the higher the stress level.

7.2.2.1. Methods

Details on experimental designs and catecholamine analyses are reported in Appendix 10: The effect of different grading equipment on stress levels in Pacific oysters, *Crassostrea gigas* (Thunberg) and Appendix 12: Background catecholamine levels in family lines of Pacific oysters *Crassostrea gigas* (Thunberg) grown in South Australia.

In this study three commonly used oyster graders were assessed by the catecholamine assay. These were the Flat Bed, the Inside/Out and the Rotary grader.

7.2.2.2. Major results and discussions

The results from this project show that:

- significant increases ($P < 0.05$) in circulating noradrenaline and dopamine levels were observed in oysters graded by the Flat Bed and the Inside/Out grader (Appendix 10);
- the circulating noradrenaline concentrations in oysters subjected to the Flat Bed grader were significantly higher ($P < 0.05$) than those observed in the Rotary graded oysters and close to being significantly higher ($P = 0.052$) than those levels measured in the Inside/Out graded oysters. The circulating noradrenaline levels in oysters subjected to the Inside/Out grader were, in turn, significantly higher than those recorded in the Rotary graded oysters. These results suggest that among the three graders used in South Australia, the Flat Bed induced the highest stress levels in oysters and the Rotary grader the lowest (Appendix 10); and
- differences in background noradrenaline and dopamine levels between 9 family lines were not significant ($P = 0.21$ and 0.07 respectively). However, a large amount of variation was found within some lines, suggesting that the sample size (3 replicates per line) used in this experiment might have been too small to differentiate the lines (Appendix 12).

7.2.3. Phenoloxidase assay

Phenoloxidase (PO) activity is often associated with host defence. Results from research by Peters and Raftos (2003) on the role of phenoloxidase suppression in QX disease outbreaks among Sydney rock oysters showed that the PO activity was significantly suppressed in oysters from the QX prone area when compared to those from the QX free rivers, and there was also a strict negative correlation between PO activity and the infection intensity.

7.2.3.1. Methods

In Mr Manning's Honours' project two previously published methods for measuring phenoloxidase activity in Sydney rock oysters were investigated, one using L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate while the other with 4-methoxyphenol (HQ). Oysters were experimentally challenged with grading, emersion, salinity and nutrition changes, which are common physical and environmental stressors associated with commercial oyster culture. Dr David Raftos was also consulted during the project to ensure the same methodology was used.

7.2.3.2. Major results and discussions

The results from Mr Manning's study show that:

- the method used in the L-DOPA assay in Sydney rock oysters does not appear to measure levels of hemolymph phenoloxidase activity in Pacific oysters (*C. gigas*);
- the method used in the HQ assay in Sydney rock oysters does not appear to measure levels of monophenolase activity of hemolymph phenoloxidase activity in Pacific oysters (*C. gigas*); and
- using the published spectrophotometric techniques in Sydney rock oysters the measurement of hemolymph phenoloxidase activity in Pacific oysters (*C. gigas*) does not provide a reliable indicator of stress.

Further experimentation with the methods used in other published studies would be able to provide the information required to improve the sensitivities of the techniques used in this study. In addition, treatments based on artificial seawater may reveal more information about what the different phenoloxidase assays are actually measuring, and what factor(s) increase the levels of HQ assayed monophenolase activity in the hemolymph of Pacific oysters (*C. gigas*). For further details refer to Mr Manning's thesis in Appendix 13: The role of phenoloxidase in stress response of Pacific oyster, *Crassostrea gigas*.

It should be noted that recently the phenoloxidase assay was advanced by Hellio at al. (2007) in Pacific oysters and a true phenoloxidase activity and activation of a proPO cascade were demonstrated *in vitro* in this species.

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8. BENEFITS

The results of the farm based survey provided insight into the occurrence and extent of Pacific oyster mortalities within South Australia, which were highly variable between farms and locations. Some farms observed mortalities of up to 50% whereas others observed as little as 5%, suggesting that except for environmental effects, oyster mortality might also relate to difference in the farming practices used on different farms.

The health survey conducted by PIRSA Aquaculture produced a pathology-based overview of the disease agents that currently occur in the South Australian oyster industry. This is the first study of this kind conducted solely on the South Australian Pacific oyster industry. This background information is critical for the identification of factors causing oyster mortality in the field.

Both catecholamine analysis and neutral red retention assay have been successfully developed for the evaluation of individual stress responses in Pacific oysters. Results from the experiments show that both assays can be used to quantify the relative stress levels created by normal farming activities and changes in the environments and animals' physiological conditions. The potential applications of these assays include:

1) development of low stress management strategies and best practice advice for Pacific oyster farmers;
2) development of an oyster health monitoring program for the oyster industry; and
3) development of a criterion for improving health related traits in the selective breeding program.

9. FURTHER DEVELOPMENT

Both a neutral red retention and catecholamine assay have been successfully applied in assessing the stressors investigated in this study. However, most of these stressors were studied separately in controlled environments. In the field these stressors often act simultaneously and repeatedly. To develop a model to monitor and/or predict the performance of oysters on farms, further investigations on the effects from both repeated application of a stressor and simultaneous application of multiple stressors (including environmental variables) would be required. In addition, the establishment of a stress response level(s) that significantly affects oyster performance is the next step toward understanding the interaction between these factors.

10. PLANNED OUTCOMES

The purpose of this project was to investigate the major factors causing oyster mortalities in South Australia and to develop best husbandry guidelines by determining low stress farming practices. The first component could not be pursued because no unusual mortality events occured in South Australia whilst the project was being conducted. The catecholamine test and neutral red retention assay developed and evaluated in this study were able to detect a range of stresses experienced by oysters including; changes in environmental parameters, animals' physiological conditions and farming practices. If the project was not terminated early all the proposed experiments could have been completed, which would have provided enough information for the development of best practice guidelines for Pacific oyster farmers. It is anticipated that this would have helped to increase the health and quality of cultured oysters.

11. CONCLUSION

The farm based survey revealed that oyster mortalities were highly variable between farms and locations, suggesting that a better understanding of oyster health and mortality could have a significant beneficial impact on the South Australian oyster industry.

The health survey conducted by PIRSA Aquaculture produced a pathology-based overview of the disease agents that currently occur in the South Australian oyster industry. The most significant pathological finding in the Pacific oysters examined was the detection of low numbers of microcell-like cells in their vesicular connective tissue from 10 of the 16 sites sampled. The detection of microcell-like cells associated with areas of haemocytosis in oysters is significant as all microcell infections in molluscs are notifiable to the OIE Molluscan Reference Laboratory (OIE 2002).

Three stress assays have been evaluated or developed in this study. Measuring levels of phenoloxidase activity has not proven to be a reliable means for detecting stress responses in Pacific oysters. Both the catecholamine analysis and neutral red retention assay (NRR) have quantified stress levels experienced by oysters in this study. Significant differences were found when animals were subjected to:

- slow and rapid temperature changes;
- exposure to different air temperatures for different durations;
- recovery after different air exposure treatments;
- starvation or food deprivation;
- gonad development;

- spawning and post spawning recovery;
- grading for different durations;
- grading with different types of graders, and
- recovery after grading.

In addition, the NRR assay detected significant differences between animals of different sizes in response to some of the above-mentioned stressors.

With the catecholamine analysis, oysters were observed to respond in a "pulse" pattern, indicating that this assay is more suitable for single stressor analysis. In comparison, when the NRR assay is applied, the oysters respond in both a "pulse" and an "accumulative" pattern depending on the nature of the stressor, indicating that the NRR test can be applied to assess both single and multiple stressors.

APPENDIX 1: INTELLECTUAL PROPERTY

N/A

APPENDIX 2: STAFF

A1. Project staff

Dr Xiaoxu Li	South Australian Research and Development Institute, SA
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A2. Project steering committee

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APPENDIX 3: PACIFIC OYSTER FARM SURVEY

A3.1. Abstract

An oyster mortality survey of 24 oyster farms in South Australia (SA) found that farmers from Coffin Bay and Cowell had not experienced any unusual oyster mortality events whilst some Denial and Smoky Bay farmers had. Some farmers reported observing higher mortalities in family lines compared with standard commercial stock. Many farmers thought that mortalities mainly occurred at the spat stage. The percentage of spat farmers expected to reach saleable size ranged from 50 to 95%. Most farmers thought that low mortality also occurred in adult stock with losses at each grade estimated to be between 1-2%. From the survey it was apparent that most farmers attributed mortalities to stock management and stress related issues, including grading, air exposure, high air and water temperatures, and the reproductive cycle of the oysters.

A3.2. Introduction

As an initial part of the project, industry based surveys were conducted to assess the occurrence and extent of Pacific oyster mortalities within SA. This information was compiled to help direct research into specific areas concerning SA oyster mortalities. Three industry surveys were conducted to outline the various issues regarding oyster mortality, quality and health. Results are presented in this and the next two chapters. Names and specific details regarding farmers and processor/wholesalers have been omitted for confidentiality reasons.

The first survey was a farm-based survey used to collect general information from oyster farmers regarding previously recorded mortality events and current farm management techniques used to reduce mortalities. Farmers from four bays around SA were surveyed to incorporate the possibility of regional based mortality issues.

The survey form was developed in consultatation with the Project Steering Committee that consisted of industry representitatives and experts in the areas of animal health, animal physiology, shellfish aquaculture, shellfish nutrition and genetics.

During the week of September 22nd-26th 2003, as many oyster farmers as possible in Denial, Smoky and Coffin Bay and Cowell (Fig. A3.1) were visited and asked to fill out a survey form regarding oyster mortalities. The questions asked included - had they experienced any unusual mortality events in previous years as well as what percentage of spat they expected to reach saleable size. In addition, they were asked about the type of farming system they used, the type of grader they had, the number of times they graded as well as other questions relating to their farming practices.

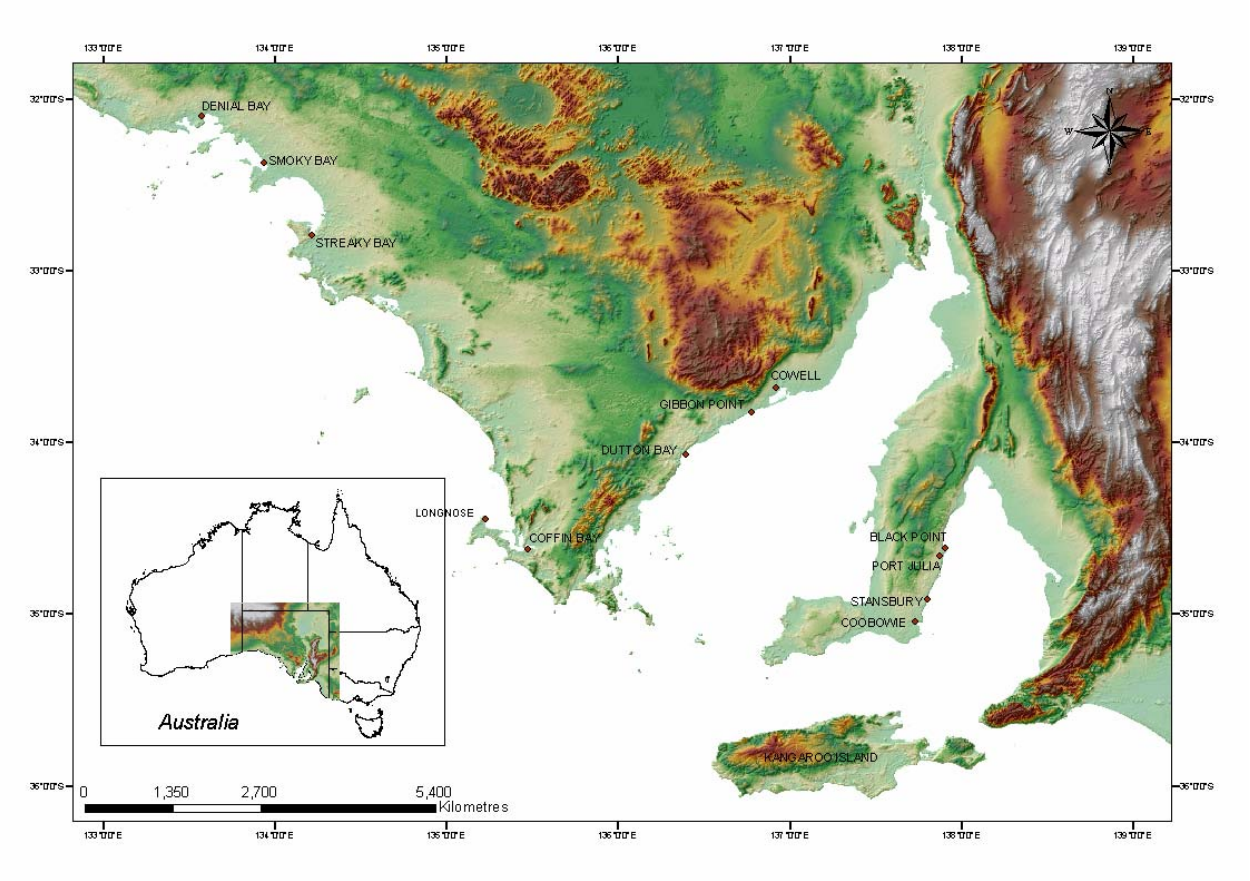

Fig. A3.1. A map of South Australia indicating the localities where samples and information were collected.

A total of 18 farmers were visited personally; four each from Smoky, Denial and Coffin Bay and six from Cowell. In addition, a mini survey was handed out at the Annual Oyster Growers Meeting held in Cowell which six farmers filled out.

A3.3. Summarized survey findings

The following information has been compiled into geographic location and the farms surveyed within each location. All information obtained from the farm has been summarized to present the key findings emphasized by the farmers.

Tables A3.1 and A3.2 were compiled from the key findings from the farm survey. Table A3.1 contains information relating to specific farming practices and observed mortalities. Table A3.2 lists the specific mortality events as documented by SA oyster farmers.

A3.3.1. Location 1: Smoky Bay

Farm: A

Farm A reported an 80% survival of spat to saleable size. This farm has experienced periods of increased oyster mortalities that occur in cycles.

Their comments were:

- Mortalities have been higher in years when there have been higher levels of food (microalgae) and oysters are growing fast.
- The mortalities occur in all size ranges (small oysters to saleable size).
- No blisters were observed in dead oysters, therefore there doesn't seem to be an association between mudworm and mortalities.
- In years when oysters haven't come back into condition as fast, less mortalities have been observed.

Farm: B

Farm B reported a 50% survival of spat to saleable size.

Their comments were:

- Deaths occur consistently from year to year and across all size classes (spat trays to saleable oysters).
- There are 3 sources of mortality: flatworm deaths, deaths from grading and deaths caused by elevated temperature.
- They stop grading in the 1st week of December until the end of February as the majority of the mortalities occur when the oysters are graded when full of spawn.
- They try not to handle their oysters at all during summer.

Farm: C

Farm C reported on average a 60% survival of spat to saleable size.

Their comments were:

- They thought the mortalities could be attributed to flatworm, mudworm and heat stress.
- They don't grade above 32ºC.

Farm: D

Farm D reported a 60% survival of spat to saleable size.

Their comments were:

- Mortalities are consistent throughout the year, even when hand grading.

A3.3.2. Location 2: Denial Bay

Farm: A

Farm A reported an 85% survival of spat to saleable size.

Their comments were:

- Flatworm occurs all year round but is most prominent in May, June and July. If the baskets are kept clean the occurrence of flatworm is lower.
- From 1988-1995 deaths occurred in spat trays due to flatworm as stock was usually left for 6-8 weeks when growth was slow. Now grading occurs every 4-6 weeks rather than 8-10 weeks.
- They have observed no mortalities in oysters that have been graded and returned the same day as opposed to when oysters are graded in one day and returned the next.

Farm: B

Farm B reported a 73% survival of spat to saleable size.

Their comments were:

- If juveniles are graded one day and can't be returned till the following day they get mortalities, however if they are taken out one day, left overnight, graded the next morning and then returned that afternoon, they get no or fewer mortalities.
- They try not to leave their oysters out overnight after having been graded.

Farm: C

Farm C reported a 70% survival of spat to saleable size.

Their comments were:

- They believe that 20% of their losses occur pre 30 mm size (length) in spat trays. They believe that these juvenile losses may be a result of oysters getting washed out of baskets or eaten by crabs.
- They experience elevated mortalities in summer, more so in larger oysters.

Farm: D

Farm D reported a 60% survival of spat to saleable size.

Their comments were:

- They believe most of their losses are juveniles and that mortalities decrease with age.
- They believe that mudworm and flatworm account for 2% loss and that they get 2% loss at each grading due to handling.

A3.3.3. Location 3: Coffin Bay

Farm: A

Farm A reported a 73-77% survival of spat to saleable size.

Their comments were:

- For the past 2 years they haven't graded their oysters from the end of November till the 3rd week of February. Since this time they have only been experiencing 1% mortality at each grade where previously when they had graded over the summer months they experienced mortalities from 10-12%.
- They now zip tie their baskets for added security after having observed juvenile oysters falling out.
- They believe that height of the baskets in the water column has no effect on mudworm levels.

Farm: B

Farm B reported a 95% survival of spat to saleable size.

Their comments were:

- They experience greater mortality in oyster seed they have purchased that has been grown in upwellers compared with seed grown in the sea. For oysters grown in the sea they have observed virtually no deaths in the seed tray stage whereas they have observed 10% mortalities in the seed tray stage for oysters grown in upwellers.
- They only grade their oysters once from seed to sale, however they run much lower stocking densities (6,000 vs 20,000 in a seed tray).

Farm: C

Farm C reported a 70-80% survival of spat to saleable size.

Their comments were:

- They reported that they experience more deaths around the start of winter due to fouling of baskets and not grading as soon as possible resulting in increased flatworm levels.

Farm: D

Farm D reported a 90% survival of spat to saleable size.

Their comments were:

- They experience higher mortalities during summer.

A3.3.4. Location 4: Cowell

Farm: A

Farm A reported a 95% survival of spat to saleable size.

Their comments were:

- They reported that they experienced bad mudworm infestation in the past but now it is virtually gone. They attributed this to growing their oysters 20.3 cm higher on the BST lines and handling them more often. They felt mudworm was purely a management issue.

Farm: B

The comments from Farm B were:

- They believed 50% of the losses they had experienced occured at the juvenile stage, as they don't see dead adult oysters.
- If they purchase 5 mm spat instead of 2.24 and 2.28 mm their mortality drops significantly (they have been forced to purchase smaller spat in the past due to spat shortages).
- During summer this farm returns the oysters the same day that they were graded, however, during winter they leave them out of the water overnight.
- They did previously experience mortality problems due to mudworm but don't anymore. They attribute this to originally growing the oysters too low, having too many in a basket and not handling them enough. To get rid of mudworm they grew their oysters at a higher level and reduced the number they stock in a basket. They also clean their baskets each time they are brought in for grading.

Farm: C

Farm C reported a 70% survival of spat to saleable size.

Their comments were:

- They occasionally get mortalities due to heat kills when there is a very hot day and a dodge tide.
- They believe that the majority of their mortalities occur between 25-60 mm when grading. After 60 mm they observe < 1% mortality at each grade.
- 1-2% of deaths occur in adult oysters transferred from Coffin Bay to Cowell.

Farm: D

Farm D reported an 80-85% survival of spat to saleable size.

Their comments were:

- They mainly experience mudworm in summer due to lowering oyster baskets to protect the oysters from heat. They believe mudworm occurs when you don't move the baskets up and down. They elevate their baskets during winter to prevent mudworm.
- To prevent mortalities during summer they try and return oysters to the water the same day they were graded and they don't bring oysters in when the temperature is above 35ºC.

Farm: E

Farm E reported an 85-90% survival of spat to saleable size.

Their comments were:

- They estimate they throw out 2% of their stock due to slow growth.
- Less than 1% of oysters are found dead at each grade.
- They don't grade above 38ºC.

Farm: F

Farm F reported that the survival of their spat to saleable size was above 80%.

Their comments were:

- They reported that greater losses occur when they transferred their oysters from Coffin Bay to Cowell than vice versa. The explanation they offered for this was that oysters grown in Coffin Bay have a weaker, more fragile shell possibly as a result of them growing faster. The consequences of this being that the shells of the oysters from Coffin Bay are more easily chipped and have a looser seal and thus are more prone to losing their water and drying out. They also reported that the muscles in oysters from Coffin Bay are much weaker than those from Cowell.
- They throw out between 5 and 7% of their oysters due to slow growth and odd shapes.
- They predict that 10% of oysters are lost through escaping out of the baskets.
- They previously graded each batch of oysters six times but now only grade four times. They have been able to do this by reducing the stocking density and through line height management.

Table A3.1. Farming practices used by the South Australian Pacific oyster industry

Bay	System currently used	Grading technique	No of grades per batch of oysters	Average time taken to reach saleable size (months)	Estimate of seed that reach saleable size (%)	Estimate of mortality found at each grade (%)	Freshwater dip for mudworm (Y/N/DN)
Coffin	BST + RR	Rotational	6	18	DN	2-5	DN
Coffin	Hybrid	Shaker	4-5	12-18	73-77	1	N
Coffin	BST	Rotational	6	12-18	90	DN	N
Coffin	BST	Rotational	3	12-18	70-80	DN	N
Coffin	RR + BST	Rotational	1	12	95	DN	N
Cowell	BST	Rotational	5-6	18	70	DN	DN
Cowell	BST	Both	6-10	12-24	80	< 3	DN
Cowell	BST	Rotational	4	15	80	DN	N
Cowell	BST	Shaking photo grader	4-5	15	85-90	< 1	N
Cowell	BST	Rotational	2-3	18	80-85	DN	N
Cowell	BST	Rotational	6-7	18	50-90	DN	N
Cowell	BST	Rotational	3	18	95	DN	N
Cowell	BST	Rotational	6	18	70	< 1	N
Denial	BST	Shaking photo grader	6-7	DN	85	DN	N
Denial	RR + BST	Shaker	DN	DN	70	DN	N
Denial	Hybrid	Shaking photo grader	8	DN	73	DN	N
Denial	RR + BST + hybrid	Rotational	8	DN	60	2	N
Smoky	Hybrid	Rotational	4	18-24	60-70	10	DN
Smoky	RR	Rotational	7	24	70	DN	N
Smoky	RR	Both	7	18-24	50	DN	N
Smoky	RR	Rotational	5-6	18-24	60	DN	N
Smoky	RR	Shaker	7-8	18-24	DN	DN	N
Smoky	RR	Rotational	6	18-24	60	DN	N
Stansbury	BST	Rotational	7	18-24	70	1-5	DN

BST = Adjustable long line system, RR = Rack and rail, DN = Don't know.

Table A3.2. Details of mortality events reported by different South Australian oyster farmers and their opinions on the suspected cause

Bay	Year	Months	Age of oysters	Condition of oysters at the time	Other comments provided by farmer	Estimated mortality level	Suspected cause
Smoky	1999	Jan, Feb, Apr, May, Nov, Dec	19 months +	Before & after spawning	High flatworm levels, faster than normal shell growth & fattening, good gonad development, average water temps	10%	DN
Smoky	2000	DN	19 months +	Prior to spawning	High flatworm levels, faster than normal shell growth & fattening, good gonad development, average water temps	10%	DN
Smoky	2001	DN	DN	DN	Don't know	DN	DN
Smoky	2002	August	13-18 months		In August more deaths occurred in a fast growth and non curl back line compared to control	5%	Genetic
Smoky	2001	August-December	All ages	Before & after spawning	Normal-mudworm & flatworm levels, water temp, shell growth, fattening rate and gonad development	50%	DN
Smoky	2002	August-December	All ages	Before & after spawning	Normal-mudworm & flatworm levels, water temp, shell growth, fattening rate and gonad development	50%	DN
Smoky	2003	May	7 month (8 & 12 mm)	NA	Fast growth line	22.8% of 12ml & 59% of 8ml	Genetic
Smoky	2002	Feb	7-12 months	NA	Low tide & high temps, all deaths found on 1 day	20%	Heat kill
Denial	2002	June & July	22 months	Prior to spawning	Normal-mudworm & flat worm levels, shell growth and gonad development, slower to fatten than usual	Up to 50%	Possibly overcrowding, oysters not as fat
Denial	2001	October/November	Adult stock	Prior to spawning	Normal oyster growth and condition	7-10%	Highly overstocked - stress?
Denial	2003	June	Adult stock	Prior to spawning		5%	Left oysters out of water for 1-2 nights, graded & then left out for another night - stress?
Denial	1988	DN	DN	DN		25%	Crab mortalities from using open top baskets

Denial	1989	Summer	25-50 mm	DN	Hot weather, no wind, low tides, temps up to 46ºC, 3 weeks above 30ºC, highest mortality in open top baskets	DN	Heat kill
Denial	1996/1997	DN	Adult stock	DN	Oysters kept down low & not checked, high level of flatworm and mudworm	20%	Mudworm and flatworm due to bad management
Denial	1997	DN	Adult stock	DN	Elevated oysters from 0.6 m to 0.9 m, oysters were a triploid line	2-3%	Stress due to management?
Denial	1997/1999	DN	All ages	DN	Algal bloom in bay, oysters weren't growing but had very good glycogen for that time of year, anyone grading at the time got worse levels of mortality	DN	Algal
Denial	2000/2001	Summer	Adult stock	DN	Under resourced and behind with grading coinciding with high summer & winter tides and calm summer conditions - bad mudworm and flatworm	36%	Mudworm and flatworm due to bad management
Denial	2001/2002	June & July	25-40 mm	Prior to spawning	Some 0ºC mornings & cool clear nights, higher level of mortality after mechanically grading	7%	Grading and cool temps?
Denial	2002/2003	DN	DN	DN	2 no curl back lines & 1 standard - treated in same way but standard line grew faster & had less mortality	DN	Genetic
Coffin Bay	1995	Jan, Feb, Dec	DN	DN		DN	Heat kills
Coffin Bay	1996	Jan, Feb, Dec	DN	DN		DN	Heat kills
Coffin Bay	DN	Summer months	30-40 mm (6 month)	DN	Deaths in triploid line only (60, 000 purchased)	30%	Genetic
Cowell	2003	July	8-12 mm	NA	Received 4mm oysters on 31st March & graded in July 2003, during grading high mortality in oysters graded into 12mm, some mortality for 8mm but none for those transferred back to 4 mm, oysters had a broader shell (only stock remaining in hatchery)	10% of 8 mm & 50% of 12 mm	Genetic

DN = Don't know, NA = Not applicable.

A3.4. Key findings from the Pacific oyster farm survey

One of the main points that was apparent from the farm survey was the large variability in the percentage of spat expected to reach saleable size. This ranged from 50 to 95% between farms and locations, suggesting that a better understanding of mortality levels in general could have a significant positive effect across the industry. Many farms thought that the majority of the losses within a given batch of oysters occurred at the spat (seed tray stage) and juvenile stage, however, obtaining a figure was difficult as no one had the time to count spat due to their small size and the large number that they receive.

In general, farmers in Coffin Bay and Cowell (Fig. A3.1) reported that they had not experienced any unusual oyster mortality events. In comparison, some incidences were reported by farmers with leases in Smoky Bay and Denial Bay (Fig. A3.1). In addition, the farmers from Coffin Bay and Cowell expect a higher percentage of spat to reach saleable size than those from Smoky Bay and Denial Bay. It should be mentioned, however, that the time for oysters to reach saleable size is faster for oysters grown in Coffin Bay (12 –18 months) than those grown in Smoky Bay and Denial Bay (18-24 months) and thus each set of oysters from Smoky Bay and Denial Bay is in general graded more times. Most farmers thought they experienced little mortality in adult stock, as they did not observe many dead shells during each grade. Estimates of percentage losses at each grade were around 1-2% but no one kept detailed records.

It was recorded that some farmers own leases in Coffin Bay and Cowell and transfer their oysters between the two locations, as the optimum growing times for each bay are complementary to one another. It was reported that greater losses occur when oysters are transferred from Coffin Bay to Cowell than vice versa. One explanation offered for this was that oysters grown in Coffin Bay have a weaker, more fragile shell possibly as a result of faster growth. The consequence of this is that the shells of the oysters from Coffin Bay are more easily chipped and thus have a reduced sealing ability making them more prone to losing water and drying out. It was also reported that the muscles in oysters from Coffin Bay are much weaker than those from Cowell.

Two farms that are holding fast growth genetic lines and one farm that has two non-curlback lines (both from the same hatchery in Tasmania) reported higher mortalities in these lines compared with their normal stock. In addition, two farms with triploid oysters from Tasmania reported higher mortalities in these oysters when compared with normal stock. Three farms reported that they had purchased rapid growth lines but had not yet graded these oysters so were unable to make comments about their mortality levels. Two other farms that did have family lines claimed that they had not observed any unusual mortality in these lines.

The majority of farmers bring their oysters in, grade them, and return them to the water the following day. Although all farmers would like to return them the same day they are unable to due to tides, so they try and return them to the water within 24 hours. Some farmers who are able to, return their oysters to the water the same day as they graded them during summer. One farmer reported bringing in the oysters the day before,

grading them the following morning, and then returning them to the water that day rather than grading them the afternoon before and leaving them out of the water all night after they had been graded. All farms stated that they no longer had a mudworm problem. Nearly all farms said that mudworm and flatworm related mortalities were the result of management issues. Nearly all farms suggested that mudworm and flatworm can be controlled by keeping their baskets clean, adjusting their levels in the water column and not overstocking. Many farms reported that they had tried mudworm treatments such as freshwater dips at least once in the past but none are doing so anymore.

In conclusion, the industry-based survey has produced a useful record of Pacific oyster mortalities within SA. One of the main points apparent from the survey is the large variability of observed mortalities between farms and locations. Some farms observed high levels of mortality whereas other farms observed little mortality. This indicates that the mortalities may be attributed to specific farm practices and the possible environmental variations between locations. It was apparent from the survey that farmers attributed the mortalities to stock management and stress related issues, including grading, air exposure, high air and water temperatures and the condition of the oyster. It was also suggested that the combination of these factors my have an added effect on oyster mortality. It is suggested that to help reduce the levels of mortality in SA these factors should be investigated.

APPENDIX 4: PACIFIC OYSTER PROCESSOR/WHOLESALER SURVEY

A4.1. Abstract

The main issues raised from the processor/wholesaler survey were spawny oysters during summer, mudworm infestation, and variability in size and condition throughout the year. Five out of eight processors/wholesalers stated that meat to shell ratio was the most important quality trait. The rest ranked uniform shell size as the most important quality trait. Four processors/wholesalers reported having observed abnormalities in a batch of oysters they had received during summer. Two processors/wholesalers reported have observed oysters with a dark or black spot in the white of their meat and an unusual smell.

A4.2. Introduction

It was suggested at the second Project Steering Committee meeting, August 2003, that in addition to the farm survey, it would be useful to conduct a survey of oyster processors to gain their perspective on oyster health and quality issues. The approach taken was that we were interested in product quality and wanting farmers to provide a better, more consistent product to processors. It was agreed that the following questions should be asked, although it was recognised that the more specific information sought may not be provided:

- What was the main complaint they had with the quality of the oysters they received and had this increased or decreased over the last 2-3 years?
- What oyster quality trait did they regard as most important from a marketing perspective?
- Had they noticed oysters with a strange odour (this was previously reported by some processors)?
- What other complaints/issues did they have with oysters they received?

A4.3. Summarized survey findings

A total of 8 processors were either visited or called during December 2003. The comments from each of these processors/wholesalers are provided below. Table A4.1 is a summary of the comments from the processors/wholesalers regarding the quality of oysters produced in SA.

Processor: 1

The main complaint expressed by Processor 1 regarding oyster product quality was the variability in shell size. This processor reported that farmers were putting 61 mm oysters in with 69 mm ones and suggested that farms needed to refine their grading practices. This processor also listed meat to shell ratio as a quality problem, but it depended on the time of the year with early or late in the year being the worst times in terms

of low meat to shell ratio. Processor 1 also believed that mudworm levels were not as much of a problem as they used to be, improvements having been observed over the last couple of years. With regard to the green oysters that are caused by algae in their gills and are in high demand in many countries such as Japan and France, Processor 1 stated that there is variability in what the market wants. Some people won't eat them while others like them. Some people like dark frilled oysters, others like light frilled oysters.

Although Processor 1 did not report having received strange smelling oysters they did report that they had observed oysters with a brown blister inside and a light brown pupil in the meat. This has been observed in oysters from several bays and the occurrence was suggested to be sporadic. Processor 1 believes it occurs when oysters are under stress. One event apparently occurred when a farmer brought his oysters out of water, graded them and left them out of the water for 36 hours, then put them back in the water. One to two days later they were then taken out and sent to the processor. Processor 1 believed that the oysters should have been left in the water for at least 10 days after grading.

According to Processor 1 2003 was a bad year for oyster quality in all bays. Processor 1 did say however, that over the last 5-7 years there has been a big improvement in the quality of oysters supplied by farms.

Processor: 2

The main complaint Processor 2 had regarding oyster quality was mudworm levels. According to Processor 2 this slowed processing and was therefore costly. Processor 2 believed that mudworm levels had increased over the last 2-3 years. The second main complaint this processor had was meat to shell ratio and the 3rd main complaint was variation in shell size. As with Processor 1, Processor 2 also said there was considerable variation in graded size. Other complaints mentioned included fouling, in particular white worm on oysters. Processor 2 said that from time to time they received batches of oysters that they believed were heat stressed. These oysters are characterised by a strange/off smell and a dark black spot in the white of the meat. Processor 2 stated that this abnormality had been observed in oysters from all bays. They also stated that on a few occasions they had received oysters that smelt bad and were heavily covered in white worm (*Galeolaira*-like) on the outside.

Processor: 3

The main complaint processor 3 had was with oysters being spawny. No other complaints were given regarding oyster quality. Processor 3 stated that they had received oysters that smelt strange, mainly in summer. The oysters were not gaping but were bluish in the middle of the white meat.

Processor: 4

The main complaint given by this processor regarding oysters they received was their spawny condition during summer. As for Processor 1 and 2 they also mentioned the non-uniformity of graded oysters and large variation in sizes provided. Processor 4 said they had not received oysters that smelt strange or were gaping.

Processor: 5

This processor didn't have any complaints other than the oysters being spawny during summer. They stated that they had never received oysters that smelt strange or were gaping.

Processor: 6

The main complaint that this processor had was that the oysters are spawny over summer. They had no other complaints. Processor 6 reported that they had not had any incidences of oysters smelling strange or gaping.

Processor: 7

The main complaint this processor had with oyster quality was that the oysters were sometimes spawny. No other complaints were given. Processor 7 said they had never received oysters that smelt strange or were gaping.

Processor: 8

The main complaint Processor 8 had with oyster quality was the substantial variation in the condition of oysters through different times of the year and from bay to bay. Processor 8 said that farmers want to produce fast growing oysters - bistros (50-60 mm in length) and plates (60-70 mm in length) rather than standards (70-85 mm in length) and larges (85-100 mm in length) so they have a faster turnover. By doing this they avoided getting mudworm (*Polydora* spp) and white worm (*Galeolaira*-like). However, he said that this is not what is required by international markets. Processor 8 said that he was approached by a Japanese buyer wanting supplies of South Australian oysters but he couldn't commit to a contract as South Australia does not produce oysters of a consistent quality and size as required by overseas markets. This processor feels that currently farmers do not care as much as they should about quality as they are able to sell everything they produce. However, he believes supply will soon exceed demand and farmers will have to start selling internationally. When this happens he suggested that they are going to have to pay a lot more attention to quality than they do now.

Processor 8 had received oysters that had a strange smell and were gaping. He reported that these incidences always occurred from October to December. He mentioned that he had complaints (December 2003) from 4 different consignments of oysters that he sent to Queensland (Townsville, Mackay, Sunshine Coast and Brisbane - about 31 bags in total). These 4 different consignments were independent of one another. Apparently the buyer complained that the oysters smelt off and were gaping. In addition to smelling unusual

and gaping they reported having observed a black spot in the meat of the oysters. Processor 8 said there are no consistencies in the reporting of these unusual smelling oyster incidences. In addition, oysters from other bays were on the same consignment (same transport) and did not have these problems.

Table A4.1. Summary of comments from oyster processors / wholesalers

Processor	Main oyster quality complaint	Has complaint increased/decreased or remained the same over last 2-3 years	Most important quality trait for marketing	Second most important quality trait for marketing
Processor 1	Variation in shell size	Remained the same	Meat: shell ratio	Colour/fatness
Processor 2	Mudworm	Increased	Meat: shell ratio	Shell size (uniformity)
Processor 3	Spawny oysters during summer	Remained the same	Shell size (uniformity)	Meat: shell ratio
Processor 4	Spawny oysters during summer	Remained the same	Shell size (uniformity)	Meat: shell ratio
Processor 5	No complaints	-	Meat: shell ratio	Shell size (uniformity)
Processor 6	Spawny oysters during summer	Remained the same	Meat: shell ratio	Colour/fatness
Processor 7	Spawny oysters during summer	Decreased	Meat: shell ratio	Colour/fatness
Processor 8	Variation in condition throughout the year	Remained the same	Quality and uniformity in shell size	-

A4.4. Key findings from the processor/wholesaler survey

In conducting this survey eight oyster processors/wholesalers were asked a series of questions regarding what they believed was the main issue with the quality of oysters they received, what they believed was the most important quality trait from a marketing perspective and if they had ever observed strange smelling or abnormal oysters. With regards to the main complaints processors/wholesalers had with the quality of oysters they received, four of the eight stated receiving spawny oysters during summer as their main concern. One of the processors/wholesalers stated mudworm as their main concern and believed this problem had increased over the last two years. Interestingly none of the other processors/wholesalers said mudworm was a problem anymore. Another of the processors/wholesalers had no complaints while another said that the huge variation in condition of the oysters throughout the year was their major complaint and a factor that would affect potential international sales. Non-uniformity/poor grading was a complaint raised by four of the processors/wholesalers with one stating that farmers were supplying too many small oysters (61 mm) in with

69 mm oysters. Five out of eight of the processors/wholesalers stated that meat to shell ratio was the most important quality trait from a marketing perspective. Interestingly the other three ranked uniform shell size as the most important quality trait.

Four of the processors/wholesalers reported having observed abnormalities in some of the oysters they have received. Apparently these incidences have a higher occurrence during the summer months. Two of the processors/wholesalers said they had observed oysters with a dark or black spot in the white of the meat and that the oysters smelt unusual. One of these said that it occurred in oysters from two bays only and felt it was a handling issue. The other stated that it had occurred mainly in one bay in previous years, but was mainly in another bay this year. This processor/wholesaler reported that they had an incident in December 2003 in which they received complaints about oysters smelling off and gaping as well as having a black spot in the meat. Oysters from several other farms in the same bay were in the same consignment but were apparently fine. Another of the processors/wholesalers said they had observed oysters from one bay with a bluish spot in the middle of the white meat. The oysters also smelt unusual but were not gaping. The fourth processor/wholesaler said they had observed oysters from several bays with a brown blister inside, like the oyster was rotting, and also with a light brown pupil in the meat. They believed this was a stress related issue.

APPENDIX 5: OYSTER HEALTH SURVEY

A5.1. Abstract

A total of 2,238 Pacific oysters from 16 sites in South Australia (SA) were collected and assessed. The pathological findings of the survey were: 1. The detection of microcell-like cells in vesicular connective tissue. 2. The abnormal hypertrophied nuclei with marginated chromatin in vesicular connective tissue cells. 3. Atrophy of digestive tubules. 4. A viral gametocytic hypertrophy-like lesion in the gonad. 5. Metaplastic changes of the digestive tubule epithelium (a lesion distinct from tubule atrophy). 6. Diapedesis through the gut epithelium. 7. Parasites and symbionts. 8. Rickettsia-like organisms. The health survey has helped to produce a pathology-based overview of the disease agents that currently occur in South Australia. This information will help to determine linkages between oyster mortalities and identified pathogens.

A5.2. Introduction

This survey was wholly funded and conducted by Primary Industries and Resources, South Australia (PIRSA) Aquaculture to produce a pathology based overview of the disease agents that currently occur in SA farmed oysters. The oyster samples were collected by PIRSA Fisheries compliance officers, mostly by Mr Benn Gramola, assisted on occasion by Dr Marty Deveney, PIRSA Aquaculture. The samples were processed and examined microscopically by Dr Ben Diggles (NIWA) under a contract between PIRSA Aquaculture and NIWA Australia Pty Ltd.

The final report (draft), from which this summary is exclusively derived, was written by Dr B Diggles (2003) and reviewed for scientific content by Dr M Hine.

It is expected that the result from this survey could be used to determine possible relationships between oyster mortalites and identified disease agents.

A total of 2,238 Pacific oysters were sampled from 16 sites in SA (Fig. A3.1): Coffin Bay, Longnose (Coffin Bay), Dutton Bay, West Streaky Bay, Gibbon Bank (Streaky Bay), Smoky Bay, Saddle Peak (Smoky Bay), Denial Bay North, Denial Bay South, Port Julia/Black Point, Coobowie, Stansbury, Cowell Bay Inner, Cowell Bay Outer, Kangaroo Island East and Kangaroo Island West.

A5.3. Summarized survey findings

The most significant pathological finding in the Pacific oysters examined was detection of low numbers of microcell-like cells (Figs. A5.1 & A5.2) in the vesicular connective tissue of oysters from 10 sites (Cowell Bay

inner & outer, Coffin Bay, Dutton Bay, Gibbon Bank, Kangaroo Island east and west, Longnose, Smoky Bay and West Streaky). The cells were between 2 and 4 μm in diameter with a nucleus around 1 μm in diameter. They were associated with focal or diffuse haemocytosis and were extracellular in most cases, though possibly intracellular in a few oysters. Overall prevalence of the microcell-like cells at the affected sites was 16.1%, and ranged from 66.4% at Longnose down to 3.3% at Cowell Bay inner and Smoky Bay. Infection intensity was low at all sites except Longnose, where 15 of 99 infected oysters had infections classed as moderate. Focal or diffuse haemocytosis was recorded at all sites and ranged from 10.7% (Coobowie) to 78.5% (Longnose) with an average prevalence of 32.6%. Prevalence of haemocytosis increased with increased prevalence of the microcell-like cells. Most haemocytosis occurred in the vesicular connective tissue (Fig. A5.3) but foci were also recorded in the mantle, gills, digestive gland, gonad and surrounding the gut.

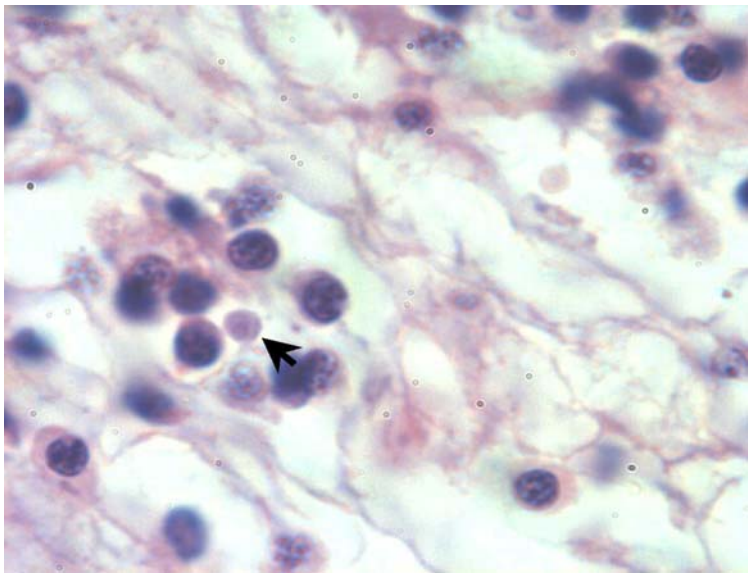

Fig. A5.1. Microcell-like cell (arrow) in vesicular connective tissue surrounded by host haemocytes in an oyster from Longnose. 1500x magnification.

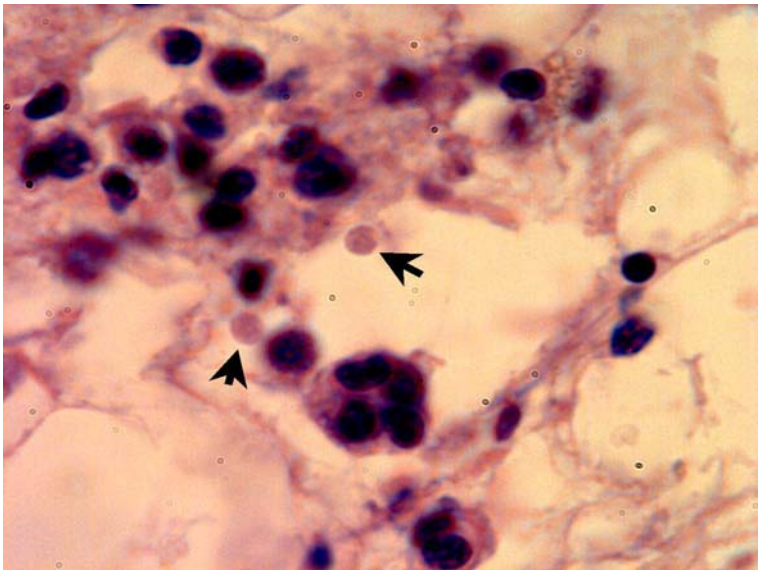

Fig. A5.2. Two extracellular microcell-like cells (arrows) adjacent to a focal area of haemocytosis in an oyster from Longnose. 1500x magnification.

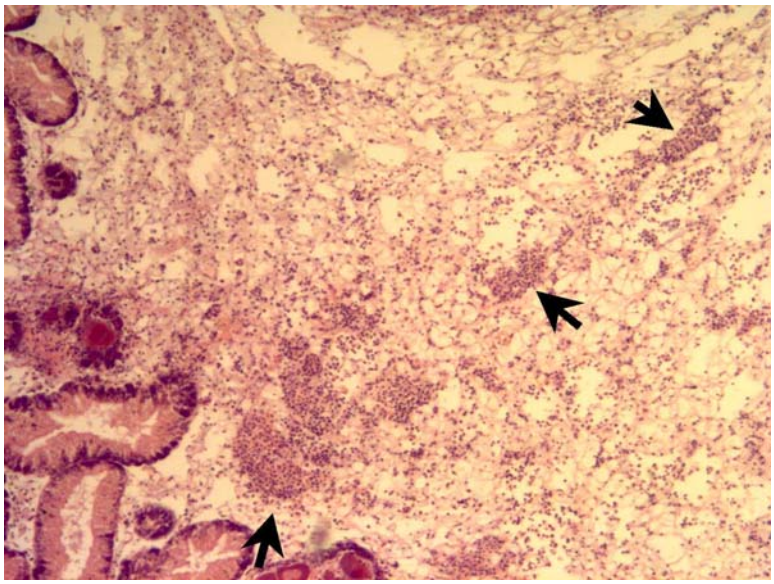

Fig. A5.3. Multiple foci of haemocytosis (arrows) in the vesicular connective tissue of an oyster from Longnose. 75x magnification.

Vesicular connective tissue cells with abnormal hypertrophied nuclei with marginated chromatin (Fig. A5.4), possibly due to infection by a virus, were evident in oysters from all sites (overall prevalence 34.3%), but particularly Coffin Bay (82.6% prevalence) and Dutton Bay (73.6% prevalence). Most of the oysters with high numbers of abnormal connective tissue nuclei (classed as moderate to heavily affected) were recorded from Coffin Bay (mean lesion intensity 1.69), West Streaky (mean intensity 1.68), and Cowell Bay inner (mean intensity 1.49).

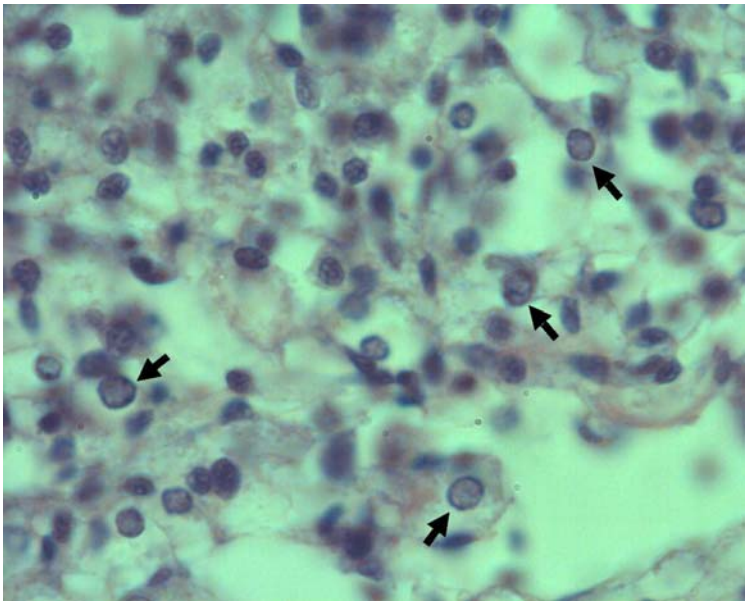

Fig. A5.4. Hypertrophied nuclei with marginated chromatin (arrows) in the connective tissues of an oyster from Denial Bay South. 1000x magnification.

Atrophy of digestive tubules (Fig. A5.5) was found at low to moderate prevalence in oysters from all sites (overall prevalence 22.1%, range 3.4-39.3%). Severity of tubule atrophy was greatest at Gibbon Bank, Port Julia/Black Point and Stansbury.

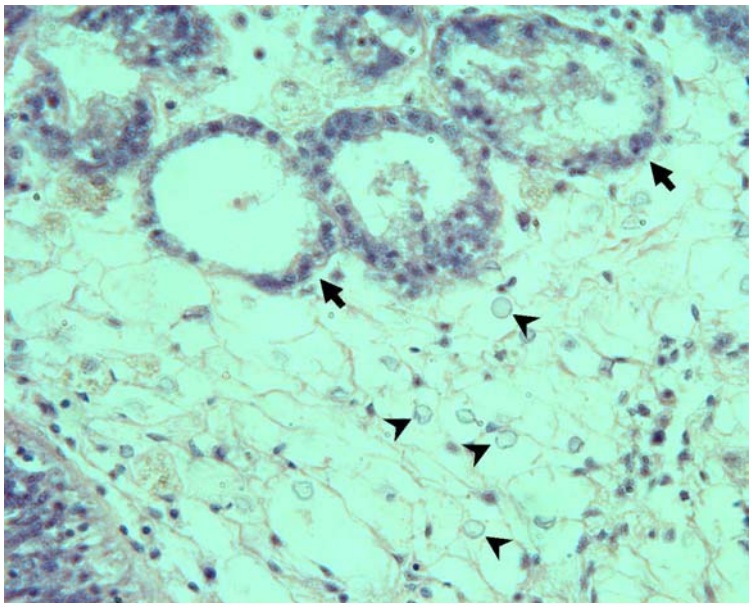

Fig. A5.5. Digestive tissue atrophy (arrows) in an oyster from site Port Julia/Black Point with numerous hypertrophied cell nuclei (arrowheads). 400x magnification.

A viral gametocytic hypertrophy-like lesion in the gonad of oysters from sites Gibbon Bank, Smoky Bay and Saddle Peak (prevalence 0.7%-1.4%) was characterized by massive hypertrophy of the nuclei of female gametes. The enlarged nuclei contained bizarre chromatin patterns (Fig. A5.6). Foci of non-specific necrosis were evident in oysters from all sites (overall prevalence 2.9%, range 0.7-9.3%).

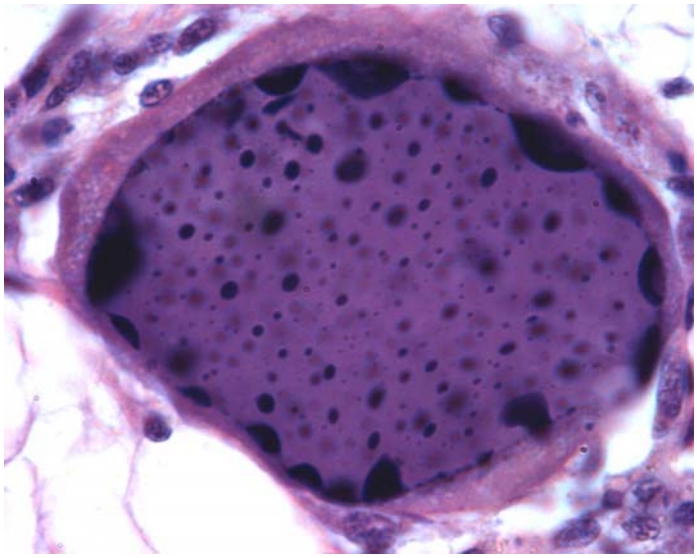

Fig. A5.6. Suspected viral gametocytic hypertrophy in the gonad of an oyster from Saddle Peak. Note bizarre chromatin patterns in the massively hypertrophied nucleus. 1000x magnification.

Metaplastic changes of the digestive tubule epithelium (a lesion distinct from tubule atrophy) were observed in one oyster from Dutton Bay, while bacterial infections were observed in oysters from Dutton Bay, Denial Bay North, Denial Bay South, and Saddle Peak (prevalence 0.7-1.3%).

Diapedesis through the gut epithelium (Fig. A5.7) was observed in oysters from all sites (overall prevalence 15.9%, range 1.3-70.7%), but occurred at particularly high prevalence (70.7%) and intensity (mean intensity 1.74) in oysters from Stansbury.

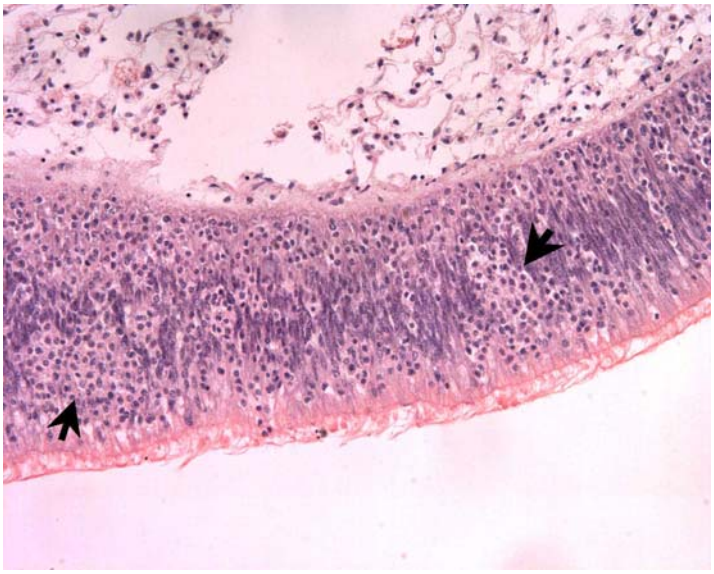

Fig. A5.7. Heavy diapedesis in the epithelium of the gut of an oyster from Stansbury. 200x magnification.

Parasites and symbionts found included *Pseudomyicola*-like copepods (Fig. A5.8) in the digestive tubules and encapsulated in host tissues (overall prevalence 5.2%, range 1.4-12.7%), *Ancistrocoma*-like ciliates in the lumen of digestive tubules (overall prevalence 17.4%, range 7.3-29.3%), and *Trichodina*-like ciliates externally in the gills and folds of the mantle (prevalence 0-8.7%).

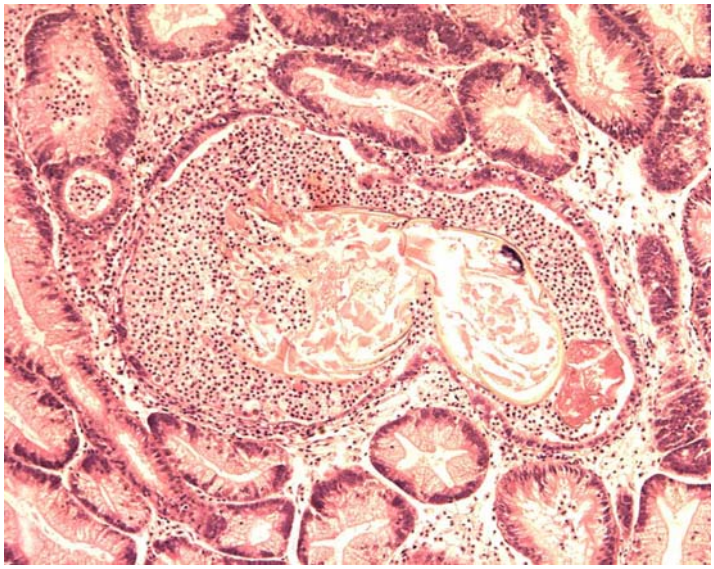

Fig. A5.8. *Pseudomyicola*-like copepod encapsulated in the connective tissue of an oyster from Smoky Bay. 100x magnification.

Rickettsia-like organisms (RLO's) (Fig. A5.9) were also present at low prevalence (overall prevalence 3.8%, range 0.7-6.7%) in and around the epithelium of the digestive tubules of oysters from all sites. Larger RLO inclusions were very occasionally observed in the gills.

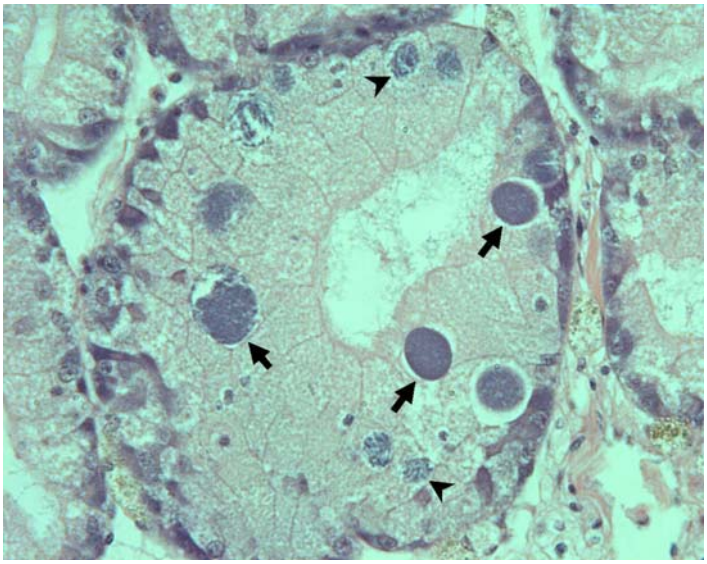

Fig. A5.9. Rickettsia-like organisms (RLOs) (arrows and arrowheads) in the digestive gland epithelium of an oyster from Port Julia/Black Point. 400x magnification.

A5.4. Discussion

The detection of microcell-like cells associated with areas of haemocytosis in oysters from 10 of the 16 sites sampled is significant as all microcell infections in molluscs are notifiable to the OIE Molluscan Reference Laboratory (OIE 2002). The preliminary detection of these organisms has been notified to the Office of the Chief Veterinary Officer (South Australia and Commonwealth). There are two described microcell genera, *Bonamia* and *Mikrocytos*. *Bonamia* spp. are primarily parasites of flat oysters, but have also been recorded in crassostreid oysters (Cochennec et al. 1998, Cochennec-Laureau et al. 2003). *Mikrocytos* spp. are parasites of crassostreid oysters and have been recorded from Pacific oysters (*M. mackini*, see Farley et al. 1988) and Sydney Rock Oysters (*Saccostrea glomerulata*), (as *M. roughleyi*, see Farley et al. 1988, but recently reclassified as *Bonamia roughleyi* (see Cochennec-Laureau et al. 2003)). *Mikrocytos mackini* is commonly found in vesicular connective tissue immediately adjacent to abscesses (foci of haemocytosis), while *B. roughleyi* and *Bonamia* spp. are most commonly intracellular within haemocytes (Hine and Wesney 1994, OIE 2000, B. Diggles, personal communication). On this basis the microcell-like cells found in the Pacific oysters from these samples appeared to possess characteristics more like those of *Mikrocytos mackini* rather than *Bonamia* spp., because the vast majority were extracellular. The microcell-like cells found in these samples were slightly larger (2-4 μm) than recorded for *B. roughleyi*, (1-4 μm, see Farley et al. 1988), but were very similar in size and appearance to *Bonamia exitiosus* when the latter are found in lightly infected New Zealand dredge oysters. In any case, due to the very small size of these cells (which at 1-4 μm are approaching the limit of resolution for light microscopy), any comparison at the light microscope level is probably meaningless at this stage. Definitive diagnosis for both *Bonamia* spp. and *Mikrocytos* spp. to satisfy OIE requirements is currently based on transmission electron microscopy (TEM) examination of the microcells. Molecular probes are available

(Adlard and Lester 1995, Cochennec et al. 2000, Carnegie et al. 2003, Diggles et al. 2003), but these have not been validated for southern hemisphere microcells and hence their use for diagnostic purposes is currently limited. This suggests that additional sampling is required to collect more material, preferentially from Longnose, Coffin Bay, specifically for TEM for initial attempts at a definitive diagnosis. At the same time, however, it would be advisable to collect samples for molecular analysis so these could be analysed at a later date. One potential problem with collecting additional samples for TEM is the low intensity of infection in the oysters examined in this sample. Even at the site with the highest prevalence of microcell-like cells (Longnose), of the 99 oysters that were recorded as infected only 15 (15%) had infections that were classified as moderate. Infected oysters from all other sites were classified as lightly infected. This is not surprising as these sites were surveyed in the absence of clinical disease, suggesting that the oysters with microcell-like cells are perhaps reservoir hosts or carriers of these cells, as suggested for *C. gigas* by Bower et al. (1994). It is considered unlikely that microcells could be visualised by TEM in lightly infected oysters, and the chances of detecting microcells by TEM in moderately infected oysters may be only slightly higher. This is because microcells are usually detected by TEM only in diseased, heavily infected oysters (M. Hine, personal communication). This suggests that before additional samples are taken for TEM, the oysters should be stressed in a quarantine facility to try and increase infection intensity, perhaps by overcrowding and/or increase in water temperature (which can promote the course of disease in the case of *Bonamia exitiosus* (see Hine et al. 2002)), and/or a decrease in water temperature, which promotes disease in the case of *Mikrocytos mackini* (see Hervio et al. 1996) and *B. roughleyi* (see Farley et al. 1988). Alternatively, some published techniques for isolating and purifying microcells (Mialhe et al. 1988, Hervio et al. 1996, Joly et al. 2001) could be utilised to attempt to obtain material for ultrastructural and/or molecular analysis.

The hypertrophied nuclei with marginated chromatin observed in vesicular connective tissue cells of oysters from all sites were almost identical to lesions described by previous workers in *C. gigas* infected by herpes-like viruses (Hine et al. 1992, Renault et al. 2000, 2001). Additional work would be required using TEM and/or molecular techniques to determine whether the cell abnormalities in these oysters were caused by a viral infection. Many of the oysters with these nuclear abnormalities were in poor condition with marked digestive tubule atrophy, suggesting they may have been diseased. However, high numbers of unusual cell nuclei in poorly conditioned oysters may not necessarily be due to infection by a viral disease agent. It is also possible these lesions could be associated with unfavourable environmental conditions such as lack of food and/or very high water temperatures. However, because of the apparent association between the nuclear lesions and poor condition, the possibility of a viral agent must be ruled out before the lesion can be attributed to an environmental cause.

The copepod and ciliate parasites found here are common symbionts of healthy oysters (McGladdery et al. 1993) and are of little pathological significance. The RLOs were present at low prevalence (0.7-6.7%) and intensity in oysters from all sites. RLOs and other related intracytoplasmic bacteria are probably ubiquitous in marine bivalves (Hine and Diggles 2002). Usually they occur at low intensities, as in the present study, and are

not associated with disease. However, if the host becomes stressed, due to factors that may include unfavourable environmental conditions or metabolic imbalances post-spawning, the RLOs can proliferate and may cause disease (Hine and Diggles 2002). Diapedesis (migration of haemocytes across epithelia) was commonly observed in the gut epithelium. Diapedesis is a normal metabolic process in oysters, and is used to remove harmful elements or metabolic by products, as well as parasites such as *Bonamia* (see McGladdery et al. 1993). However, diapedesis also occurs in healthy oysters, and hence increased prevalence and intensity of diapedesis at some sites may not necessarily be related to the presence of disease agents or pollutants. Focal areas of cell necrosis were also encountered at all sites and appeared to occur in the absence of obvious disease agents in most cases (non-specific necrosis). In very rare cases bacterial infections were associated with areas of necrosis and haemocytosis, particularly in small, poorly conditioned oysters. Due to their low prevalence and intensity of the bacterial infections observed, it is assumed they resulted from the poor condition of these oysters (i.e., secondary infections), rather than the alternative of them playing a direct causative role in a disease process.

Viral gametocytic hypertrophy (VGH) due to a *Papillomavirus*-like papovirus has been recorded in both the male and female gonads of *Crassostrea virginica* (see McGladdery et al. 1993) and *C. gigas* (see Farley 1985). In *C. virginica* the virus causes hypertrophy of gametes and germinal epithelium, but intensity of infection is usually low and the infection is not associated with mortality or reduced fecundity (McGladdery et al. 1993). The nuclear changes observed in *C. gigas* in the present study were very suggestive of viral infection, however viral involvement would again require confirmation by TEM. In any case, given the viruses involved with VGH are apparently ubiquitous, and that they appear to have minimal adverse effects on oyster health, prevention of spread of such viruses by restriction of oyster movements appears unnecessary (McGladdery et al. 1993).

A5.5. References

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APPENDIX 6: EFFECTS OF WATER TEMPERATURE AND AIR EXPOSURE ON THE LYSOSOMAL MEMBRANE STABILITY OF HEMOCYTES IN PACIFIC OYSTERS, *CRASSOSTREA GIGAS* (THUNBERG)

This chapter published as:

Zhang, Z., Li, X., Vandepeer, M., Zhao. W, 2006. Effects of Water Temperature and Air Exposure on the Lysosomal Membrane Stability of Hemocytes in Pacific Oysters, *Crassostrea gigas* (Thunberg). Aquaculture 256, 502-509.

A6.1. Abstract

Neutral red retention (NRR) assay was used to evaluate lysosomal membrane integrity in hemolymph from Pacific oysters subjected to rapid and gradual water temperature changes and exposure to different air temperatures. Recovery of the lysosomal membrane after air exposure was also evaluated.

The NRR times of oysters held in 5, 15 and 25°C seawater for 7 days were 60.0 ± 0.0, 145 ± 5.0, and 66.3 ± 8.5 min, respectively. The rapid water temperature change experiment showed that the recovery of lysosomal membrane integrity took longer than destabilization in Pacific oysters. The gradual temperature change experiment indicated that 13 to 17°C was the optimal water temperature range for maintaining lysosomal membrane integrity. Further assays showed that the NRR time at 5, 15 and 25°C was not influenced by the speed at which the temperature was changed.

The results from the air exposure experiment suggest that within the first 1.5 hrs the integrity of the lysosomal membrane was mainly affected by air temperature. From 3 hrs onward both exposure duration and air temperature affected the integrity of lysosomal membrane. Higher air temperatures resulted in shorter NRR times than lower temperatures did at every time point after 12 hrs. During recovery in 15°C water after 72 hrs air exposure, significantly shorter time periods were required for NRR times to reach control levels in oysters exposed to 5°C than those exposed to 15 and 25°C, indicating that exposure to lower air temperatures caused less damage to lysosomal membranes in Pacific oysters.

Key words: Neutral red retention; Lysosomal membrane stability; Stress; Temperature; Air exposure; *Crassostrea gigas*

A6.2. Introduction

Pacific oyster mortality has become a recurrent problem in the world (Glude, 1975). The literature indicates that the oyster mortality might result from the effects of multiple factors or stressors, including infection, temperature changes, low dissolved oxygen levels, air exposure, mechanical damage and other xenobiotic stressors, and the physiological stresses associated with reproduction (Glude, 1975; Perdue et al., 1981; Friedman et al., 1991; Shpigel et al., 1992; Cheney et al., 1998; Cho and Jeong, 2005; Zhang and Li, submitted).

In molluscs lysosomes play a cental role in innate defence (Cheng, 1981). They can release lysosomal enzymes, reactive oxygen species (ROS) (Cheng, 1983; Austin and Paynter, 1995), etc to destroy foreign invaders such as bacteria and viruses. However, many stressful conditions can destabilise the lysosomal membrane resulting in increased permeability to substrates (Moore, 1980). This response can be enhanced and lead to lysosomal membrane fusing making larger lysosomes, and can cause deleterious cellular effects (Lowe et al., 1981; Winston et al., 1996; Tremblay and Pellerin-Massicotte, 1997).

Lysosomal membrane stability has historically been assessed by lysosomal latency and NRR assay. The latency method determines alterations in the permeability of the lysosomal membrane to the passage of substrates from the cytosol into the lysosome (Moore, 1976; Schneider, 1984; Viarengo et al., 1995). The neutral red retention (NRR) assay (Lowe et al., 1995a, b) or neutral red uptake (NRU) assay (Borenfreund and Puerner, 1985; Chu et al., 2002), on the other hand, measures the speed of the efflux of the lysosomal contents into the cytosol (Lowe et al., 1995a, b). The two methods produced comparable results in an investigation into the effects of contaminants on mussels (Lowe et al.; 1995b). Because NRR assay is simpler, more convenient and more cost efficient, the method has been widely applied to evaluate the effects of environmental and physiological changes and mechanical stresses on molluscan species (Lowe and Pipe, 1994; Lowe et al., 1995a, b; Fernley et al., 2000; Grøsvik et al., 2000; Da Ros et al., 2002; Castro et al., 2004; Harding et al., 2004a, b; Zhang and Li, submitted).

Published NRR assay data on oysters indicates that the stability of the lysosomal membrane is associated with the stresses the animals are experiencing, such as temperature and salinity changes, spawning, bacterial inoculation, environmental contaminants, grading, etc (Hauton et al., 1998, 2001; Butler and Roesijadi, 2001; Ringwood et al., 2002; Cho and Jeong, 2005; Zhang and Li, submitted).

Temperature has been suggested to be the main environmental limiting factor for the wild production of Pacific oysters (Shatkin et al., 1997). Within certain water temperature ranges the growth rate in oysters increases (Askew, 1972; Dame, 1972; Malouf and Breese, 1978), with the highest growth rates at 15-19°C

(Walne, 1979). It has also been confirmed that 15°C is the most favoured temperature of *C. gigas* when lactate: succinate ratios or NRR times were used as indicators (Hauton et al., 2001; Laing et al., 2004).

The main objective of this study is to provide an integrated view of the response of lysosomes to rapid and gradual water temperature changes, and exposure to different air temperatures, using NRR time as the indicator. The recovery of lysosomal membrane stability after air exposure is also assessed.

A6.3. Materials and Methods

A6.3.1. Oysters

Two batches of Pacific oysters were imported from an oyster farm in Smoky Bay, South Australia and transported to South Australian Research and Development Institute's (SARDI) Aquatic Sciences Centre over night in a refrigerated container. On arrival the oysters were cleaned and put into a 500L tank on a flow through system (120 L h^{-1}) at 15°C, where the oysters were acclimated for 7 days prior to being used in the experiments. During this period the animals were fed with a mixed microalgal diet of *Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans*. The water salinity was kept at 37‰.

The first batch was imported in November 2004 (late spring in Australia) and used in the water temperature change and air exposure experiments while the second batch was imported in late January 2005 (summer in Australia) for the post-air exposure recovery experiments. Their height (mean ± S.E, n = 100 oysters), length (mean ± S.E, n = 100 oysters) and condition index (CI, mean ± S.E, n = 12 oysters) were 106.51 ± 1.00 mm, 51.90 ± 0.77 mm and 70.43 ± 1.92 and 101.59 ± 1.08 mm, 46.81 ± 0.61 mm and 73.93 ± 2.11 for the first and second batches, respectively.

A6.3.2. Condition index (CI)

The condition index was calculated using the equation used by Crosby and Gale (1990):

CI = [dry soft tissue weight (g) × 1000] / internal shell cavity capacity (g)

The dry soft tissue weight was obtained by drying the soft tissues individually at 60°C for 48 hrs. The shell cavity capacity was determined by subtracting dry shell weight (g) from the total whole live weight (g).

A6.3.3. Neutral red retention (NRR) assay

The Neutral red retention assay used in this study was modified from the methods used by Lowe et al. (1995a, b) and Hauton et al. (1998, 2001).

The neutral red stock solution was made by dissolving 2.28 mg of neutral red powder in 1 mL of dimethyl sulphoxide (DMSO). The working solution (0.04mg mL^{-1}) was prepared by diluting 17µL of the stock solution with 1 mL of oyster saline solution consisting of 0.48g L^{-1} $CaCl_2$, 1.45g L^{-1} $MgSO_4$, 2.18g L^{-1} $MgCl_2 \cdot 6H_2O$, 0.31g L^{-1} KCl, 11.61g L^{-1} NaCl, and 0.35g L^{-1} $NaHCO_3$ (Buchanan et al., 2001).

Each oyster used in the experiments was opened with an oyster knife, 0.2 mL hemolymph was drawn from the pericardial cavity into a 1 mL syringe. The oyster was then discarded. The hemolymph was then placed into a 2 mL siliconised Eppendorf® tube containing 0.2 mL of the oyster saline solution and gently mixed. The temperatures of the oyster saline solution and the Eppendorf tubes were adjusted to the temperatures the oysters were experiencing prior to sampling to minimise the possible impacts of sudden temperature change on the NRR assay. Four oysters were sampled each time and each sample was collected within 1 min.

A 40 µL mixture of hemolymph sample and oyster saline solution was placed onto a microscope slide that had been treated with poly-L-lysine (20 µL in 100 µL distilled water) to enhance cell adhesion. The slide was then suspended on a rack above ice water in a lightproof humidity chamber (a modified 20 kg Styrofoam box) to allow the blood cells to adhere to the slide (the temperature on the rack was 10°C). After 15 min the slides were removed from the rack and the excess hemolymph was poured off. A 20 µL neutral red working solution (10°C) was added to the cell layer and incubated in the humidity chamber for another 15 min. A cover slide (22 × 22 mm) was then put on the slide and the hemocytes were examined under a BX 60 Olympus compound microscope (× 600 magnification) using low light intensity. The slide was examined every 15 min for the first 60 min and then every 20 min. Twenty-five granulocytes were examined each time from an individual oyster. Once 50% of the hemocytes had started to lose dye from their lysosomes the assay was stopped and the time of the previous examination was recorded as the neutral red retention (NRR) time for that oyster. The observer was not informed of the origin of the sample under examination to minimise the possibility of a biased assessment.

A6.3.4. Effects of water temperature changes on stability of lysosomal membrane

A6.3.4.1. Rapid water temperature changes

Two experiments were conducted. In the 1st experiment the oysters that had been acclimated in a 15°C tank for 7 days were split into three groups. The first two groups were transferred into a 5°C and a 25°C tank respectively while the third was maintained in the 15°C tank as a common control for both experiments. In the 2nd experiment the oysters that had been held in 5 or 25°C water for 7 days were put directly into a 15°C tank. Hemolymph samples were collected at 0, 0.5, 1.5, 3, 6, 12, 24, 48, 72 hrs and then on days 5, 7 and 12 for temperature treatment groups. Control hemolymph samples were collected on each sampling day.

A6.3.4.2. Gradual water temperature changes

The temperatures used in the two rapid water temperature change experiments (5°C and 25°C, and 15°C) were also used as the final temperatures in the two experiments on the effects of gradual water temperature changes. In the gradual temperature change experiment, the water temperatures were raised or lowered by 2°C per day until the final temperature was reached. The hemolymph was sampled every day at 12 hrs after the water temperature required for that day was reached. Blood samples were also collected on days 2, 7 and 12 after the final water temperatures had been reached. On each sampling day oysters were taken directly from the 15ºC tank and used as a control.

A6.3.5. Effects of exposure to different air temperatures on NRR times

After acclimation in 15°C water for 7 days the oysters were exposed to 5, 15 and 25°C air temperatures directly. Hemolymph was sampled at 0, 0.5, 1.5, 3, 6, 12, 24, 48 and 72 hrs after the start of each air temperature treatment.

A6.3.6. Recovery after air exposure

After exposure to 5, 15 and 25°C air temperatures for 72 hrs the oysters were put back into the 15°C tank to recover. Hemolymph was collected at 0, 0.5, 1.5, 3, 6, 12, 24, 48 and 72 hrs, and then on days 5 and 7 in each treatment. The animals were considered dead when gaped.

A6.3.7. Statistical analysis of data

Two-way ANOVA, Tukey's *b* and Dunnett test multiple comparisons were used to defect significant differences between treatments after first checking for normality and homogeneity for the data (SPSS 10.0 statistical software). A probability level of $P < 0.05$ was considered statistically significant.

A6.4. Results

A6.4.1 Rapid water temperature changes

For the duration of the experiment the mean NRR times in the control oysters remained at approximately 145 min.

Within the first half hour of the rapid temperature change from 15°C to 5°C and 25°C the NRR times in both the 5°C and the 25°C groups dropped significantly from the initial level of 145.0 ± 5.0 min to 105.0 ± 5.0 min ($P < 0.05$. Fig. A6.1), and then down to about 65 min at 3 hrs. The NRR times then fluctuated at this level in both groups until the completion of the experiment.

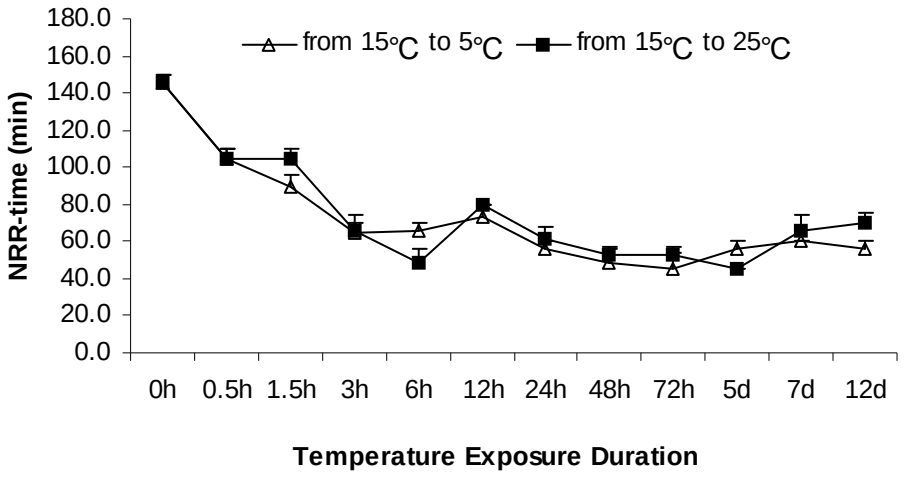


Fig. A6.1. Comparison of NRR times in lysosomes of *C. gigas* with rapid change in temperature from 15°C to 5°C and 25°C, respectively. Bars express the mean + S.E., n = 4 oysters.

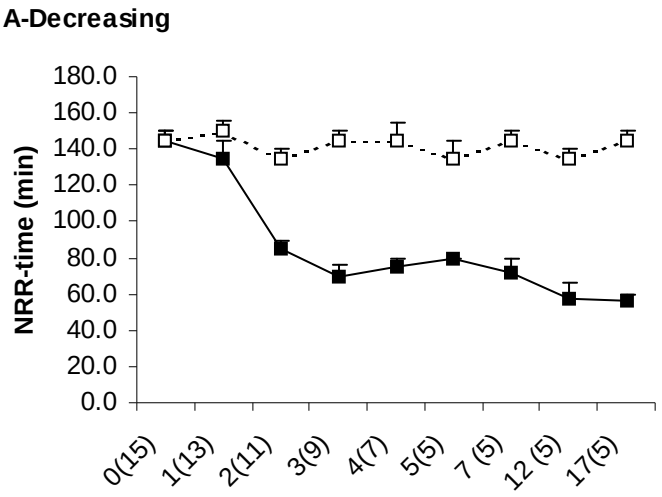
When the oysters were transferred directly from 5 and 25°C (where they had been acclimated for 7 days) to 15°C the NRR times increased gradually from 60 ± 0.0 to 115 ± 15.0 min and 66.3 ± 8.5 to 125 ± 5.0 min

within the first 12 hrs for the 5 and 25°C groups, respectively, and then increaseded slowly to approximately 145 min on day 7 and day 5 for these two groups respectively.

A6.4.2 Gradual water temperature changes

The NRR times in the control group remained at approximately 145 min for the duration of these experiments.

In the experiments where the water temperature was gradually lowered or raised by 2°C per day from 15°C to 5°C or 25°C the NRR times remained relatively unchanged in the 15-13°C and 15-17°C intervals and then dropped significantly at 11 and 19°C, respectively ($P < 0.05$). The NRR times decreased slightly thereafter and then fluctuated at 60 and 75 min in the 5°C and 25°C groups respectively until day 12 (Figs. 10.2A and B).



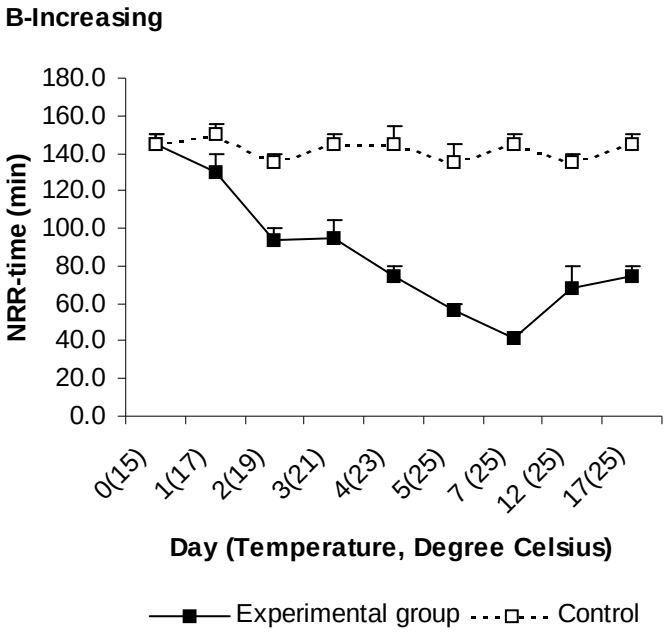


Fig. A6.2. Comparison of NRR times in lysosomes of *C. gigas* with gradual change in temperature: (A) decreasing from 15°C to 5°C, (B) increasing from 15°C to 25°C, (2°C per day). Bars express the mean + S.E., n = 4 oysters.

In the reverse experiments where the water temperatures changed gradually from 5 and 25°C to 15°C the NRR times in the 5 to 15°C group increased from 56.3 ± 3.8 min to the control level when the water temperature reached 13°C on day 4 and then fluctuated at this level until the end of the experiment on day 17. The NRR times in the 25 to 15°C group increased from 70.0 ± 5.8 min to the control level when the water temperature reached 17°C on day 4 and then remained at this level until the completion of the experiment on day 17. The temperature was changed to 15°C on day 6 in both experiments.

ANOVA analyses of the NRR values at 5, 15, and 25°C indicated that the lysosomal membrane stability at these final temperatures was not significantly affected by the rate at which water temperatures were changed in achieving those temperatures (gradually or rapidly) (P = 0.773, 0.721, and 0.888, respectively).

A6.4.3. Exposure to different air temperatures

Air temperature, air exposure duration and their interaction had significant effects on the stability of lysosomal membrane in oysters ($F_{3, 81}$ = 18.753, P < 0.001; $F_{6, 81}$ = 138.342, P < 0.001; $F_{18, 81}$ = 5.186, P < 0.001) (Fig. A6.3).

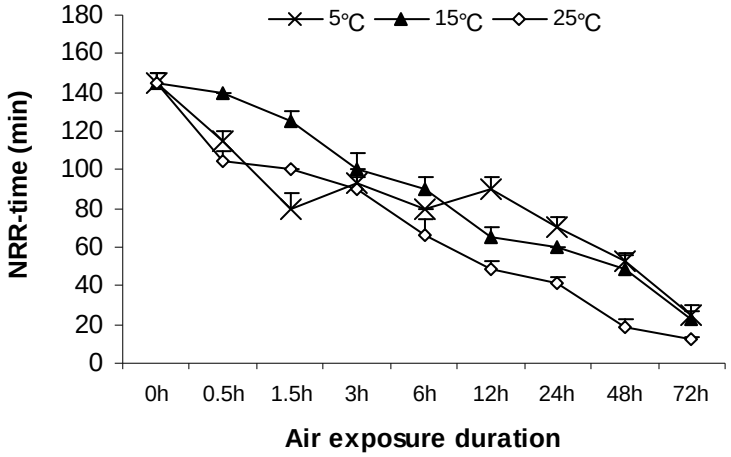


Fig. A6.3. Effects of exposure to different air temperatures on NRR times in lysosomes of *C. gigas*. Oysters were acclimated in 15°C tank. Bars express the mean + S.E., n = 4 oysters.

The NRR times in the oysters exposed to 15°C air temperature dropped gradually from 145 ± 5.0 (control) to 125.0 ± 5.0 min within the first 1.5 hrs and remained significantly longer than that in the 25°C group (100.0 ± 0.0 min), which was in turn significantly longer than the 5°C group (80.0 ± 8.2 min; $P < 0.05$). At 3 and 6 hrs the NRR times in all groups were similar. Exposure to 25°C resulted in the shortest NRR time of 12.5 ± 1.4 min at the completion of the experiment (72 hrs), which differed significantly from the 5 and 15°C groups at the same time (25.0 ± 5.0 and 22.5 ± 4.3 min, respectively ($P < 0.05$)).

A6.4.4. Recovery after air exposures

Air temperature and the re-immersion duration had a significant effect on lysosomal membrane stability in *C. gigas* ($F_{2, 96} = 19.422$, $P < 0.001$; $F_{10, 96} = 20.786$, $P < 0.001$). The lysosomal stability of oysters exposed to 5°C air temperature recovered much quicker than the oysters exposed to higher air temperatures (Fig. A6.4. $P < 0.05$).

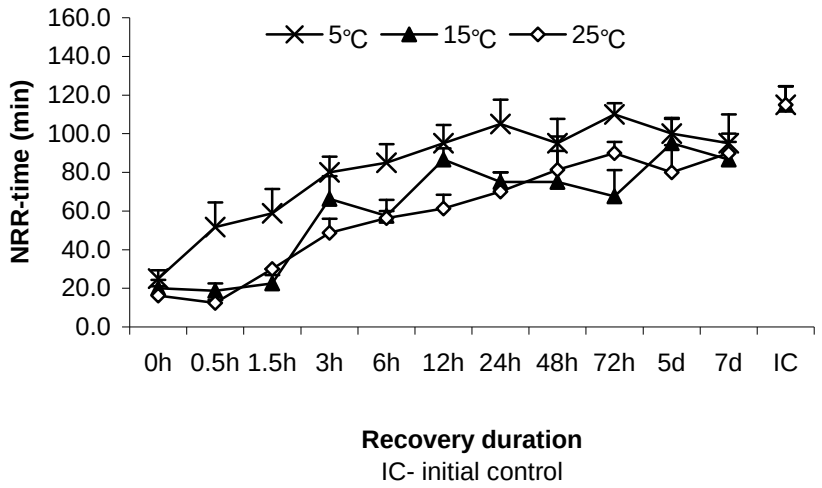


Fig. A6.4. Effects of re-immersion in 15°C water on NRR times in oysters after exposure to 5, 15, or 25°C air temperatures for 3 days. Oysters were originally acclimated in 15°C tank for 7 days. Bars express the mean + S.E., n = 4 oysters.

Within the first 24 hrs after re-immersion the NRR times in the 5°C air exposure group increased from 25.0 ± 4.3 min to 105.0 ± 12.6 min, which was not significantly different from the initial control of 115.0 ± 9.6 min ($P > 0.05$). The NRR times then fluctuated at this level until the completion of the experiment on day 7. The NRR times in the 15 and 25°C air exposure groups increased with time, but remained shorter than the NRR times of the 5°C group at every sample collection time point. In addition, 14% mortality occurred in the 25°C air temperature group during this period whereas no mortalities occurred in the other groups.

A6.5. Discussion

Neutral red is a lipophilic compound that can permeate the cell membrane freely (Lowe et al., 1992). Lysosomes in unstressed cells are largely impermeable to many substrates. However, when the cells are stressed the lysosomal membrane will be destabilized, resulting in increased permeability to substrates (Moore, 1980). As a weak cytotoxic compound neutral red is an additional stressor to the cells (Lowe et al., 1995a). In unstressed cells lysosomes will accumulate and retain the neutral red dye for an extended period of time. Once destabilized (stress response) the neutral red dye will leak into the cytosol of the cell through damaged membranes (Moore, 1980; Pipe, 1987; Lowe et al., 1995a, b).

Previous research into bivalves has shown that water temperature alteration and air exposure could lead to metabolic stress (Widdows and Shick, 1985; McMahon, 1988; Shpigel et al., 1992) and complex metabolic coordination was required to maintain cellular homeostasis associated with regulating energy producing pathways, changes in the total amount of enzyme activity, alterations in allosteric control of regular enzymes,

etc (Brooks and Storey, 1997). Lysosomes, as a major organelle that produces enzymes in bivalves, could be the site of the earliest detectable stress response (Harding et al., 2004a, b).

It can be seen from the rapid water temperature change experiments that when oysters were transferred from 15°C to 5°C and 25°C water the NRR times decreased to levels corresponding with 5 and 25°C water temperatures, within 3 hrs. This was substantially less than the 5 days required in the reverse experiment in which the oysters were transferred from 5 and 25°C to 15°C water. These results indicated that with a rapid water temperature change the recovery of the lysosomal membrane integrity took longer than its destabilization.

Our study of gradual water temperature changes from 15°C to 5 and 25°C showed that at 37‰ salinity (the normal salinity of South Australian seawater) 13-17°C was the optimal water temperature range for maintaining lysosomal membrane stability in Pacific oysters, although the NRR time at 15°C was slightly longer than those at 13 and 17°C. This result was similar to the finding of Hauton et al. (2001). Hauton et al. (2001) found that in *C. gigas* the lysosomal stability of hemocytes was maximal at 15°C in 32‰ salinity water. However, the NRR times at 15°C in this study (approximately 145 min) were longer than the results published by Hauton et al. (2001) at the same temperature (approximately 80 min). The discrepancy between these two results might be due to the differences in salinities used in these two studies, in the genetic makeup of the oysters, and in the condition of the oysters (Zhang and Li, 2006).

Our results also showed that the lysosomal membrane stabilities at the final temperatures (5, 15, and 25°C) were not affected by whether the water temperatures were changed gradually or rapidly.

Stress responses within the first 1.5 hrs of air exposure mainly reflected the change in temperature rather than the change from water to air. The NRR times of the oysters exposed to air temperature equivalent to the water temperature in which the oysters were acclimated (15°C) did not change significantly during this period, whereas the NRR times of the oysters exposed to 5 and 25°C air temperatures, on the other hand, decreased significantly. Studies by Qu (2004) showed that the body temperature of oysters exposed to different air temperatures dropped significantly within the first half hour, and reached the final air temperature in 1 hr. Research with wild cultivated mussels also showed that air temperatures within 5°C of ambient seawater temperatures had little additional effects on mussel stress response during their experimental length of 8 hrs (Harding et al., 2004b). Significant decline in NRR time in 15°C air exposure group at 3 hrs suggested that the stability of lysosomal membrane started to be affected by the combined effects of air exposure and temperature.

The results from the recovery after air exposure showed that the lysosomal stability in oysters exposed to 5°C air temperature recovered faster than the oysters exposed to the 15°C air temperature, which in turn recovered faster than the oysters exposed to 25°C air temperature for the same period. In addition, 14% mortality was recorded in the 25°C air exposure group whereas no mortality was found in the 5 and 15°C air exposure groups. The results from the recovery experiments confirmed the findings in the air exposure experiments that exposure to lower air temperatures caused less damage to lysosomal membranes in oysters.

In this study the NRR times of the initial control oysters (115 ± 9.6 min) used in the recovery after air exposure experiments (the second batch) were significantly lower than those oysters used in the other experiments (the first batch; 145 ± 5.0 min). This might be due to the animals in the two batches being of different age, size, genetic make-up, gametogenesis development stage or a combination of these factors. It was observed that the second batch was smaller than the first batch. The second batch was also obtained in January 2005, just prior to the natural spawning cycle in Smoky Bay. Bertheline et al. (2000) found that gametogenesis could result in a fragile physiological condition in Pacific oysters.

From this study and the study conducted by Zhang and Li (2006) it can be concluded that varying water temperatures, air exposure and some farming practices could impose strong stresses to oysters. Therefore, wherever possible minimising the effects from these activities could improve oysters' performances in the field and thus productivity.

A6.6. Acknowledgements

This work was partially supported by the Fisheries Research and Development Corporation. The authors are grateful to Mr Kriston Bott for his assistance in algal culture and experiment maintenance. We also thank Mr Gary Zippel of Zippel's Enterprises Pty Ltd for providing the oysters used in this study and the two anonymous reviewers of this manuscript.

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APPENDIX 7: DIFFERENCES IN RESPONSE OF TWO SIZE CLASSES OF PACIFIC OYSTERS, *CRASSOSTREA GIGAS* (THUNBERG) TO CHANGES IN WATER TEMPERATURE AND AIR EXPOSURE

This chapter submitted as:

Song, L., Li, X., Clarke, S., Wang, T., Bott, K. Differences in response of two size classes of Pacific oysters, *Crassostrea gigas* (Thunberg) to changes in water temperature and air exposure.

A7.1. Abstract

In this study the neutral red retention assay was used to investigate the effects of size and/or age on the response of Pacific oysters, *Crassostrea gigas,* to changes in water temperature and recovery after exposure to different air temperatures. Results from moving oysters directly between different water temperatures showed that differences in the NRR times between large and small oysters were not significant when they were transferred from 15°C to 5°C and 25°C and from 10°C to 20°C, but were significant when they were transferred from 5°C and 25°C to 15°C and from 20°C to 10°C. The air exposure experiments showed that after exposure to an air temperature of 5°C, 15°C or 25°C the lysosomal membrane integrity of the large oysters recovered at a slower rate than that of the small oysters in 15°C water. The lysosomal membrane stability in both size classes exposed to 5°C air temperature recovered at a faster rate than when exposed to higher air temperatures. Within the same size class the differences in NRR times between the 15°C and the 25°C air exposure were not significant.

Key words: Neutral red retention; Lysosomal membrane stability; Size; Temperature; Air exposure; *Crassostrea gigas*

A7.2. Introduction

The Pacific oyster, *Crassostrea gigas*, is the most commercially important oyster species cultivated and has been ranked second among the top ten most cultured aquatic species around the world (FAO, 2003). The attractiveness of this species for aquaculture is due to its high growth rate and high tolerance to temperature, salinity and sediment load (Shpigel and Blaylock, 1991). However, periodic mass mortalities have occurred in some farming regions (Cheney et al., 2000) resulting in severe economic losses. Previous research has shown that these mortalities could be caused by many internal and external factors and their combinations, which

may include sexual maturation, water temperature, dissolved oxygen level, water nutrient level, air exposure, mechanical damage, bacterial infestation and other xenobiotic factors (Lipovsky and Chew, 1972; Glude, 1975; Beattie et al., 1980; Perdue et al., 1981; Friedman et al., 1991; Shpigel et al., 1992; Cheney et al., 2000; Patrick et al., 2006). Therefore, it is critical to know how and when these factors could become detrimental.

Water temperature changes have been suggested as one of the main environmental factors affecting the distribution, growth, feeding, respiration and reproduction in many marine bivalves (James and Hartwick, 1988; Widdows and Bayne, 1971; Wieser, 1973; Bayne et al., 1976a, b; Newell and Branch, 1980; Shumway, 1982; Bayne and Newell, 1983), particularly for those species inhabiting shallow waters, such as Pacific oysters (Soletchnik et al., 2005). Within certain water temperature ranges the growth rate of oysters increases, with the highest rates at 15°C - 19°C (Walne, 1979). Water temperature also plays a key role in gonial mitosis regulation and maturation of germ cells (Fabioux et al., 2005).

Air exposure could lead to metabolic changes and imposes strong stresses on molluscs (Widdows and Shick, 1985; McMahon, 1988; Shpigel et al., 1992; Zhang et al., in press). These stresses could be further enlarged if general farming activities such as grading, washing and declumping were undertaken at the same time (Dare, 1974; Slabyj and Hinkle, 1976; Slabyj, 1980; Prochazka and Griffiths, 1991; Zhang and Li, 2006).

In Pacific oysters it has been noted that size/age may also influence the stress response of the animal. Wang et al. (accepted) demonstrated that smaller Pacific oysters recover more quickly from stress imposed by spawning when compared to larger animals, suggesting that size/age could play an important role in the ability of an animal to respond and recover from various stressors. Various authors have also noted similar relationship between size/age and stress response in other molluscs. In the blue mussel *Mytilus edulis* the response to environmental stressors such as low salinity, elevated temperature and air exposure are suggested to be related to size (Sukhoin et al., 2003). Wells and Baldwin (1995) demonstrated that the stress experienced by the two species of New Zealand abalone *Haliotis iris* and *H. australis* during air exposure was also related to animal size, with larger animals experiencing less stress in comparison to smaller individuals.

Previous studies have shown that the lysosome is the earliest site of detection for the response of bivalves to various stressors (Harding et al. 2004) and can be measured using the neutral red retention (NRR) assay (Lowe et al., 1995). When a stressor impairs the lysosomal membrane its capacity to retain neutral red dye in its compartments is reduced, thus losing the dye into the cytosol at a faster rate than non-stressed cells. Therefore the relative value of the NRR assay can be correlated to the overall stress of the animal (Harding et al. 2004).

The main objective of this study is to use the NRR assay to investigate the effects of size and/or age on the response of the lysosomal membrane in Pacific oysters to changes in water temperature and recovery after exposure to different air temperatures.

A7.3. Materials and Methods

A7.3.1. Experimental animals

Two size classes of farmed Pacific oysters were provided by an oyster grower in Smoky Bay, South Australia and transported live to the South Australian Research and Development Institute's Aquatic Sciences Centre at West Beach, Adelaide, South Australia in a refrigerated container. The large oysters were approximately 24 months old and 103.21 ± 1.83 mm, 55.88 ± 1.98 mm, 38.80 ± 0.88 mm and 103.71 ± 7.39 g in height, length, width and whole wet weight respectively. The small oysters were approximately 14 months old and 52.65 ± 3.18 mm in height, 34.65 ± 1.10 mm in length, 24.76 ± 0.97 mm in width and 17.91 ± 1.60 g in whole wet weight (mean ± SE, n = 100 individuals). On arrival the oysters were cleaned and placed into a series of 450 L flow through tanks set at a temperature of 15°C for 7 days prior to being used in the experiments. During this period and the acclimation periods in the experiments the oysters were fed with a mixture of three microalgae species, *Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans*.

A7.3.2. Neutral red retention (NRR) assay

The NRR assay used in this study was the same as that used by Zhang et al. (2006), which was modified from the methods used by Lowe et al. (1995) and Hauton et al. (2001).

The neutral red stock solution was made by dissolving 2.28 mg of neutral red powder in 1 mL of dimethyl sulphoxide. The working solution (0.04 mg mL^{-1}) was prepared by diluting 17 µL of the stock solution with 1 mL of oyster saline solution (Buchanan et al., 2001).

Each oyster used in the experiments was opened with an oyster knife; 0.2 mL of hemolymph was then drawn from the pericardial cavity using a 1 mL syringe. The hemolymph was then placed into a 2 mL siliconised Eppendorf® tube containing 0.2 mL of the oyster saline solution and gently mixed. Three oysters per replicate (tank) per size group (small and large) were sampled each time and each sample was collected within 1 min.

A 40 µL mixture of hemolymph sample and oyster saline solution was placed onto a microscope slide pre-treated with poly-L-lysine (20 µL in 100 µL distilled water). The slide was then suspended on a rack above ice water in a lightproof humidity chamber to allow the blood cells to adhere to the slide. After 15 min the slides

were removed from the rack and the excess hemolymph was poured off. A 20 μL neutral red working solution was added to the cell layer and incubated in the humidity chamber for another 15 min. A cover slide was then put on the slide and the hemocytes were examined under a compound microscope (× 600 magnification). The slide was examined every 15 min for the first 60 min and then every 20 min. A total of 30 granulocytes per individual were examined at each time interval. Once 50% of the hemocytes had started to lose dye from their lysosomes the assay was stopped and the time for the previous examination was recorded as the NRR time. The observer was not informed of the origin of the sample under examination to minimise the possibility of a biased assessment.

A7.3.3. Effects of water temperature changes on lysosomal membrane stability

The experiment consisted of three treatments. In the first treatment the large and small oysters were acclimated in three 5°C or six 15°C 450 L tanks for 7 days. The oysters from the three 5°C tanks were then transferred to three 15°C tanks, whereas the oysters from three 15°C tanks were transferred to the three 5°C tanks. The oysters in the remaining three 15°C tanks were used as controls.

The experimental design for the second treatment was the same as the first apart from substitution of the three 5°C tanks with three 25°C tanks.

In the third treatment the large and small oysters were acclimated in three 10°C tanks, three 20°C tanks and three 15°C tanks for 7 days. The oysters in the three 10°C tanks were transferred to three 20°C tanks and vice versa. The oysters in the 15°C tanks were used as controls.

After being transferred into the new temperature tanks hemolymph samples were collected for NRR analysis at 0 hr, 0.5 hr, 1.5 hr, 3 hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr and 5 days post transferral. The hemolymph of the controls was collected on days l, 2, 3 and 5.

A7.3.4. Effects of air exposure on lysosomal membrane stability

After acclimation in 15°C water for 7 days three sets of oysters (each with 40 large and 40 small oysters) were exposed to 5°C air temperature for 72 hr and then placed into three 15°C water tanks. These procedures were repeated for 15°C and 25°C air temperatures. The un-exposed oysters in the three-acclimation tanks were used as controls. During the re-immersion period the hemolymph from the air-exposed oysters was collected at 0 hr, 0.5 hr, 1.5 hr, 3 hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr and 5 days post re-immersion. The hemolymph in the controls was sampled on days 1, 2, 3 and 5.

A7.3.5. Statistical analysis

All data was analysed using SPSS 13.0 software. ANOVA and Tukey's *b* multiple comparisons were used to determine differences between treatments after first testing for normality and homogeneity of the data. A probability level of $P < 0.05$ was considered statistically significant.

A7.4. Results

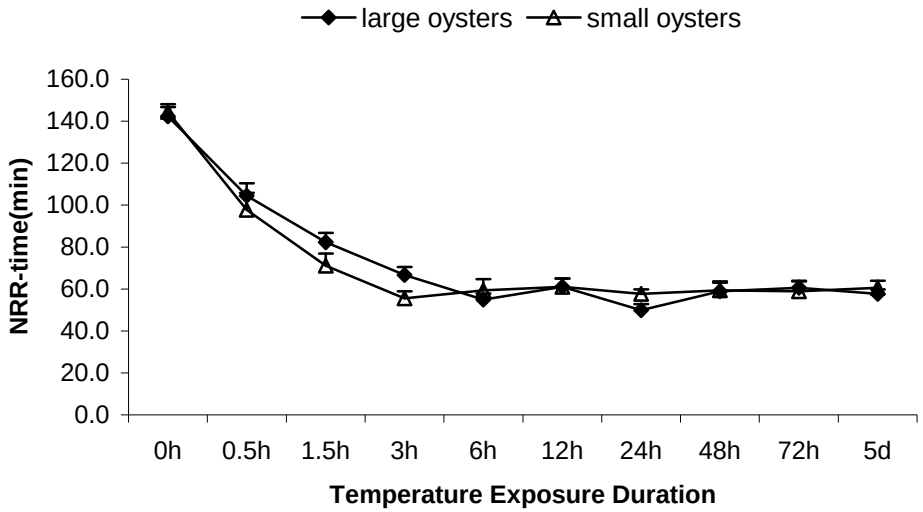
A7.4.1. Effects of water temperature changes on stability of lysosomal membrane

In this experiment the mean NRR times in the control oysters were similar at the different sampling times within each treatment, between treatments and between size classes ($P > 0.05$), remaining at approximately 140 min.

In the first treatment when the water temperature was changed directly from 15°C to 5°C (Fig. A7.1A) the NRR times of both the large and small oysters dropped significantly ($P < 0.05$ in both cases) from the initial level of 142.22 ± 5.88 min and 144.45 ± 2.22 min to 55.0 ± 2.89 min and 55.56 ± 3.38 min at 6 hr and 3 hr respectively. The NRR times then fluctuated around this level in both size classes until the end of the experiment ($P = 0.110$ and 0.460). The difference in the NRR times between the large and small oysters was not significant ($F_{1, 40} = 0.007$, $P = 0.933$).

With the reverse water temperature change from 5°C to 15°C, the NRR times (Fig. A7.1B) in the large and small oysters increased significantly ($P < 0.05$ in both cases) from 51.67 ± 1.67 min and 50.0 ± 2.89 min to 135.56 ± 4.44 min and 135.56 ± 2.22 min at 24 hr and 12 hr respectively. These times were not significantly different from the controls ($P = 0.259$ and 0.189, respectively). The NRR times then remained at this level until day 5. A significant difference in NRR times between the large and small oysters was observed ($F_{1, 40} = 4.351$, $P = 0.043$).

A



B

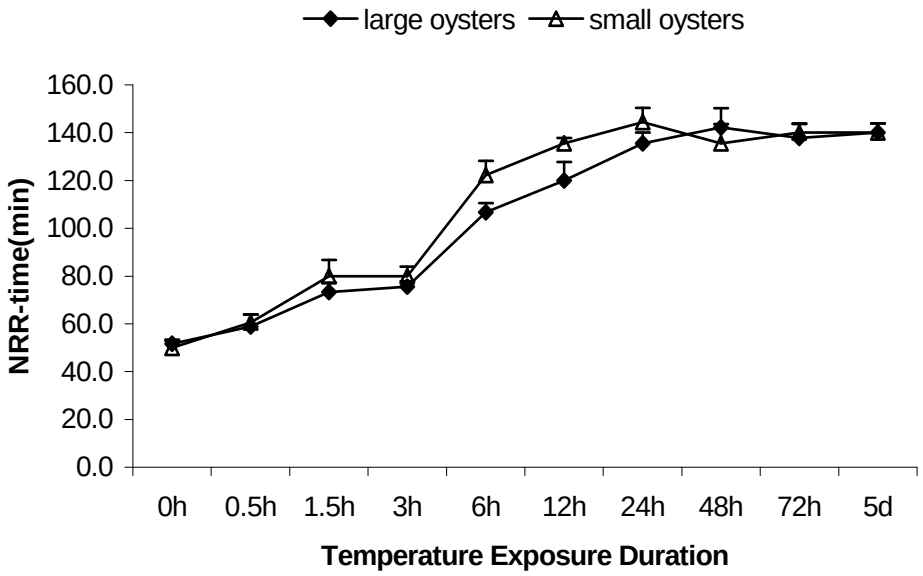
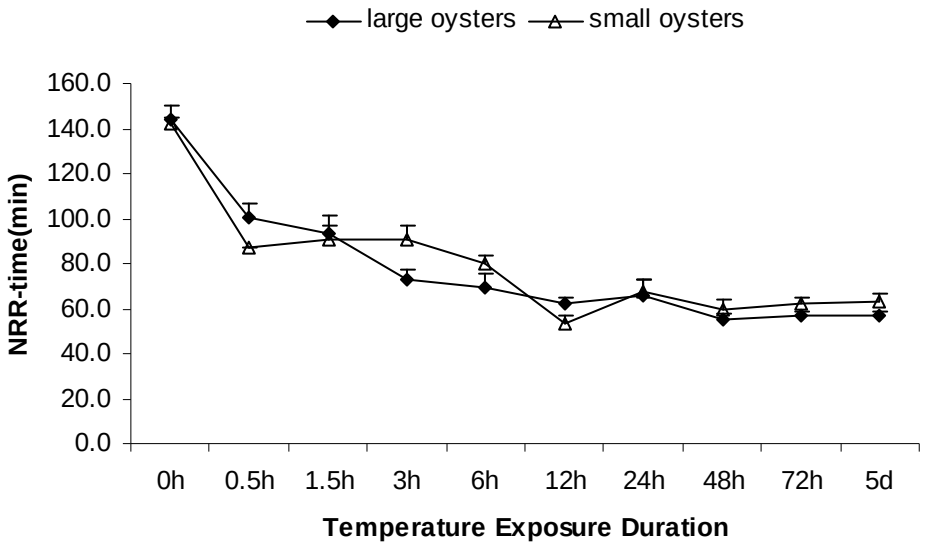


Fig. A7.1. Comparison of NRR time changes between large and small Pacific oysters after being transferred directly from 15°C to 5°C water (A) or from 5°C to 15°C water (B). Bars express the mean + S.E., n = 9 oysters.

In the second treatment when the large and small oysters were transferred directly from 15°C to 25°C the NRR times in the large oysters (Fig. A7.2A) declined significantly ($P < 0.001$) from 144.44 ± 5.88 min to 73.33 ± 3.85 min at 3 hr, followed by a fluctuation around this level until day 5 ($P = 0.307$), whereas the NRR times in small oysters decreased significantly ($P < 0.001$) from 142.22 ± 2.22 min to 53.33 ± 3.33 min at 12 hr and then fluctuated around this level until the completion of the experiment ($P = 0.166$). No significant

difference was found in NRR times between the large and small oysters ($F_{1, 40}$ = 0.806, P = 0.375).

A



B

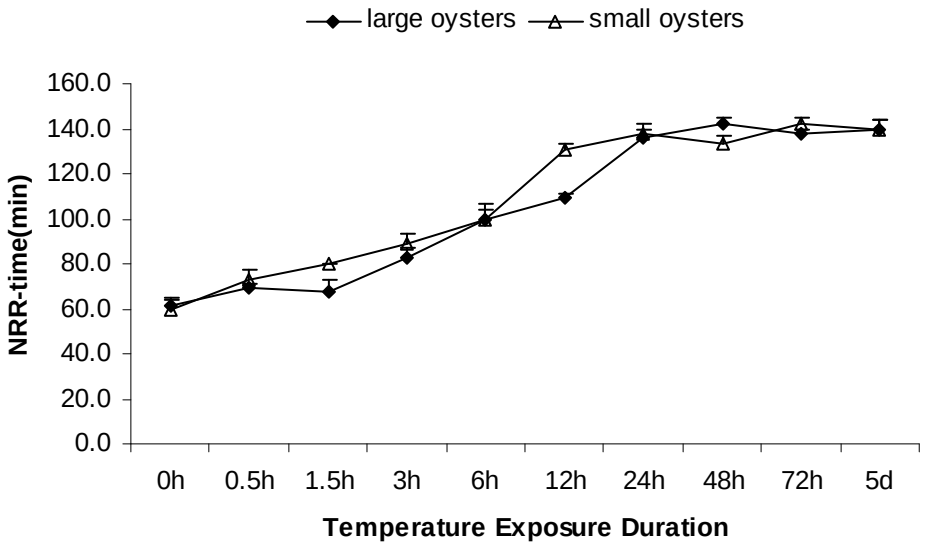


Fig. A7.2. Comparison of NRR time changes between large and small Pacific oysters after being transferred directly from 15°C to 25°C water (A) or from 25°C to 15°C water (B). Bars express the mean + S.E., n = 9 oysters.

In the reverse treatment when the water temperature was changed from 25°C to 15°C (Fig. A7.2B), the NRR times in the large animals only varied slightly within the first 1.5 hr from 61.67 ± 2.89 min to 67.22 ± 6.11 min (P = 0.219), and then climbed significantly (P < 0.001) to 135.56 ± 4.44 min. This retention time was not

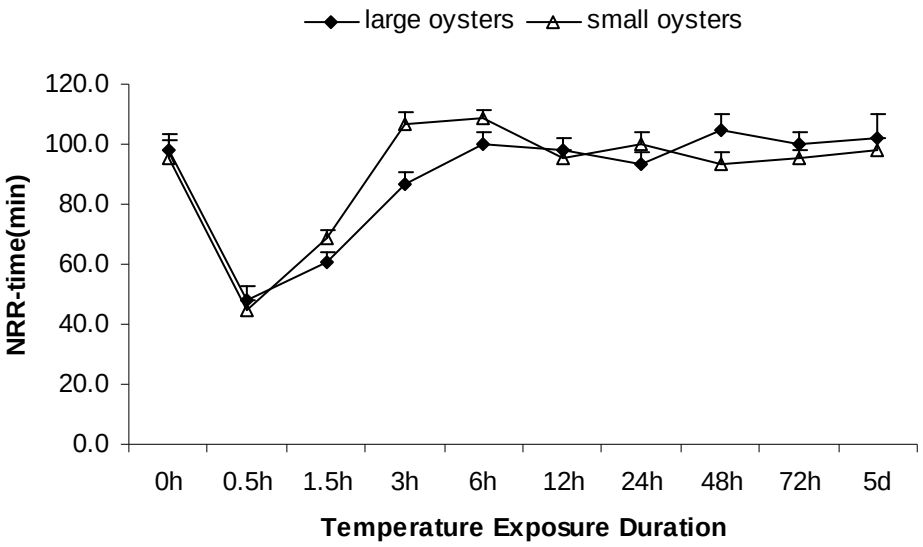
significantly different from that of the control group at 24 hr (P = 0.488). It then remained at this level until day 5. The NRR times in the small oysters gradually increased from 59.45 ± 4.94 min to 100.0 ± 3.85 min at 6 hr and then sharply increased to 137.78 ± 4.45 min, which was also similar (P = 0.759) to the control at 24 hr. It then fluctuated around this level until day 5. The difference in the NRR times between the two oyster size classes was significant ($F_{1, 40}$ = 5.731, P = 0.021).

When the water temperature was changed from 10°C to 20°C (Fig. A7.3A) in the third treatment the NRR times in both large and small oysters fell significantly (P < 0.05) within the first 0.5 hr, from 97.78 ± 5.88 min and 95.56 ± 5.88 min to 48.33 ± 4.41 min and 45.0 ± 2.89 min, respectively. The NRR times in the large oysters then gradually increased to 86.67 ± 3.85 min at 3 hr and kept gradually increasing until day 5 (P = 0.302). The NRR times in the small oysters increased significantly to 106.67 ± 3.85 min at 3 hr and then decreased slightly to 95.55 ± 2.22 min at 12 hr, followed by a fluctuation around this level until day 5 (P = 0.121). No significant difference in the NRR times was found between large and small oysters ($F_{1, 40}$ = 0.701, P = 0.407).

When the large and small oysters were transferred from 20°C to 10°C water (Fig. A7.3B), their NRR times dropped significantly (P < 0.05 in both cases) within the first 0.5 hr, from the initial level of 91.11 ± 2.22 min and 93.33 ± 3.85 min to 45.0 ± 2.89 min and 50.0 ± 2.89 min, and then increased back to 95.56 ± 8.01 min and 95.55 ± 2.22 min at 6 hr and 3 hr, respectively. The NRR times in both size classes then fluctuated around this level until day 5. A significant difference in NRR times was found between large and small oysters ($F_{1, 40}$ = 17.171, P < 0.001).

No mortality occurred in any of these treatments.

A



B

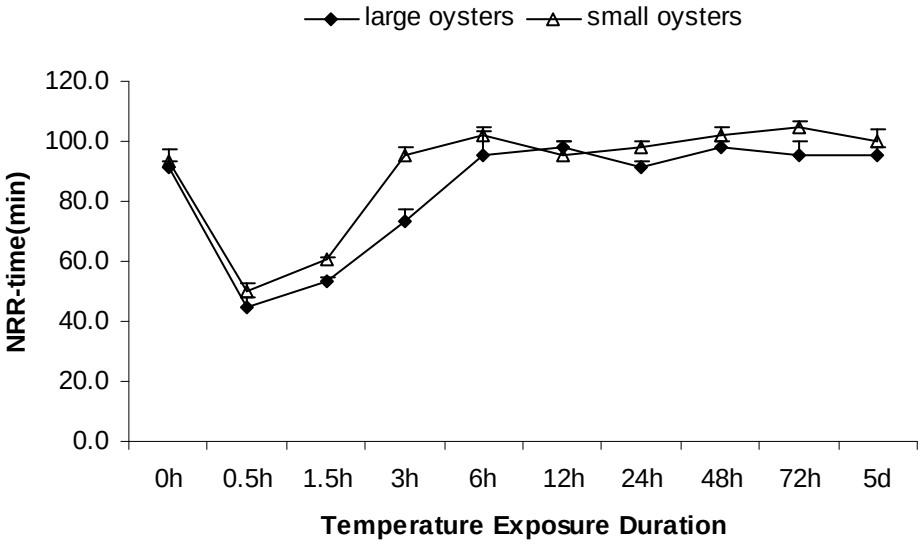


Fig. A7.3. Comparison of NRR time changes between large and small Pacific oysters after being transferred directly from 10°C to 20°C water (A) or from 20°C to 10°C water (B). Bars express the mean + S.E., n = 9 oysters.

A7.4.2. Effects of air exposure on lysosomal membrane stability

After 72 hr air exposure oyster size class, re-immersion duration and their interaction had a significant effect on lysosomal membrane integrity ($F_{1, 40}$ = 31.825, P < 0.001; $F_{9, 40}$ = 137.280, P < 0.001 and $F_{9, 40}$ = 4.935, P < 0.001 for 5°C air exposure; $F_{1, 40}$ = 20.070, P < 0.001; $F_{9, 40}$ = 260.310, P < 0.001 and $F_{9, 40}$ = 4.495, P < 0.001 for 15°C air exposure and $F_{1, 40}$ = 20.793, P < 0.001; $F_{9, 40}$ = 127.945, P < 0.001 and $F_{9, 40}$ = 2.489, P = 0.023 for 25°C air exposure) (Fig. A7.4).

After exposure to an air temperature of 5°C, 15°C or 25°C the lysosomal membrane integrity of the large oysters recovered in the 15°C water at a slower rate than did that of the small oysters.

After 72 hr exposure to 5°C air the NRR times in both large and small oysters increased significantly from initial levels of 25.0 ± 2.89 min and 28.33 ± 4.41 min to 135.56 ± 4.44 min and 140.0 ± 3.85 min within 24 hr and 12 hr of re-immersion in 15°C water respectively. These levels were not significantly different from those of the control (P = 0.784 and 0.860) (Fig. A7.4A).

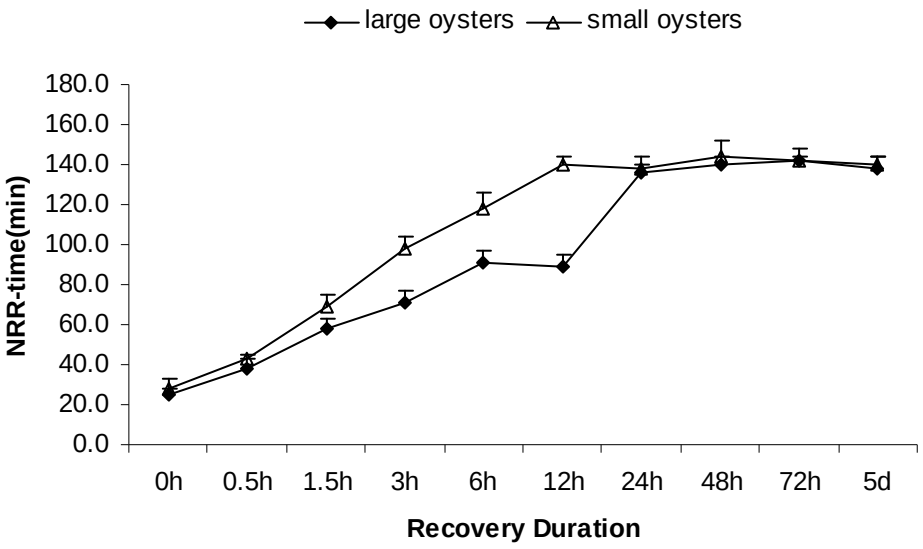
After 15°C air exposure the NRR times of the two size classes changed slightly (P = 0.478 and 0.173) within the first 1.5 hr of re-immersion in 15°C water (Fig. A7.4B), from 23.33 ± 4.41 min and 30.0 ± 2.89 min to 28.33 ± 4.41 min and 35.0 ± 2.89 min in large and small oysters, respectively. The NRR times in large oysters then increased to 140.0 ± 3.85 min, which was not significantly different (P = 0.738) from the control at 48 hr, and remained at this level until day 5; whereas the NRR times in the small oysters increased to 137.78 ± 2.22 min, which was similar (P = 0.238) to the control within 12 hr, then fluctuated slightly around this level until day 5.

After exposure at 25°C for 72 hr the NRR times (Fig. A7.4C) in the large oysters remained steady within the first 1.5 hr of re-immersion and then increased to a level not significantly different from the control (P = 0.350) at 24 hr (from 26.67 ± 4.41 min to 131.11 ± 5.88 min). The NRR times in small oysters increased significantly within the first 0.5 hr, from 25.0 ± 5.77 min to 43.33 ± 6.01 min and remained at this level until 1.5 hr. The NRR times then increased significantly to 137.78 ± 2.22 min at 12 hr, which was similar to the control (P = 0.329).

After exposure to 25°C air, the mortality in large oysters was 12.2%, which was significantly higher (P = 0.007) than the 6.7% observed in the small oysters. No mortality occurred in the oysters exposed to the other air temperatures.

Air temperature and the re-immersion duration had a significant effect on lysosomal membrane stability in large and small oysters, respectively ($F_{2, 60}$ = 8.708, P < 0.001; $F_{9, 60}$ = 263.717, P < 0.001 for large oysters and $F_{2, 60}$ = 15.358, P < 0.001; $F_{9, 60}$ = 220.504, P < 0.001 for small oysters). The lysosomal membrane stability recovered at a faster rate in both sizes of oysters exposed to 5°C air than those exposed to higher air temperatures (P = 0.01 and P < 0.001 for large and small oysters, respectively). Within the same size class the differences in NRR times between the 15°C and the 25°C air exposures were not significant (P = 0.771 and 0.08 for large and small oysters, respectively).

A



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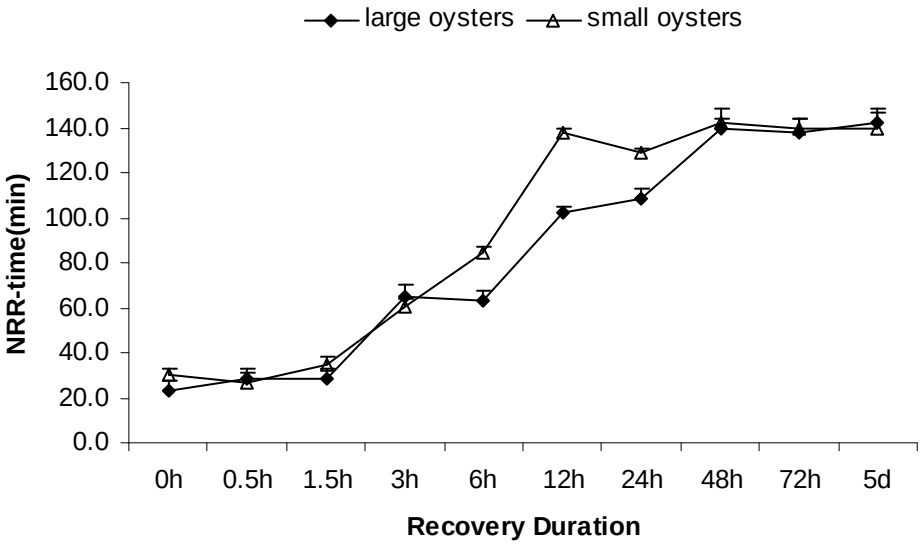

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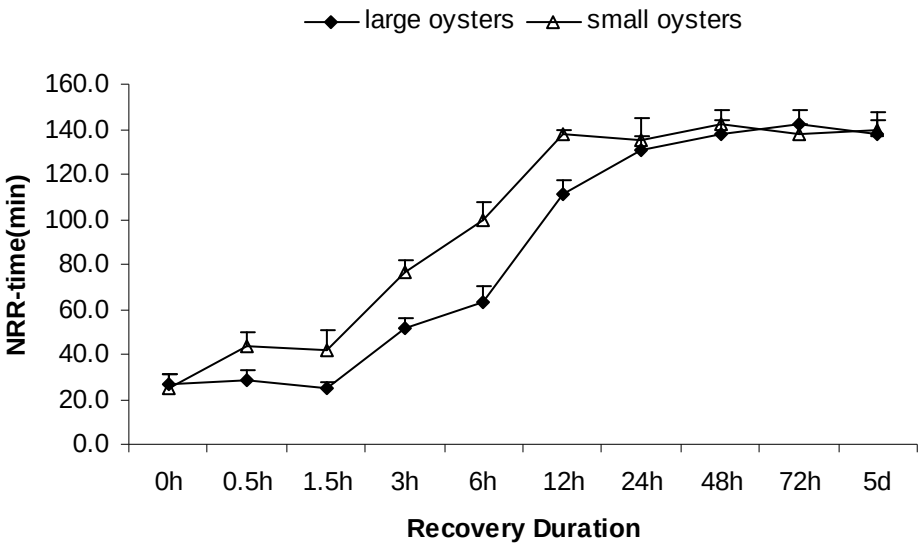

Fig. A7.4. Comparison of NRR time changes between large and small Pacific oysters in 15°C water after exposure to 5°C air (A), 15°C air (B) or 25°C air for 72 hr. Bars express the mean + S.E., n = 9 oysters.

A7.5. Discussion

Oysters may experience strong water temperature variations over the seasons, which in turn can influence their metabolic activity and energy budget allocation, especially in oysters inhabiting temperate and subtropical waters (Kinne, 1971; Widdows, 1973; Widdows and Bayne, 1971). Air exposure is another factor that could impose strong stress on oysters (Zhang et al., 2006). In this study the influence of size and/or age on the responses of Pacific oysters to these environmental changes was investigated.

Fifteen degrees Celsius was reported to be the optimal water temperature for maintaining the lysosomal membrane integrity in Pacific oysters (Zhang et al., 2006). In the first and second treatments when the water temperature was changed directly from 15°C to 5°C and 25°C, the lysosomal membranes of both large and small oysters responded in the same manner to these two temperature changes respectively (i.e. differences in the NRR times between large and small oysters were not significant). The NRR times of the larger animals declined to the levels corresponding with the 5°C and 25°C water temperatures in 3 hr to 6 hr, which was similar to the results in the study by Zhang et al. (2006) on this species. The size of the oysters (106.51 ± 1.00 mm in height, 51.90 ± 0.77 mm in length) used in their study was similar to the large oysters used in this study. They found that when oysters were transferred from 15°C to 5°C and 25°C water, the NRR times decreased to the levels corresponding with 5 and 25°C water temperatures within 3 hr.

With the reverse water temperature changes from 5 and 25°C to 15°C, differences in the NRR times between the two oyster size classes were significant ($F_{1, 40}$ = 4.351, P = 0.043 and $F_{1, 40}$ = 5.731, P = 0.021, respectively). Large oysters required a longer duration to recover their lysosomal integrity than did the small animals. The NRR times of the large oysters in both temperature changes increased to the level corresponding with the 15°C water temperature within 24 hr, which was much longer than the duration required in the reverse water temperature changes from 15°C to 5°C and 25°C (3 hr to 6 hr). This was similar to the finding by Zhang et al., (2006) who reported that with rapid temperature change the recovery of the lysosomal membrane stability took longer than its destabilization. However, instead of 24 hr, the oysters in their study required 5 days to recover their lysosomal membrane stability when transferred from 5°C and 25°C to 15°C water. The discrepancy between these two studies might be due to differences in the physiological condition and genetic makeup of the oysters used.

In the third treatment when oysters were subjected to direct water temperature changes between 10°C and 20ºC their NRR time decreased to levels significantly lower than those previously recorded for oysters acclimated at these temperatures. It was also observed that after an initial decrease, their NRR time gradually increased to the levels corresponding with 10ºC or 20ºC water, indicating that the lysosomal membrane had the ability to recover from this extra stress. A similar phenomenon was also found by Wang et al. (accepted) in blacklip abalone *Haliotis rubra* during direct water temperature changes between 7°C and 25ºC and between 11.5°C and 20.5ºC.

In the rapid temperature change experiment there was a 10ºC difference between the two temperatures for each treatment (5°C and 15ºC, 15°C and 25ºC, and 10°C and 20ºC). However, only the 10°C - 20ºC treatment produced a two-phased response. As suggested by Wang et al. (accepted) different ranges of water temperature change could have different effects on the integrity of the lysosomal membrane.

In the third treatment when the water temperature changed from 10°C to 20°C the differences in NRR times between large and small oysters were not significant ($F_{1, 40}$ = 1.701, P = 0.407). In the reverse temperature change from 20°C to 10°C, however, these differences were significant ($F_{1, 40}$ = 17.171, P < 0.001). The large oysters recovered from the 10°C water temperature change at a slower rate than did the small ones (6 hr versus 3 hr).

The 72 hr air exposure experiment showed that the recovery of lysosomal membrane stability in 15°C water was significantly affected by both oyster size class and exposure temperature (5°C, 15°C and 25°C). At an air temperature of 5°C, 15°C or 25°C the lysosomal membrane integrity of the large oysters recovered at a slower rate than did that of the small oysters ($F_{1, 40}$ = 31.825, P < 0.001; $F_{1, 40}$ = 20.070, P < 0.001 and $F_{1, 40}$ = 20.793,

$P < 0.001$ for 5°C, 15°C and 25°C air temperatures, respectively). The lysosomal membrane stability in both oyster size classes exposed to 5°C air temperature recovered quicker than when exposed to higher air temperatures ($P < 0.05$). The differences in the NRR times between the oysters exposed to 15 and 25°C air were not significant ($P = 0.771$ and 0.080 for large and small respectively). These results partially agree with the findings by Zhang et al. (2006), which showed that after air exposure, the lysosomal stability in oysters exposed to 5°C air temperature recovered faster than those exposed to 15°C air temperature, which in turn recovered faster than the oysters exposed to 25°C air temperature. The oysters used in their experiment were obtained just prior to their natural spawning cycle. It was reported that gametogenesis could result in a fragile physiological condition in Pacific oysters (Bertheline et al., 2000) and impose stress on oysters (Song et al., submitted). The oysters at later gonad developmental stages might be more sensitive to air temperature changes.

The significantly higher mortality in the large oysters exposed to 25°C air temperature than in the small oysters exposed to the same air temperature, further confirmed our results with the NRR assay that 25°C air exposure imposed a significantly stronger stress on the larger oysters.

The results from this study and the study by Song et al. (submitted) show that different size classes of oysters can respond differently to stresses imposed by certain environmental changes and spawning. It therefore appears important to standardize the size and age of oysters used in any experiments where the NRR assay is used to improve existing Pacific oyster farming practices.

A7.6. References

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APPENDIX 8: APPLICATION OF THE NEUTRAL RED RETENTION ASSAY TO EVALUATE DIFFERENCES IN STRESS RESPONSE TO SEXUAL MATURATION AND SPAWNING BETWEEN DIFFERENT SIZED PACIFIC OYSTERS, *CRASSOSTREA GIGAS* (THUNBERG)

This chapter submitted as:

Song, L, Li, X, Clarke, S, Wang, T, Bott, K. Application of the neutral red retention assay to evaluate differences in stress response to sexual maturation and spawning between different sized Pacific oysters, *Crassostrea gigas* (Thunberg).

A8.1. Abstract

Neutral red retention (NRR) assay was used to evaluate the effects of sexual maturation, spawning and post spawning recovery on lysosomal membrane integrity in the hemolymph of two size classes of Pacific oysters (large - 102.23 mm in height and 24 months old and small - 52.47 mm in height and 14 months old). The two size classes of oysters were each divided into two groups: Group H was fed a high quantity of microalgae (approximately 2×10^6 cells mL^{-1} at a rate of 1.0 L per oyster per day) to enhance gonad development, while Group L was fed a low quantity of microalgae (same cell concentrations but at a rate of 0.15 L per oyster per day), sufficient to maintain existing dry meat weight, whole body weight, condition index (CI) and maturation index (MI). The results showed that prior to spawning the decrease in lysosomal membrane stability in Group H was negatively correlated with the dry meat weight, CI and MI. After spawning the dry meat weight, whole body weight and CI reduced to levels not significantly different from the values on day 0. These results indicate that the increase in dry meat weight, whole body weight and CI prior to spawning mainly resulted from the growth of gonad tissues, and thus any stress experienced by the oysters during this period was mainly related to gametogenesis and the related metabolic alterations that it causes. The results also showed that spawning further impaired lysosomal membrane stability ($P < 0.05$). After spawning the NRR times reached the lowest levels recorded before recovering to levels corresponding with the water temperature in which the animals were maintained. The smaller oysters recovered from the stress of spawning much faster than the larger animals ($P < 0.05$). Prior to spawning the dry meat weights of both large and small oysters increased by approximately 85% in the first 42 days, whereas after spawning the dry meat weights only increased by 20% during the same time period, suggesting that available energy was used to recover from the stress created by spawning.

Key words: Neutral red retention; Lysosomal membrane stability; Stress; Sizes; Sexual maturation; Spawning; Pacific oysters; *Crassostrea gigas*

A8.2. Introduction

Reproduction is a basic biological function found in all animals. In Pacific oysters, *Crassostrea gigas* (Thunberg) the success of reproduction not only influences the structure of the wild population but can also determine the success of commercial farming enterprises as it is considered to be one of the major factors contributing to abnormally high mortality levels during summer, the so-called "summer mortality" (Lipøvsky and Chew, 1972; Beattie et al., 1980; Perdue et al., 1981; Berthelin et al., 2000). In France, losses of up to 80% in some areas have been recorded (Berthelin et al., 2000). Studies on the seasonal variation in the biochemical composition of Pacific oysters have shown that gametogenesis and spawning are associated with changes in lipid, protein and glycogen content (Perdue et al., 1981; Gabbott, 1983; Berthelin et al., 2000). During gametogenesis glycogen content decreases reaching its lowest level after spawning (Perdue et al., 1981; Berthelin et al., 2000). This low energetic state could then reduce the animal's capability to respond to other stressors such as elevated water temperature, high water nutrient level, mechanical stress and bacterial or viral infection (Mori et al., 1965; Perdue et al., 1981; Shpigel et al., 1992; Berthelin et al., 2000).

As a major site of intracellular digestion for the sequestration and detoxification of xenobiotics, lysosomes play a central role in the innate defence of molluscs (Cheng, 1981; Moore, 1990; Viarergo and Nott, 1993; Lowe et al., 1995a, b; Petrović, et al., 2004) and therefore would be the site of the earliest detectable response to stresses imposed both internally and externally (Harding et al., 2004a, b). Neutral red retention (NRR) assay measures the retention time of the neutral red dye in the lysosomes. In unstressed cells lysosomes will accumulate and retain neutral red dye for an extended period of time. Once destabilized by a stress response the neutral red dye will leak into the cytosol of the cell through the damaged lysosomal membrane (Moore, 1980; Pipe, 1987; Lowe et al., 1995a, b). As stressed cells lose their dye at a faster rate than non-stressed cells the rate of NRR in the lysosome can therefore be correlated to the overall stress of the animal (Harding et al. 2004a). NRR assay has proven to be a useful method of evaluating responses to environmental, physiological and mechanical stresses in oysters, mussels, freshwater snails and abalone (Lowe and Pipe, 1994; Lowe et al., 1995a, b; Svendsen and Weeks, 1995; Fernley et al., 2000; Hauton et al., 1998, 2001; Petrović et al., 2004; Mamaca et al., 2005; Cho and Jeong, 2005; Zhang et al., 2006; Zhang and Li, 2006, Wang et al., submitted for publication).

By using the NRR assay it has been found that seasonal patterns in the stress response of mussels are mainly associated with their reproductive cycle (Harding et al., 2004b). A significant decrease in lysosomal membrane stability was also observed by Cho and Jeong (2005) in spawned Pacific oysters. However, this observed

destabilization of the lysosomal membrane might also be the result of other environmental factors such as water temperature changes, air exposure, salinity fluctuations, etc. In fact, the lysosomal membrane stability of oysters is strongly influenced by the above-mentioned factors (Hauton et al., 1998; Zhang et al., 2006).

Published data also shows that in marine bivalves the energy cost of reproduction increases with size and age (Griffiths and King, 1979; Bayne and Worrall, 1980; Griffiths, 1981; Thompson, 1984). Deslous-Paoli and Héral (1988) found in *C. gigas* that the average energy usage in reproduction increased by approximately 10% per year. However, the response of oysters to energy usage between sizes and/or age classes remains poorly documented. Size and/or age class effects on other biological functions and stress responses have been documented in other molluscs. In the blue mussel *Mytilus edulis* L. stress responses to low salinity, elevated temperature and air exposure were size-dependant, whereas pumping rates were significantly affected by both size and age (Sukhotin et al, 2003). In the New Zealand abalone, *Haliotis iris*, larger animals were less stressed and could live longer out of water when compared to smaller ones (Ryder et al., 1994).

The main purpose of this study was to use the NRR assay to investigate: 1) whether gonad development imposes any stress on Pacific oysters, 2) whether there are any differences in the stress response to spawning between different sized oysters, and 3) how long it takes for different sized oysters to recover from the stress produced by spawning.

A8.3. Materials and Methods

A8.3.1. Experimental animals

Two sizes classes of Pacific oysters were obtained from an oyster farm in Smoky Bay, South Australia, in early September 2005. The large oysters were approximately 24 months old and 102.23 ± 1.79 mm, 54.46 ± 0.98 mm and 33.96 ± 1.50 mm in height, length and width, respectively (mean ± SE, n = 100), while the small oysters were approximately 14 months old and 52.47 ± 2.08 mm in height, 33.43 ± 1.16 mm in length and 24.69 ± 0.99 mm in width (mean ± SE, n = 100). The oysters were transported live to the South Australian Research and Development Institute's (SARDI) Aquatic Sciences Centre at West Beach, Adelaide, South Australia in a refrigerated container. On arrival the animals were cleaned and placed into six 450L flow-through tanks (flow rate 120L h^{-1}, salinity 37‰) filtered to 10 µm. The oysters were acclimated at a temperature of 15°C for 7 days prior to being used in the experiment. The oysters were fed with a mixture of three microalgae species, *Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans*.

A8.3.2. Effects of sexual maturation and spawning on lysosome membrane stability

After acclimation the large and the small oysters were divided into two groups: Group H and Group L, each group was divided into three 450L tanks to provide 3 replicates per group. During the experimental period Group H were fed with a microalgal mixture of approximately 2×10^6 cells mL^{-1} at a rate of about 1.0 L per oyster per day to enhance gonad development. A microalgal mixture of the same concentration was also supplied to Group L at a reduced rate of 0.15 L per oyster per day to maintain existing condition. Results from preliminary experiments showed that when oysters of both size classes were fed at the rate used in Group L the reproductive condition and condition indices were maintained at a constant level for 40 days.

The experiment was split into two physiological periods: the pre-spawning and the post-spawning periods. The first period ended prior to the induction of spawning (day 70) while the second covered the duration from spawning (day 0S or day 71 if counted continuously) to the completion of the experiment (day 42S). During the experimental period the water temperatures in both Group H and L were changed simultaneously. The temperatures were set at 15°C at the start of the experiment. When more than 60% of the males had developed into the mature stage (for details refer to *2.4. Maturation index and condition index*) in Group H on day 63 the water temperatures in both groups were then increased by 1°C per day to 20°C to day 67 and maintained at this temperature until day 70. On the next day (day 0S) the oysters in both groups were induced to spawn by adding fresh oyster gametes. On day 27 (27S) post-spawning, 6 days after the NRR times of large oysters in Group H had reached NRR times corresponding with those in 20°C water temperature, the water temperature in both groups was decreased immediately to 15°C and maintained at this level until the completion of the study. The experiment lasted for 113 days in total.

Whole body, dry shell and dry meat weight were determined and a hemolymph sample collected at predetermined time points - day 0, day 21, day 42, day 63 and day 70 during the pre-spawning period and every 3 days from day 0S to day 42S during the post-spawning period.

At each sampling time three oysters were randomly selected in each replicate and sampled individually with the methods described in sections 12.3.3. and 12.3.4.

A8.3.3. Neutral red retention (NRR) assay

The methods described in Zhang et al. (2006) for neutral red retention assay were applied in this study.

The neutral red stock solution was made by dissolving 2.28 mg of neutral red powder in 1 mL of dimethyl sulphoxide (DMSO). The working solution (0.04mg mL^{-1}) was prepared by diluting 17 μL of the stock

solution with 1 mL of oyster saline solution consisting of 0.48 g L^{-1} $CaCl_2$, 1.45 g L^{-1} $MgSO_4$, 2.18 g L^{-1} $MgCl_2 \cdot 6H_2O$, 0.31 g L^{-1} KCl, 11.61 g L^{-1} NaCl, and 0.35 g L^{-1} $NaHCO_3$ (Buchanan et al., 2001).

At each sampling time point 0.2 mL of hemolymph was drawn individually from the oysters pericardial cavity into a 1 mL syringe. The hemolymph was then placed into a 2 mL siliconised Eppendorf® tube containing 0.2 mL of oyster saline solution and gently mixed. Each sample was collected within 1 min.

A 40 µL sample of the hemolymph and the oyster saline solution mixture was then placed onto a microscope slide that had been treated with poly-L-lysine (20 µL in 100 µL distilled water) to enhance cell adhesion. The slide was then suspended on a rack above ice water in a light proof humidity chamber to allow the blood cells to adhere to the slide (the temperature on the rack was 10°C). After 15 min the slides were removed from the rack and the excess hemolymph was poured off. A 20 µL neutral red working solution (10°C) was added to the cell layer and incubated in the humidity chamber for another 15 min. A cover slide (22 × 22 mm) was then placed onto the slide and the hemocytes were examined under a BX 60 Olympus compound microscope (× 600 magnification). The slide was examined every 15 min for the first 60 min and then every 20 min. Fifty granulocytes were examined at each time interval for an individual oyster. Once 50% of the hemocytes had started to lose dye from their lysosomes the assay was stopped and the time for the previous examination was recorded as the neutral red retention (NRR) time for that oyster. The observer was not informed of the origin of the sample under examination to minimise the possibility of bias.

A8.3.4. Maturation index (MI) and condition index (CI)

During sampling each oyster was dried with a paper towel and weighed to the nearest 0.01 g. The oyster was then opened with an oyster knife for hemolymph collection. After hemolymph collection the gonad was examined visually and one drop of gonad tissue was taken from right side at the front of the heart by using a clean Pasteur pipette. After dilution with fresh seawater for 10 min the percentage of motile sperm was determined microscopically by counting the number of moving sperm out of a sample of 100. Sperm moving forward were counted as motile sperm while those vibrating or not moving at all were treated as immotile. The reproductive condition of the male oysters was assessed only when their sex was identifiable and could be classified into one of the following stages:

Indeterminate: The gonad is transparent. The stomach is visible. No identifiable sperm or eggs are present.

Early development: The sperm motility is less than 40%.

Late development: The sperm motility is between 40% and 80%.

Mature: The gonad has a dense and creamy appearance. More than 80% of the sperm are motile.

Spawned: The gonad is soft and the stomach is visible.

After the reproductive condition was assessed the soft tissue was removed from the shell and individually dried in a 60°C oven for 48 hr. The shell was also dried at the same temperature for 24 hr. The dried tissue was then weighed individually and the two valves from the same individual weighed together to the nearest 0.01 g.

The condition index was calculated according to the equation used by Walne (1976):

CI = [dry meat weight (g) × 1000] / dry shell weight (g)

A8.3.5. Statistical analysis

All statistics were calculated using SPSS 13.0 software. ANOVA and Tukey's *b* multiple comparisons were were used to detect significant differences in data after first checking for normality and homogeneity. Correlations were calculated using Pearson's correlation coefficients. A probability level of $P < 0.05$ was considered statistically significant.

In this study the data used in analyzing the correlations between NRR times and gonad development (sperm mobility) were from male oysters only as the stocks used had approximately 70% males in the small size class and 50% in the large size class. In addition, the morphological categories used for classification of gonad development could only be standardized within a sex class (eg. males).

A8.4. Results

A8.4.1. Effects of sexual maturation and spawning on lysosome membrane integrity

During the entire experimental period (113 days) the differences in NRR times between the large and small oysters in Group L were not significant ($F_{1, 320} = 0.07$, $P = 0.79$) (Fig. A8.1).

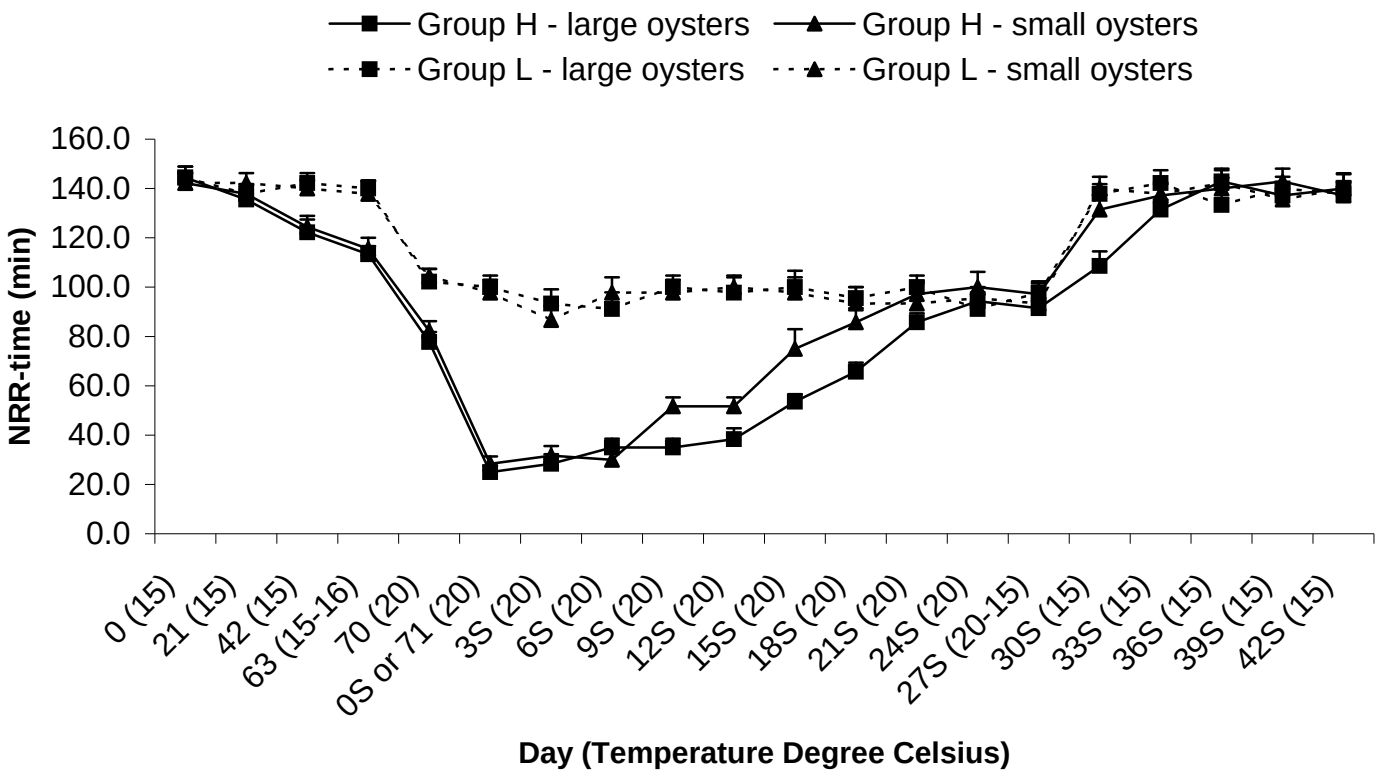

Fig. A8.1. Relationship between NRR times and oyster size (large vs small), water temperature, feeding regime (1 L per oyster per day in Group H vs 0.15 L per oyster per day in Group L) and spawning. Bars represent mean + S.E. (n = 9 oysters). S is days post-spawning.

In the pre-spawning period both feeding regime and duration and their interaction significantly affected lysosomal membrane integrity in large ($F_{1, 80}$ = 29.83, P < 0.001; $F_{4, 80}$ = 45.70, P < 0.001; $F_{4, 80}$ = 3.77, P < 0.001) and small ($F_{1, 80}$ = 24.92, P < 0.001; $F_{4, 80}$ = 46.84, P < 0.001; $F_{4, 80}$ = 3.14, P < 0.05) oysters, respectively.

In Group L the NRR times were approximately 140 min at day 0 in both large and small oysters and only varied slightly from this level (P > 0.05) for the first 63 days at 15°C. The NRR time then decreased significantly (P < 0.05) to 102.22 ± 5.21 min and 104.44 ± 2.94 min, respectively on day 70 after the water temperature was raised to 20°C. During the same period the NRR times of large and small oysters in Group H decreased significantly to 113.33 ± 3.33 min and 115.56 ± 4.44 min on day 63 and then to 77.78 ± 4.01 min and 82.22 ± 4.01 min on day 70, respectively. These values were significantly lower than those for the same sized oysters in Group L on the same days (P < 0.05 in all cases). The differences in NRR times between the large and small oysters in Group H during the pre-spawning period were not significant (P > 0.05).

During the post-spawning period (Fig. A8.1) the lysosomal membrane stability was significantly affected by both spawning and post-spawning recovery duration and their interaction in large ($F_{1, 220}$ = 261.18, P < 0.001; $F_{14, 220}$ = 76.60, P < 0.001; $F_{14, 220}$ = 17.23, P < 0.001) and small ($F_{1, 220}$ = 105.50, P < 0.001; $F_{14, 220}$ = 62.47, P < 0.001; $F_{14, 220}$ = 13.53, P < 0.001) oysters respectively. Lysosomal membrane stability was also significantly affected by oyster size, post-spawning recovery duration and their interaction ($F_{1, 200}$ = 24.26, P < 0.001; $F_{14, 200}$ = 181.99, P < 0.001; $F_{14, 200}$ = 1.89, P < 0.05).

From day 0S when spawning was induced to day 27S the NRR times in Group L remained around 100 min in both oyster size classes. The NRR times then increased to approximately 140 min on day 30S when the water temperature was decreased to 15°C and remained at this level until the end of the experiment (Fig. A8.1). In comparison, the NRR times in Group H dropped significantly (P < 0.001) after spawning on day 0S, from 77.78 ± 4.01 min to 25.00 ± 2.50 min and from 82.22 ± 4.01 min to 28.33 ± 3.00 in the large and small oysters, respectively. These two post-spawning NRR times were the lowest levels recorded for the two size classes in this experiment. The NRR times increased slightly at the next two sampling times (P > 0.05). The lysosomal membrane stability in small oysters then recovered significantly faster (P < 0.05) than in large oysters and reached a level (85.71 ± 5.71) that was not significantly different (P = 0.34) from Group L on day 18S (Fig. A8.1). The NRR time in the small oysters further increased slightly on day S21 and fluctuated around that level until day 27S when the water temperature was decreased to 15°C. In large oysters the NRR times reached a similar level (85.71 ± 3.69 min; P = 0.450) to the large oysters in Group L on day 21S and then increased slightly until day 27S. On day 27S the NRR times of both large and small oysters in Group H were significantly higher (P = 0.01 and 0.04, respectively) than on day 70, and similar (P = 0.26 and 0.22, respectively) to the values in Group L on the same day (day 70) (Fig. A8.1).

On day 27S when the water temperatures in both groups was changed to 15°C, the NRR times of the small oysters in Group H and the small and large oysters in Group L increased at a similar rate and reached levels corresponding with a water temperature of 15°C on day 30S (Fig. A8.1). In comparison, the NRR time of large oysters in Group H increased at a slower rate and was significantly lower ($P = 0.001$) than that of the small oysters in Group H on day 30S, reaching a similar level to the small oysters on day 33S. After the water temperature was changed to 15°C the lysosomal membrane stability of both size classes in both groups recovered to levels not significantly different ($P > 0.05$) from those in Group L on day 63, but were significantly different ($P < 0.05$) from those in Group H on the same day (day 63).

A8.4.2. Maturation index (MI) and condition index (CI)

On day 0, four out of 9 large oysters and 2 out of 9 small oysters were at the indeterminate stage while the other 8 males (5 small and 3 large oysters) were all at the early development stage. In Group L the gonads developed into or remained at the early development stage (< 40% sperm motility) until the end of this study. In Group H the gonads of all the 11 male oysters sampled on day 21 (18 oysters in total) were at the early development stage. Seven out of 11 on day 63 and 10 out of 11 males on day 70 developed into the mature stage. On day 0S the oysters in Group H started to spawn within 1 hr of the addition of fresh gametes and spawning was completed within 10 hr. During the post-spawning period only 2 large un-spawned or partially spawned individuals were found and removed from the study.

The dry meat weight (Fig. A8.2) of the large oysters in Group L exhibited a marginal increase ($P = 1.00$) during this study with an initial weight of 2.33 ± 0.12 g on day 0 and a final weight of 2.62 ± 0.07 g on day 42S, while the small oysters in Group L exhibited a slight increase from day 0 to day 21S ($P = 0.137$). On day 24S the dry meat weight (0.60 ± 0.01 g) was significantly higher ($P = 0.033$) than on day 0 (0.55 ± 0.01 g). The dry meat weight then increased further to 0.63 ± 0.00 on day 42S ($P > 0.05$), which was similar to the dry meat weight of the same size oysters in Group H on day 21 (0.66 ± 0.02 g; $P > 0.05$).

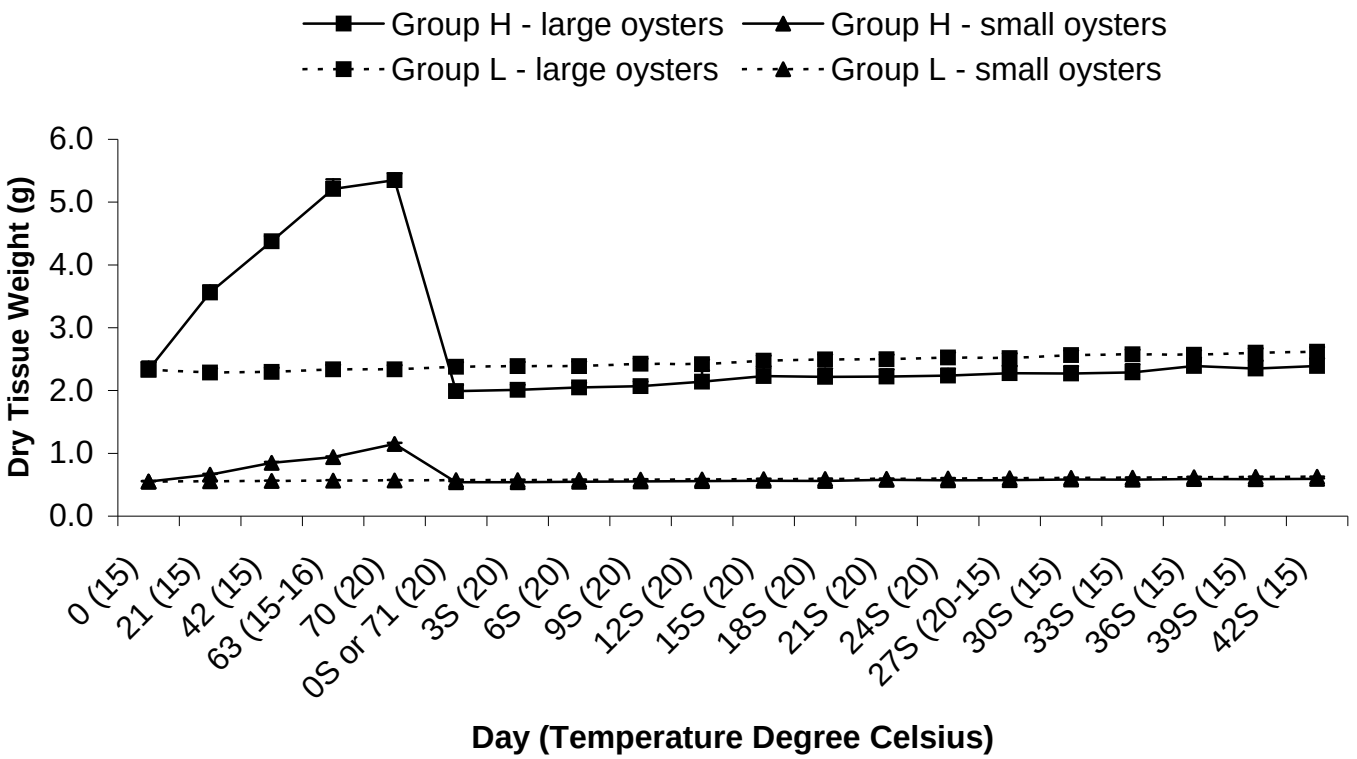

Fig. A8.2. Relationship between dry meat weight and oyster size (large vs small), water temperature, feeding regime (1 L per oyster per day in Group H vs 0.15 L per oyster per day in Group L) and spawning. Bars represent mean + S.E. (n = 9 oysters). S is days post-spawning.

In Group H the dry meat weights in both large and small oysters increased significantly ($P < 0.05$) at each sampling time from 2.33 ± 0.12 g and 0.55 ± 0.01 g on day 0 to 5.21 ± 0.12 g and 0.94 ± 0.01 g on day 63, respectively. On day 70 the dry meat weight in the large oysters increased slightly ($P = 1.00$) to 5.23 ± 0.11 g, while those in the small oysters increased significantly ($P = 0.013$) to 1.15 ± 0.02 g. After spawning the dry meat weights dropped significantly ($P < 0.001$) to 1.99 ± 0.10 g and 0.54 ± 0.02 g in large and small oysters respectively on day 0S. These meat weights were not significantly different from the initial values at day 0 ($P > 0.05$). The dry meat weights then increased gradually ($P > 0.05$) to 2.39 ± 0.12 g and 0.59 ± 0.01 g on day 42S for large and small oysters, respectively. After spawning the dry meat weights in large and small oysters reduced by 62.8% and 53.04%, respectively. The difference in percentage dry meat weight losses between these two size classes was significant ($P < 0.001$). During the pre-spawning period the dry meat weights of large and small oysters increased by 87.7% and 85.0% respectively during the first 42 days, while during the post-spawning period the dry meat weights increased by 20.2% and 9.9% in large and small oysters, respectively, in the same number of days.

During the entire experimental period (113 days) the dry shell weights of large and small oysters in Group L increased slightly ($P > 0.05$) from 33.79 ± 0.56 g and 12.28 ± 0.63 g to 34.50 ± 1.13 g and 13.20 ± 0.59 g, respectively. The dry shell weight of large oysters in Group H increased significantly to 43.34 ± 0.97 g on day 63 ($P < 0.001$) and then remained at this level until day 42S. The dry shell weights of small oysters in Group H increased slightly ($P > 0.05$) to 15.58 ± 0.79 g by 42S, with increases mainly occurring prior to spawning (15.00 ± 0.45 g by day 70).

The CI's of both large and small oysters in Group L increased slightly ($P > 0.05$) from 69.44 ± 4.54 and 45.98 ± 2.83 on day 0 to 76.38 ± 2.67 and 48.24 ± 2.25 on day 42S, respectively (Fig. A8.3).

The CI's of both large and small oysters in Group H significantly increased ($P < 0.05$) to 128.91 ± 4.83 and 77.25 ± 2.67 on day 70, and then dropped significantly ($P < 0.05$) to 44.54 ± 2.14 and 36.80 ± 1.43, respectively, on day 0S (or day 71) after spawning (Fig. A8.3). These levels were not significantly different from the initial values on day 0 ($P > 0.05$). The CI's then increased slightly ($P > 0.05$) to 54.94 ± 4.48 and 38.85 ± 1.09 in large and small oysters, respectively, on day 42S.

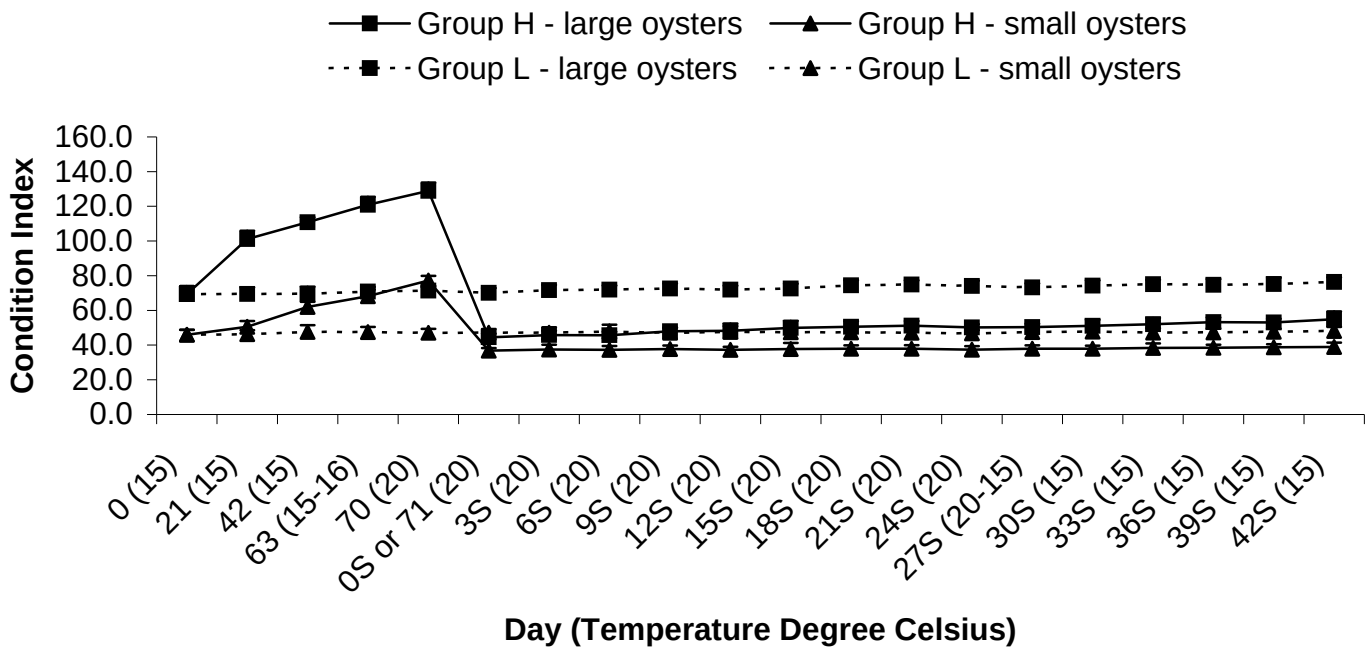

Fig. A8.3. Relationship between CI and oyster size (large vs small), water temperature, feeding regime (1 L per oyster per day in Group H vs 0.15 L per oyster per day in Group L) and spawning. Bars represent mean + S.E. (n = 9 oysters). S is days post-spawning.

The whole body weights of large and small oysters in Group H increased significantly ($P < 0.05$) from 108.94 ± 3.95 g and 25.83 ± 1.94 g on day 0 to 137.99 ± 5.45 g and 38.64 ± 1.99 g on day 70 and then decreased significantly ($P < 0.05$) to 109.23 ± 3.37 g and 26.45 ± 1.56 g on day 0S post spawning. The values were not significantly different from the initial values on day 0 ($P > 0.05$). The whole body weights remained at this level until the end of this study. The whole body weights of the large and small oysters in Group L only increased slightly ($P > 0.05$) during the experimental period.

During the pre-spawning period from day 0 to day 63 when the water temperature was maintained at 15°C the correlations between NRR time and CI, NRR time and dry meat weight and NRR time and MI were negative but significant for large (r = -0.533, -0.616 and -0.604) and small (r = -0.489, -0.665 and -0.651) oysters in group H ($P \leq 0.002$ and n = 36 for the first two correlations; $P = 0.013$, n = 16 and $P < 0.001$, n = 26 respectively for large and small oysters in the correlation between NRR time and MI).

A8.5. Discussion

In marine bivalves the neutral red retention (NRR) assay has been shown to represent a measure of the adaptive capacity of cellular processes following an exposure to stressful conditions (Lowe and Pipe, 1994), thus reduced NRR times reflect the onset of cell death in sensitive cells following the imposition of stresses (Hauton et al., 1998). In this study the NRR assay was used to assess change in the integrity of the lysosomal membranes during sexual maturation, spawning and post-spawning recovery in large and small Pacific oysters.

During the experimental period the NRR times of both small and large oysters in Group L were not significantly different from each other at each sampling time and were approximately 140 min and 80 min in 15°C and 20°C water temperatures, respectively. These values were similar to the finding of Zhang et al (2006) when investigating the temperature effects on lysosomal membrane stability in this species. The experiment conducted by Zhang and Li (2006) also showed that if oysters were starved their ability to retain neutral red was impaired significantly. This indicates that the feeding regime used in Group L created minimal, if any, stress on the experimental animals, and was adequate to maintain a constant body condition. After spawning at 20°C the NRR times in Group H dropped significantly to less than 30 min. These results were consistent with the findings of Cho and Jeong (2005) that spawning could lead to a significant decrease in lysosomal membrane stability in this species. In mussels NRR times were clearly affected by the reproductive cycle and decreased significantly following spawning events, resulting in a gradual increase over time (Harding et al., 2004b). The results from this study also showed that in the first 6 days post spawning, recovery was minimal and that it can take more than 18 days for the lysosomal membranes to recover to the levels of integrity corresponding to the water temperature the oysters were held in.

In the present study the rate of lysosomal membrane recovery after spawning was significantly different between large and small oysters. Smaller oysters recovered at a significantly faster rate than larger ones. In addition, the lysosomal membrane of small oysters in Group H responded to a direct water temperature decrease from 20°C to 15°C at a similar speed to oysters in Group L, while the lysosomal membranes of large oysters responded significantly slower, indicating a reduced capacity to react to further temperature change. These differences in recovery rates might be due to the fact that after spawning the percentage of energy loss in smaller oysters is significantly lower than that of larger oysters. This size difference response to different stressors has also been documented in other molluscs. In blue mussels, *M. edulis,* responses to environmental stress such as low salinity, elevated temperature and air exposure were suggested to be size dependant (Sukhoin et al., 2003). In the abalone, *Haliotis iris*, larger animals took longer to recover from anesthetic treatment than smaller ones (Sharma, et al., 2003). The stress response of *H. iris* to air exposure was also related to animal size, with larger animal experiencing less stress when compared to smaller animals (Wells and Baldwin, 1995).

The CI (dry meat weight: dry shell weight ratio) used in this study was recommended by Lucas and Beninger (1985) as a preferable static index because it can be easily standardized and low CI values can reflect the energy deficits resulting from either environmental stress or loss of gametes. After spawning the CI's and the dry meat weights in Group H dropped to the lowest levels for the two size classes of oysters used in this study and were not significantly different from their initial values on day 0. During the pre-spawning period the dry shell weights of both size classes of oysters in Group H did not decrease and the gonads were at an indeterminate or early developmental stage when this experiment was started. Therefore, this rapid increase in CI's and the dry meat weights during this period resulted from the growth of gonad tissue. The rapid loss in dry meat weight and CI after spawning has also been observed in other studies on seasonal variation in the reproductive activity and biochemical composition of farmed and wild Pacific oysters (Ruiz et al., 1992; Kang et al., 2000; Ren et al., 2003). Deslous-Paoli and Héral (1988) and Patrick et al. (2006) also confirmed in their studies that increases in dry meat weight and CI were correlated with the gametogenesis during the gonad development stages.

From Fig. A8.1 it can be seen that prior to spawning in Group H the NRR times of the large and small oysters on day 63 at 15°C were significantly lower than their initial value on day 0 and the NRR times on day 63 at 15°C and on day 70 at 20°C were significantly lower than the values of the same sized oysters in Group L on these two days respectively. A similar phenomenon was also found by Zhang and Li (2006) in their study on starvation with this species. They observed that NRR times in oysters fed a higher level of microalgae decreased significantly after 42 days. They suggested that this decrease in NRR time could have been caused by gametogenesis, a nutritional imbalance or a combination of both. The same microalgae species and a similar density and volume were also used in this study. We observed that after spawning, when oysters were held at the spawning temperature of 20°C, the NRR times in Group H first recovered to the levels

corresponding with the oysters in Group L held at 20°C on day 70. When the water temperature was decreased to 15°C on day 27S the NRR times in Group H then increased to the levels corresponding with the oysters in Group L held at 15°C on day 63. These NRR values were significantly higher than those in the oysters in Group H on day 63 and 70, respectively, indicating that the decrease in NRR time in Group H before spawning was mainly caused by gonad development. This was further supported by the significant negative correlations between NRR time and CI, dry meat weight and male MI in Group H during this period.

During the post-spawning period the oysters in Group H were fed with the same amount of microalgae per animal per day as in the pre-spawning period. Therefore, the per unit dry meat weight microalgal access rate during this period should have been the same as that in the pre-spawning stage, because the dry meat weights of large and small oysters on day 0S post-spawning were similar to their initial values at the start of this experiment. However, after spawning the dry meat weights of large and small oysters increased by less than 20% in 42 days. In comparison, the dry meat weights of these two sized oysters increased by approximately 85% during the first 42 days prior to spawning. It should also be noted that during the post-spawning period the increase in dry meat weights was not significant. This lower efficiency in food conversion is most likely due to the redirection of energy to recover from the stress imposed by the spawning and/or to repair their immune system. This assumption was also supported by a study on fish (Barton and Dwyer, 1997) where during a response to stress, energy was directed away from non-essential functions such as growth and towards essential functions such as the immune system. However, the slow increase in dry meat weights could have also resulted from a reduction in feeding efficiency after spawning. This could be investigated by comparing the food intake of recently spawned oysters with oysters of the similar size and age, but at an earlier gonad developmental stage.

In *C. gigas* it has been found that a significant amount of pre-stored glycogen is converted into lipid reserves during gametogenesis and lost during spawning (Gabbott, 1975; Gabbott, 1983; Bertheline et al., 2000), and that low glycogen levels often coincide with mortality events (Bertheline et al., 2000, Patrick et al. 2006). It is suggested that animals at this pre- or post-spawning stage are probably in a fragile condition (Mori et al., 1965; Bertheline et al., 2000), which could in turn increase their sensitivity to different stresses (thermal stress, hypoxia, etc) during this time. Perdue et al. (1981) reported that oyster mortality consistently occurred after spawning events in Puget Sound (USA), while Patrick et al. (2006) demonstrated that mortality events occurred before spawning in Marennes-Oléron Bay (France). It is expected the discrepancy between these two studies could possibly be clarified if the effect of gonad development and spawning could be separated from the low glycogen levels reported during these reproductive stages. The results from this and other studies (Patrick et al., 2006) indicate the NRR assay in combination with other biochemical analyses has the potential to resolve these issues.

In summary, it can be concluded that the effects imposed on the lysosomal membrane stability by gonad development became stronger towards the end of the mature reproductive stage, resulting in the highest effects occurring during spawning. After spawning the lysosomal membrane integrity recovered at a much faster rate in small oysters compared to large oysters and the NRR times remained at the lowest level for a period of 6 and 12 days for small and large oysters respectively before increasing. The results from this experiment also showed that the NRR assay could be further applied to investigate the correlation between summer mortality and reproduction in Pacific oysters.

A8.6. Acknowledgements

This work was partially supported by the Fisheries Research and Development Corporation. The authors are grateful to Mr Gary Zippel of Zippel's Enterprises Pty Ltd for providing the oysters used in this study.

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Zhang, Z., Li, X., 2006. Evaluation of the effects of grading and starvation on the lysosomal membrane stability in Pacific oysters, *Crassostrea gigas* (Thunberg) by using neutral red retention assay. Aquaculture 256, 537-541.

APPENDIX 9: EVALUATION OF THE EFFECTS OF GRADING AND STARVATION ON THE LYSOSOMAL MEMBRANE STABILITY IN PACIFIC OYSTERS, *CRASSOSTREA GIGAS* (THUNBERG) BY USING NEUTRAL RED RETENTION ASSAY

This chapter published as:

Zhang, Z., Li, X., 2006. Evaluation of the effects of grading and starvation on the lysosomal membrane stability in Pacific oysters, *Crassostrea gigas* (Thunberg) by using neutral red retention assay. Aquaculture 256, 537-541.

A9.1. Abstract

In this study the neutral red retention (NRR) assay was used to evaluate the effects of simulated rotation grading, starvation, and their combination on lysosomal membrane stability in Pacific oysters. NRR results showed that both duration of simulated grading and duration of subsequent recovery after grading in 15°C water had a significant effect on lysosomal stability. In general, the oysters that underwent longer durations of grading had lower NRR times at most time points. The NRR times in the oysters graded for shorter periods returned to the control level more quickly than those graded for longer periods during the post-grading recovery in 15 °C water.

After 42 days starvation, the condition index and NRR times in starved oysters were significantly lower than those in fed animals and the control animals. The NRR times in the oysters that were fed during this period also decreased significantly in comparison with day 0 controls although the animals' CI increased slightly. Significant differences in responses to 3 min simulated grading were found between the oysters that were starved and fed for 42 days.

Key words: Neutral red retention; Lysosomal membrane stability; Stress; Simulated grading; Starvation; Pacific oysters

A9.2. Introduction

Lysosomal responses to stresses can be divided into three categories: changes in lysosomal components, changes in fusion events, and changes in membrane permeability (Hawkins, 1980). As an indicator for

evaluating lysosomal membrane integrity neutral red retention (NRR) assay has been extensively applied in marine bivalves to assess their response to contaminants (Lowe and Pipe, 1994; Lowe et al., 1995a, b), to seasonal and environmental changes associated with the reproductive cycle, temperature, air exposure and food availability (Harding et al., 2004b), and to mechanical disturbances related to post-harvest processing activities and storage conditions (Harding et al., 2004a). NRR assay applications in oysters have revealed that lysosomal membrane stability is associated with water temperature changes (Hauton et al., 1998, Zhang et al., submitted), air exposure (Zhang et al., submitted), hyposalinity (Hauton et al., 1998), spawning (Cho and Jeong, 2005), bacterial inoculation (Hauton et al., 2001), and environmental contaminants (Butler and Roesijadi, 2001; Ringwood et al., 1999, 2002).

Starvation is another stressor encountered by oysters farmed in an intensive system, especially nutrient levels may be low and cause limitation in food availibility. Published results showed that partial or complete food deprivation could not only alter the biochemical composition of oysters (Whyte et al., 1990) but also decrease their metabolic activity (Rodhouse and Gaffney, 1984). Starvation could also change the immune capability of oysters, resulting in an increased respiratory burst, and decreased phagocytosis, aggregation and hemocyte count, thereby possibly making them more susceptible to disease and parasites (Funakoshi, 2000; Hégaret et al., 2004).

Apart from the aforementioned environmental changes that oysters normally experience in the wild, farmed oysters are also challenged by the mechanical disturbances introduced during sorting, grading, transportation, etc. These farming practices could further compromise the oysters' ability to perform essential life functions in the field (Li and Vandepeer, 2002). It has been demonstrated in mussels that some post-harvest processing activities can significantly impair the stability of the lysosomal membrane (Harding et al., 2004a).

In this study we use NRR time as an indicator to evaluate the effects of starvation, simulated rotational grading, and their combinations on lysosomal membrane stability in Pacific oysters.

A9.3. Materials and Methods

A9.3.1. Oysters

Two batches of oysters farmed in Smoky Bay, South Australia were used in this study. The first batch was imported in early October 2004 and used in the simulated rotational grading experiments while the second batch was imported in late October 2004 for the starvation experiments. Their height (mean ± S.E, n = 100 oysters), length (mean ± S.E, n = 100 oysters) and condition index (CI, mean ± S.E, n = 12 oysters) were 110.89 ± 6.12 mm, 53.99 ± 0.69 mm and 64.13 ± 4.54; and 97.63 ± 1.02 mm, 49.79 ± 0.70 mm and 75.24 ±

5.31 for the first and second batches, respectively. Methods for oyster transportation and maintenance prior to experiments were the same as those used by Zhang et al. (2006).

The condition index was calculated with the same method described by Zhang et al. (2006).

A9.3.2. Neutral red retention assay

The neutral red retention assay used in this study was the same as Zhang et al. (2006). Four oysters were opened and sampled each time point. After sampling the oyster was discarded.

A9.3.3. Simulated rotational grading

After acclimation for 7 days the oysters were subjected to simulated grading in a plastic barrel (45 cm in length and 27.5 cm in diameter) by hand for one of the following 4 durations: 1, 1.5, 3 and 9 minutes. The barrel was rolled at a rate of approximate 50 rpm along the ground in a 15°C room. After simulated grading had ceased the oysters were put back into their acclimation tank and maintained there until haemolymph samples were collected at 0, 10, 20 and 30 mins, and then at 1, 2, 3 and 6 hrs. At the start of each treatment haemolymph samples were collected from the oysters in the acclimation tank and used as a control.

A9.3.4. Starvation

At the start of the experiment the NRR times (0 day control) were determined using the haemolymph sampled from the oysters that had acclimated in the laboratory condition for 7 days. The starvation and feeding experiments were then started and lasted for 42 days. During this period oysters belonging to different experiments were maintained separately in 15°C tanks on a flow through system with seawater filtered to 1 μm by using a 10 μm and a 1 μm cartridge sequentially. The algal mixture of *Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans* (approximately 2×10^6 cells mL^{-1}) was harvested and supplied to the fed group at a rate of about 1.0 L per oyster per day.

On day 42 both the starved and fed oysters were subjected to 3 min simulated grading and then maintained in 15°C tanks before being sampled. The procedures for haemolymph sampling were the same as described in *A9.3.3. Simulated rotational grading*. The haemolymph collected from the oysters prior to the simulated grading treatment were used as experimental controls.

A9.3.5. Statistical analysis

ANOVA, Tukey's *b* and Dunnett test (2-sided) multiple comparisons were conducted using SPSS 10.0 statistical software after first checking data for normality and homogeneity. The level for statistical significance was set at $\alpha = 0.05$.

A9.4. Results

A9.4.1. Simulated rotational grading

Simulated grading duration and post grading recovery duration had significant effects on lysosomal membrane stability in Pacific oysters ($F_{3, 101} = 35.456$, $P < 0.001$, $F_{8, 101} = 65.335$, $P < 0.001$, respectively) (Fig. A9.1). The differences in NRR times between controls of different treatments were not significant ($P = 0.29$).

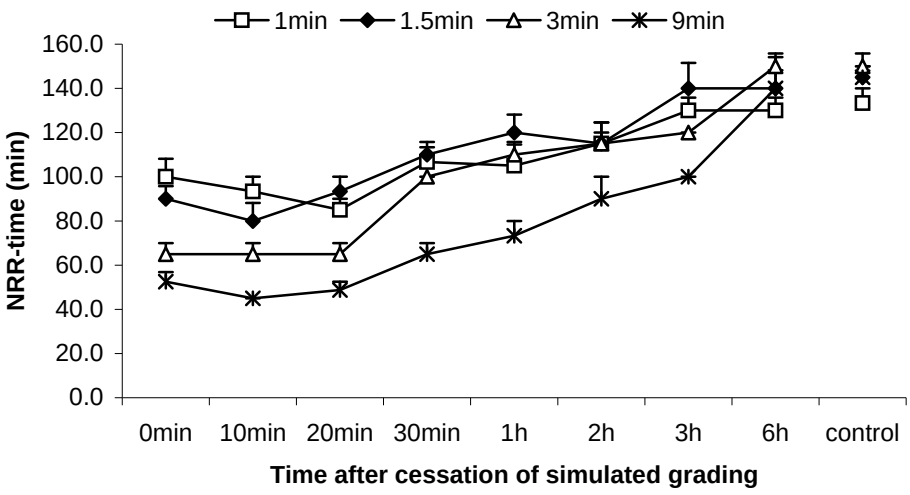

Fig. A9.1. Effects of simulated rotational grading on NRR times in lysosomes of *C. gigas*. Oysters were acclimated and allowed to recover (post-grading) in 15°C tank. Bars express the mean + S.E., n = 4 oysters.

The oysters that underwent longer durations of grading had lower NRR times at most time points. The lowest retention times for all treatments occurred at approximately 10 to 20 min after cessation of grading. After this period the NRR time for all treatments began to increase gradually with time. Within the first 20 min post-grading the NRR times in the 1 and 1.5 min grading groups remained at similar levels, but significantly higher than those in the other grading duration groups ($P < 0.05$). For example, the NRR times at 0 min were 52.5 ± 4.3, 65.0 ± 5.0, 90.0 ± 5.8, and 100.0 ± 8.2 min for 9, 3, 1.5, and 1-minute grading treatments, respectively.

The NRR times in the oysters graded for 1 and 1.5 min returned to control levels 3 hr after the cessation of simulated grading, while the NRR times in the 3 and 9 minute grading groups required an extra 3 hr to return to control levels.

A9.4.2. Starvation

A9.4.2.1. Condition index (CI)

The condition index (mean ± S.E, n = 12 oysters) in starved oysters decreased significantly ($P < 0.05$) from 75.24 ± 5.31 on day 0 (controls) to 57.86 ± 4.01 after 42 days. The CI in fed oysters, on the other hand, increased from 75.24 ± 5.31 to 82.16 ± 4.63 during the same period, although the increase was not significant ($P > 0.05$).

A9.4.2.2. Effects of starvation on lysosomal stability

After treatment for 42 days the NRR times in both starved and fed oysters decreased significantly ($P < 0.001$), dropping from 140.0 ± 0.0 at the start of the experiment (initial control) to 85.0 ± 9.6 and 115.0 ± 5.0 in starved and fed groups, respectively, although the ability to retain neutral red in fed oysters was still significantly higher than that in starved animals ($P < 0.05$, n = 4 oysters).

A9.4.2.3. Effects of 3 min simulated rotational grading on starved and fed oysters

Both starvation and post-grading recovery duration had significant effects on lysosomal responses to 3 min simulated grading, although the effect from their interaction was not significant ($F_{1, 57}$ =33.071, $P < 0.001$, $F_{9, 57}$ = 10.429, $P < 0.001$, $F_{9, 57}$ = 1.223, $P > 0.05$, respectively).

Fig. A9.2 shows that the 3 min grading treatment resulted in a significant decrease in NRR times in both fed and starved groups. As was the case in the simulated rotational grading experiments in *13.4.1* the lowest retention times appeared around 20 min after cessation of grading, after which the NRR time rose gradually with time. After 3 min grading the ability to retain neutral red in lysosomes in starved oysters was significantly lower than that in the fed animals at most time points ($P < 0.05$).

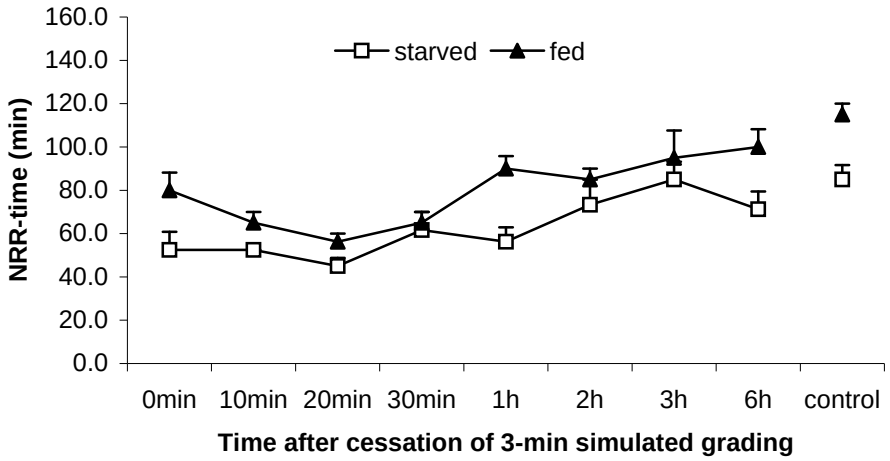

Fig. A9.2. Effects of 3 min simulated grading on NRR times in lysosomes of oysters that were starved for 42 days or fed for 42 days. Oysters were maintained and allowed to recover (post-grading) in 15°C tank. Bars express the mean + S.E., n = 4 oysters.

A9.5. Discussion

The parameters used in the simulated rotational grading experiments in this study, such as rotational speed (50 rpm), diameter of the barrel (27 cm) and grading in air were selected according to the settings currently adopted by oyster growers to grade oysters in South Australia. Results from NRR assay showed that the duration of grading and the duration of the subsequent recovery after grading had a significant effect on the stress response in Pacific oysters. In general, the oysters undergoing longer durations of grading had lower NRR times. The NRR times in the oysters graded for shorter periods returned to the control level more quickly than those graded for longer periods. These results are similar to the findings of Lacoste et al. (2001) by using circulative noradrenaline and dopamine concentrations as indicators to assess the effect of mechanical stress (simulated grading) in Pacific oysters. The study conducted by Lacoste et al. (2001) showed that the animal's level of response reflected both the mechanical stress intensity (100 rpm or 300 rpm) and duration (1 min, 15 min or 60 min) at a speed of 300 rpm. It is noted that higher speeds were used in that study and their experiments were conducted in seawater. In mussels, Harding et al. (2004a) found that lysosomal stability was also associated with the post-harvest processing activities such as washing, declumping and storage practices, etc.

At the start of the starvation experiment the NRR time of the oysters acclimated in 15°C water for 7 days was 140 min (the 0 day control). This NRR time was similar to the highest stabilised NRR times (about 145 min in 15°C seawater) found in our recent investigation into the temperature effects on lysosomal stability (Zhang et al., submitted).

After 42 days the condition index and NRR time of starved oysters were significantly lower than those of fed oysters and the 0 day control, dropping from 75.24 ± 5.31 and 140.0 ± 0.0 min on day 0 to 57.86 ± 4.01 and 85.0 ± 9.6 min on day 42, respectively. The decrease in CI after starvation found in this experiment was similar to results in the starvation experiments conducted by Riley (1976) on the same species. The 3 min grading experiments with starved and fed oysters showed that the NRR assay was suitable for assessing the effects of multiple stressors. After simulated grading the ability to retain neutral red in lysosomes decreased significantly in both starved and fed groups. Furthermore the NRR times in starved oysters were significantly lower than that in the fed animals.

In the fed oyster group, although their condition index increased after 42 days, their NRR times decreased significantly within the same period, from 140 ± 0 min to 115.0 ± 5.0 min. The decrease in NRR time was probably caused by gametogenesis or nutritional imbalance during this period, or a combination of both. On day 42 the fed oysters appeared to have substantially developed gonad with creamy-white colour and were ready to spawn (the un-treated animals spawned two days later when the water supply was changed to ambient seawater of about 19°C). It was found in cultivated mussels that the seasonal pattern of stress response in lysosomes was mainly associated with reproduction cycle (Harding et al., 2004b). However, high lysosomal enzyme activity could also be associated with stressful periods of reduced food quality in the field experiments on mussels (Tremblay et al., 1998). Only three algal species were used in the current study. Therefore, further research is required to investigate the reasons for the decrease in the lysosomal stability in Pacific oysters during this period.

A9.6. Acknowledgements

This work was partially supported by the Fisheries Research and Development Corporation. The authors are grateful to Mr Kriston Bott for his assistance in algal culture and experiment maintenance. We also thank Mr Gary Zippel of Zippel's Enterprises Pty Ltd for providing the oysters used in this study and the two anonymous reviewers of this manuscript.

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APPENDIX 10: THE EFFECT OF DIFFERENT GRADING EQUIPMENT ON STRESS LEVELS IN PACIFIC OYSTERS, *CRASSOSTREA GIGAS* (THUNBERG)

This chapter was submitted as:

Qu, Y., Li, X., Yu, Y., Vandepeer, M., Babidge, P., Clarke, S., Bott, K., Li, H. The stress effects of some grading equipment on Pacific oysters, *Crassostrea gigas* (Thunberg).

A10.1. Abstract

Variations in oyster survival rates on South Australian farms have been attributed mainly to differences in grading practices. In this study the effects of three commonly used graders in South Australia - Rotary, Flat Bed and Inside/Out were investigated using post-grading catecholamine levels as a stress indicator. Significant increases ($P < 0.05$) in circulating noradrenaline and dopamine levels were observed in oysters graded by the Flat Bed and the Inside/Out graders. The circulating noradrenaline concentrations in oysters subjected to the Flat Bed grader were significantly higher ($P < 0.05$) than those observed in the Rotary graded oysters and close to being significantly higher ($P = 0.052$) than those levels measured in the Inside/Out graded oysters. The circulating noradrenaline levels in oysters subjected to the Inside/Out grader were, in turn, significantly higher than those recorded in the Rotary graded oysters. These results suggest that among the three graders used in South Australia, the Flat Bed induced the highest stress levels in oysters and the Rotary grader the lowest.

Keywords: Catecholamine; Grading equipment; Pacific oyster

A10.2. Introduction

The Pacific oyster, *Crassostrea gigas*, is the most commercially important molluscan species cultivated and has been ranked second among the top ten most cultured aquatic species in the world (FAO, 2003). This species was first introduced to Australia, from Japan in the 1940s and currently is mainly farmed in Tasmania and South Australia (Roch, 1999; ABREA, 2003).

The equipment currently used in oyster farming has mainly been developed to improve production efficiency. It has been demonstrated that some of this equipment could cause stress to animals, resulting in poor performance in the field (Spencer et al., 1992). The ability to detect a stress response in oysters is therefore of

interest as it could be used as a tool to help farmers select those practices that cause the least stress, thereby possibly improving production efficiency.

When animals are exposed to a stress, a physiological response is initiated stimulating the sympathetic nervous system to release catecholamines - a group of chemicals acting as neurotransmitters that are amine derivatives of catechol (2-hydroxyphenol), including adrenaline, noradrenaline and dopamine (Montpetit and Perry, 1999). Release is rapid and circulating levels can change instantly with stress (Randall and Perry, 1992). Recent studies have shown that when oysters are stressed their neuroendocrine responses involve the release of noradrenaline and dopamine in their hemolymph (Lacoste et al., 2001b, Lacoste et al., 2001c, Lacoste et al., 2002). Furthermore, the physiological changes imposed by stress have been observed to influence host-pathogen interactions in juvenile oysters and increase their vulnerability to *Vibrio splendidus* (Lacoste et al., 2001a).

In Australia, mechanical grading is the main technique used by the oyster industry to separate stock according to their size. Due to variation in growth rates animals are graded multiple times throughout the production cycle, depending on the stocking densities used by farms at different stages. Once oysters are separated according to various size ranges, they are either harvested for sale or subsequently returned back to the lease for on-growing. During each grade oysters are subjected to multiple mechanical disturbances that include handling, transportation, and the grading itself. These disturbances could produce a transient state of stress in oysters (Lacoste et al, 2002). It was also suggested that differences in oyster survival between farms were partially due to the difference in the grading practices between them. However, it is not known if these grading methods produce any stress to oysters and if they do, whether the levels produced differ between graders. To investigate this we measured catecholamine levels in the blood from oysters subjected to different graders using the same operational settings as on the farms.

A10.3. Methods

A10.3.1. Experimental animals

The Pacific oysters used in this study were obtained from Smoky Bay, South Australia. The animals used in each experiment were originally from the same batch of spat and had been maintained as a group during the grow-out stage. The average size of the oysters used in the simulated grading experiment was 63.63 ± 0.53 mm (SE; n = 50 oysters) in height. The oysters used in the on-farm grading experiments were 101.59 ± 1.08 mm (SE; n = 50 oysters) in shell height and approximately 3 years of age.

The animals used in the air exposure and simulated grading experiments were transported to the South Australian Research and Development Institute's Aquatic Sciences Centre over night in a refrigerated container. On arrival the oyster were cleaned with a brush, and then put into a 450 litres tank on a flow through system where the oysters were acclimated at 15°C for 20 days prior to being used in the experiment. During this period the animals were fed with an algal mixture of *Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans*.

The oysters used in the on-farm grading experiments were collected directly from the intertidal lease each morning prior to emersion and transported to a shed equipped with air-conditioning. The time from when the oyster baskets were taken out of the water until they reached the first shed was approximately 30 minutes and the transportations were completed prior to sunrise. Upon arrival the oysters were taken out of the baskets and randomly divided by hand into three equal groups, with 200 individuals per group. Each group was then stored in a separate plastic tub prior to being used in the grading experiment. The three graders used in this study were owned by commercial oyster farmers and kept in different sheds. The sheds were approximately 2 minutes drive apart. The oysters were transported to the different sheds by car prior to the start of the experiment. During the 6-day experimental period the air temperature in the first shed (with air-conditioning) varied less than one degree Celsius, with an average temperature of 17.9°C. The maximum difference between water temperature at the time when oysters were collected and the air temperature in the first shed visited on the same day was 2.2°C (less than 1.5°C on other days). The maximum temperature difference between the first shed and other sheds on any given day was 2.7°C (less than 2°C on other days).

A10.3.2. Air exposure and simulated grading experiments

In the air exposure experiment, the animals that had been acclimated at 15°C for 20 days were randomly divided into two groups and were respectively exposed to 15°C and 27.5°C air temperatures. The animals were sampled at 0 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h and 48 h post air exposure.

In the simulated grading experiments oysters were taken from the acclimation tank (15ºC) and put into a plastic barrel (45.0 cm in length and 27.5 cm in diameter). The barrel was rolled by hand at a rate of approximately 50 rpm along the ground in a 15ºC temperature controlled room. After 3 min simulated grading the oyster were taken out of the container and left in the temperature-controlled room until being sampled. Blood samples were collected at 0 min, 5 min, 10 min, 15 min, 20 min, 30 min, 40 min, 50 min and 60 min post-grading. Blood collected from the oysters taken directly from the acclimation tank was used as a control.

A10.3.3. On-farm grading experiment

Three commonly used graders in South Australia were selected for the grading experiment: the Inside/Out (Fig. A10.1), the Rotary (Fig. A10.2) and the Flat Bed graders (Fig. A10.3). The Inside/Out grader consists of three different mesh size wire cylinders, with the centre cylinder having the largest mesh size. When grading the oysters are placed into the centre cylinder via a conveyor belt and the smaller oysters fall through onto the second and then third cylinders depending on their size. The oysters larger than the mesh size of the centre cylinder will be removed via the end opening. The centre cylinder is 74 cm in diameter and rotates at a speed of 15 rpm. The Rotary grader used in this study consists of a long PVC cylinder with even size holes at each of its five sections and set on a slight downward angle. The diameters of the holes increase along the length of the cylinder. During grading the oysters are placed into the top of the cylinder via a conveyor belt. When the cylinder rotates the oysters travel down falling through the holes similar or larger to their size. The oysters larger than the largest holes in the cylinder are removed via the end opening. The cylinder is 30 cm in diameter (internal) and rotates at a speed of 26 rpm. The Flat Bed grader consists of two flat mesh screens (86 cm wide and 250 cm long) that are attached to an electric motor-driven shaking device, with the larger mesh screen being placed on the top. The shaking device induces a reciprocating vertical lift of 1 cm at a frequency of 360 times per minute. The screens are longitudinally divided by a frame (6 cm wide) in the middle. When grading, the oysters are placed onto the top screen via a conveyor belt. The smaller oysters fall through onto the lower screen and then the base depending on the size of the oysters. Oysters then are deposited in size specific collection channels. In this study the largest mesh size and hole size were standardised between graders according to their grading range and an oyster size class that could not fall through the largest mesh size or holes was used. The grading duration for each grader was determined by averaging the results from three trials. In each trial 200 oysters per grader were used and delivered into the grader by a conveyer belt used on the farm. The grading duration was measured from when the last oyster fell into the grader until when the last oyster had emerged from the grader. The average grading duration calculated for each grader was: 1 min 30 sec for the Inside/Out grader, 3 min for the Rotary grader and 34 sec for the Flat Bed grader.

Fig. A10.1. The Inside/Out grader used in this study.

Fig. A10.2. The Rotary grader used in this study.

Fig. A10.3. The Flat Bed grader used in this study.

The experiment was repeated 6 times over 6 days. Each day the order in which the graders were used was rotated to account for any possible air exposure duration effect on oysters. The first grading trial was conducted at approximately 8.30 am. The three grading trials in each day were completed within 6 h from the time when the oysters were taken from the lease. After each grading, the oysters were placed in a shaded section of the shed until the blood was sampled. Blood samples were collected at 5 min, 10 min, 15 min and 3h post-grading. Blood samples collected before each grading trial were used as the control for that trial.

A10.3.4. Blood collection, catecholamine extraction and analyses

At each sampling time point, nine oysters were randomly selected and opened individually with an oyster knife within 30 sec. The hemolymph was then collected individually within the next 30 sec from the pericardial cavity using a 1 mL syringe with a 29-gauge needle. Hemolymph collected from 3 oysters (0.2 mL/individual) was pooled into one tube (replicate) and three replicates were collected at each time point. The blood samples (replicates) were then kept on ice and extracted immediately. The methods described by Li and Vandepeer (2004) for catecholamine extraction and HPLC analysis were used in this experiment. After centrifugation at 600 g at 4°C for 10 min, 0.5 ml supernatant was carefully removed from each tube and placed into a clean Eppendorf tube. Then 0.5 mL of TRIS buffer (1.5 M, pH 8.6) containing 0.07 M EDTA, 50 µL 5 nM sodium metabisulfite, 100 µL internal standard – dihydroxybenzylamine (DHBA) of 10 ng mL^{-1}

in concentration and 10 mg acid-washed alumina were added to each tube. The sample was mixed for 15 min and then centrifuged at 1000 g for 2 min. The supernatant was discarded and 1 mL of distilled water added to the alumina. The sample was mixed for 10 min and then centrifuged for 2 min at 1000 g. The supernatant was discarded and 100 µL of 0.2 M acetic acid was added. The sample was then mixed for 10 min and centrifuged for 2 min at 1000 g. The supernatant was stored at below -20°C. The extracts were analysed within 2 weeks. Chemical separation employed a Waters Symmetry C_{18} column (150×3.9 mm, 5 µ) with a mobile phase containing 50 mM sodium dihydrogenphosphate, 50 mM citric acid, 0.05 mM EDTA, 3 mM sodium chloride, 0.4 mM octanesulfonic acid and 5% methanol at a flow rate of 1 mL/min. The pH in the solution was 3.0. A Waters 2465 electrochemical detector was used at 0.7 V with sensitivity set at 5 nAFS.

A10.3.5. Statistical analysis

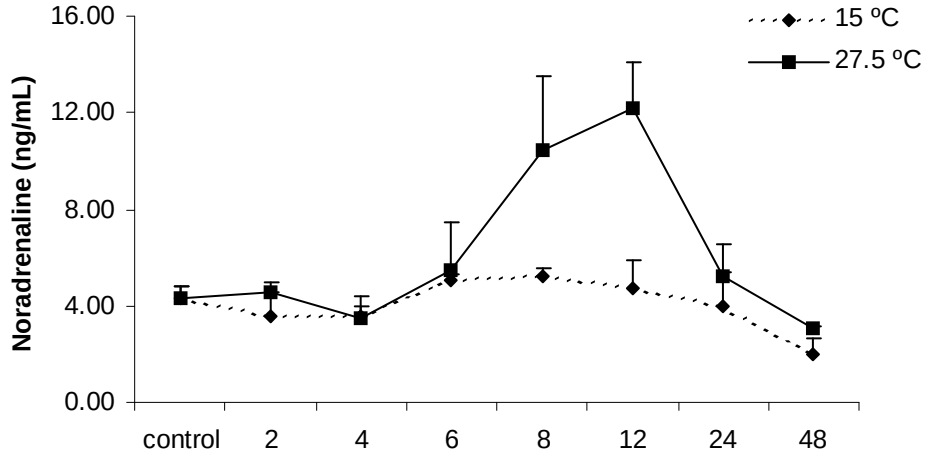
Data were analysed by ANOVA using SPSS 13.0 software, after first testing for normality and homogeneity. Where a significant difference was found Tukey's *b* multiple comparisons were used to compare means. All tests were performed at the 5% significance level. The results are presented as means ± standard errors.

A10.4. Results

A10.4.1. Air exposure and simulated grading experiments

The circulating noradrenaline levels of oysters exposed to 15°C air temperature fluctuated around a similar level to the control (4.30 ± 0.53ng/mL) for the duration of the experiment (Fig. A10.4A). In comparison, the noradrenaline levels in oysters subjected to 27.5°C air exposure remained at the control level for the first 6 h and then increased to 10.46 ± 3.04 ng/mL at 8 h post air-exposure. The level increased further to 12.17 ± 1.90 ng/mL at 12 h, which was significantly different from the control ($P < 0.01$), before returning to the control level at 24 h (Fig. A10.4A). Noradrenaline levels were affected significantly by both air exposure duration and air temperature and their interactions ($F_{7, 32} = 5.42$, $P < 0.001$; $F_{1, 32} = 10.86$, $P = 0.02$; $F_{7, 32} = 2.41$, $P = 0.04$, respectively). The dopamine levels in the blood collected from the oysters exposed to 15ºC and 27.5ºC air temperatures generally remained slightly higher than the control level (0.16 ± 0.02 ng/mL) over the experimental period ($P > 0.05$ for both temperatures; Fig. A10.4B).

A.



B.

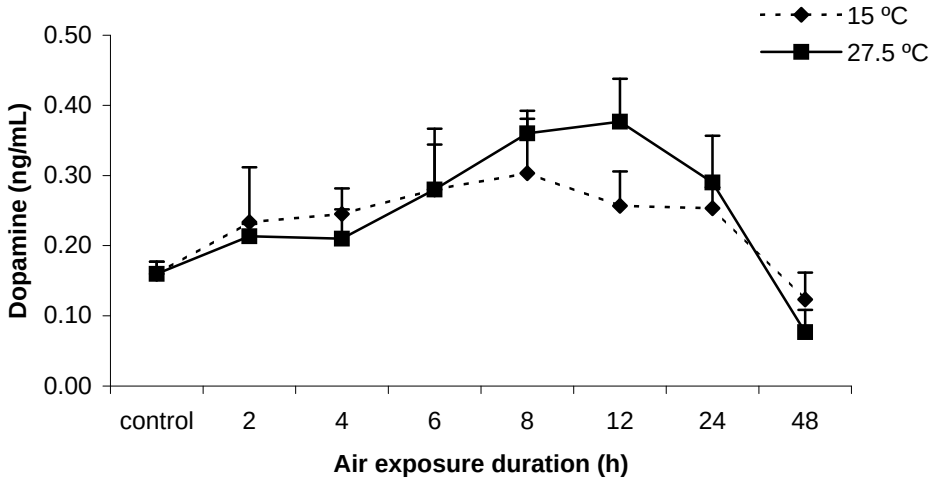
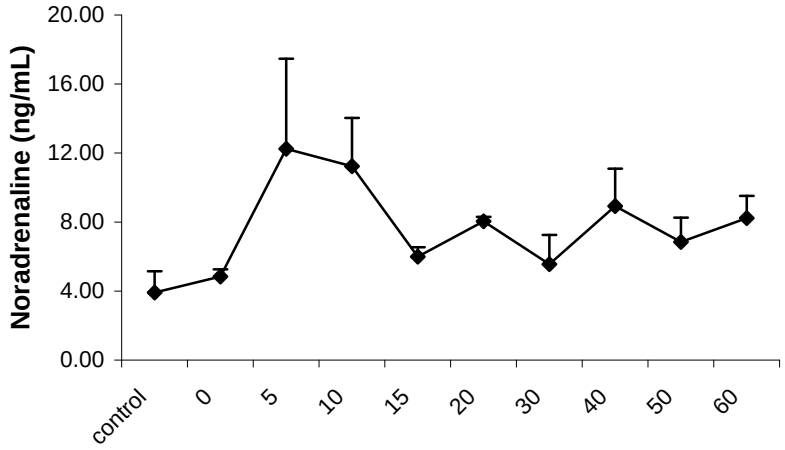


Fig. A10.4. The effects of exposure to 15ºC or 27.5ºC air temperatures on catecholamine levels in the blood of Pacific oysters (*C. gigas*). The animals had been acclimatised at 15ºC water prior to being used in the experiments. Data are mean + standard error (n = 3 samples). A: Noradrenaline; B: Dopamine.

Simulated grading elicited significant noradrenaline responses in oyster hemolymph (Fig. A10.5A). After 3 min of grading the noradrenaline level increased to 12.25 ± 5.22 ng/mL at 5 min post grading, which was significantly higher ($P < 0.05$) than the level in the control (3.92 ± 1.24 ng/mL). Noradrenaline then returned to a similar level to the control at 15 min post grading and remained slightly higher than the control until the end of this experiment at 60 min. The dopamine levels remained around the control level (0.42 ± 0.06 ng/mL) for the duration of the experiment ($P > 0.05$; Fig. A10.5B).

A.



B.

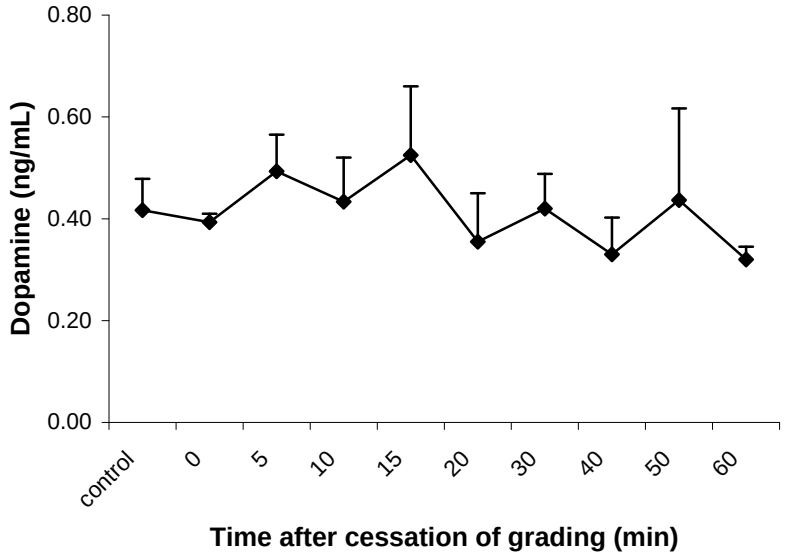


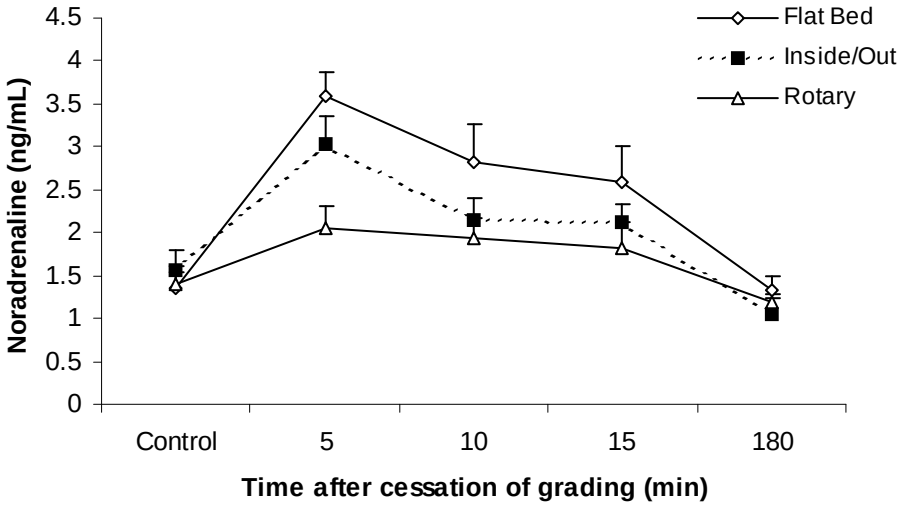
Fig. A10.5. The effect of 3 min simulated grading on catecholamine levels in the blood of Pacific oyster (*C. gigas*). The experimental oysters were acclimatised and graded at 15ºC. Data are mean + standard error (n = 3 samples). A: Noradrenaline; B: Dopamine.

A10.4.2. On-farm grading experiment

No significant differences were found among the control catecholamine values collected from different graders on the same day or among values collected from the same graders on different days ($P > 0.05$). As

such, pooled data from the 6 days were used to analyse the effects of different graders on circulating catecholamine levels. Grading by the Flat Bed and the Inside/Out graders elicited significant increases in circulating noradrenaline 5 min post-grading ($P < 0.05$). Levels ranged from 1.35 ± 0.20 and 1.57 ± 0.22 ng/mL (controls) to 3.59 ± 0.29 and 3.02 ± 0.32 ng/mL, respectively (Fig. A10.6A). The noradrenaline levels in the Flat Bed graded oysters returned to the control level ($P = 0.066$) at 15 min post-grading while the levels in the Inside/Out graded oysters returned to the control level at 10 min post-grading ($P = 0.47$). The Rotary grader also elicited an increase in noradrenaline from 1.40 ± 0.22 ng/mL to 2.06 ± 0.25 ng/mL at 5 min post-grading (Fig. A10.6A), however, this increase was not significant ($P = 0.28$). Similar patterns were also observed for the circulating dopamine levels. The Flat Bed and the Inside/Out graders elicited a significant increase ($P < 0.05$) while the Rotary grader induced slight but insignificant changes ($P > 0.05$; Fig. A10.6B). Differences in circulating noradrenaline concentrations were significant between the oysters graded by the Inside/Out and Rotary, and the Flat Bed and Rotary graders ($F_{1, 168} = 3.98$, $P = 0.048$ and $F_{1, 167} = 13.609$, $P < 0.001$, respectively; Fig. A10.6A). The difference in noradrenaline between the oysters graded by the Flat Bed and Inside/Out was very close to being significant ($F_{1, 169} = 3.837$, $P = 0.052$; Fig. A10.6A). No significant differences were observed in dopamine values between the oysters graded by different graders ($P > 0.05$ for all cases; Fig. A10.6B).

A.



B.

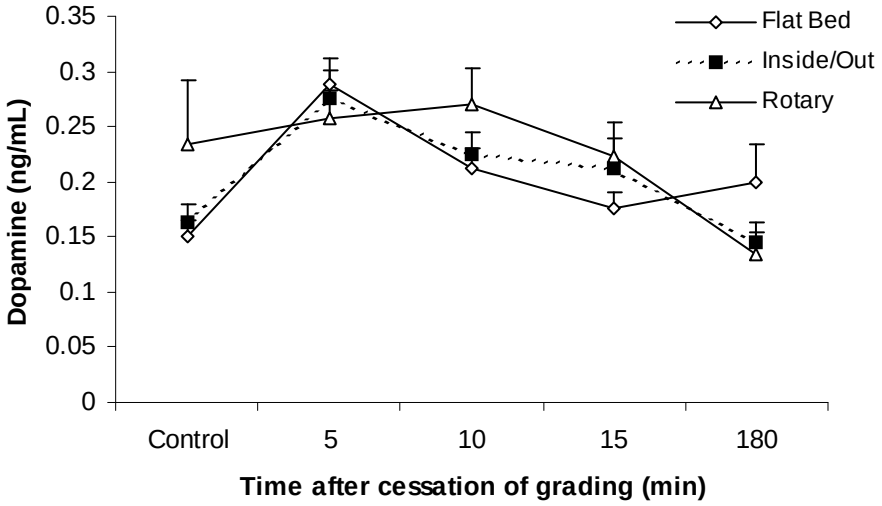


Fig. A10.6. The effects of different grading equipment on catecholamine levels in the blood of Pacific oysters (*C. gigas*). A: Noradrenaline; B: Dopamine. Data are means and standard errors (n = 18 samples).

A10.5. Discussion

Currently grading and/or sorting are the most labour intensive practices in oyster farming. Several types of equipment have been developed to improve the efficiency and accuracy of grading, however, the use of this equipment could also create extra stress(es) to oysters, which could in turn affect their performance in the

field, increasing their susceptibility to mortality, especially after spawning. To minimise the potential effects caused by graders, a tool that can quantify stressors at sublethal levels is critical. The study by Lacoste et al. (2001c) showed that the catecholamine response to stress in Pacific oysters reflected both intensity and duration of the stressor, indicating that this response could potentially be used as an indicator for this purpose. However, prior to its application in commercial settings, environmental factors that could influence this assessment need be minimised or standardised. These include air exposure duration, air temperature and the sampling time points.

The circulating noradrenaline concentrations in the oysters used in this study fell within the ranges reported for bivalve blood in previous studies (1-20 ng/mL), while the dopamine concentrations were slightly lower than the range reported in previous reports (0.4-3 ng/mL) (Osada and Nomura, 1989; Dietz et al., 1992; Lacoste et al., 2001b).

The air exposure experiment showed that when oysters were exposed to an air temperature (15°C) similar to the water temperature they had been acclimated to, their circulating catecholamine levels remained at the control level until the end of the experiment. In comparison, noradrenaline levels in oysters exposed to 27.5ºC air temperature changed slightly for the first 6 h and then increased significantly before returning to the basal level. Dopamine concentrations followed similar patterns to noradrenaline in the same treatments. However, the changes were not significant. Results from experiments conducted by Lacoste et al. (2001c) showed that a direct water temperature increase from 15ºC to 28ºC immediately elicited significant catecholamine responses in the Pacific oyster hemolymph which remained at high levels for nearly two days. The delays in the catecholamine response to air temperature changes in this study might be because the speed of heat exchange between oysters and the surrounding air were much slower than that between oysters and the surrounding water. The results from Qu (2004) showed that when oysters were exposed to an air temperature that was different from the water temperature they had been acclimated to, it took more than 2 h for water inside their shells to change to the new temperature. This means that when oysters are exposed to a new air temperature they experience a gradual temperature change rather than an immediate temperature change that they normally experience in water. Results from the air exposure experiments in this study also indicated that the effect from air exposure in the simulated grading experiment and the on-farm grading experiments were minor. Further study would be required to clarify if catecholamines in oyster hemolymph respond to gradual and rapid temperature changes differently.

In this study 3 min of simulated grading induced a nearly threefold increase in circulating noradrenaline concentration, which is consistent with the report by Lacoste et al. (2001c). They found that 1-minute shaking was sufficient enough to elicit a fivefold increase in circulating noradrenaline and a two-fold increase in circulating dopamine in oysters. It was noted that the rotation rate used in their study was 300 rpm, which was much higher than the rate used in this study (50 rpm). In addition, their experiment was conducted in water.

The circulating noradrenaline and dopamine levels in the oysters subjected to the Inside/Out and the Flat Bed grading increased significantly from control levels 5 min after grading. In comparison, the levels of noradrenaline and dopamine in oysters graded by the Rotary grader only increased slightly. These results suggested that when the Flat Bed and the Inside/Out graders were used according to the settings applied on the farms they created significant stresses to oysters. Circulating noradrenaline levels in the oysters graded by the Flat Bed grader were significantly higher or very close to significantly higher than those graded by the other two graders. The noradrenaline levels in the oysters graded by the Inside/Out grader were, in turn, significantly higher than those in the oysters graded by the Rotary grader. The experiments conducted by Lacoste et al. (2001c) showed that the catecholamine response to stress in oysters reflected both the intensity and duration of the stressor. It is, therefore, reasonable to conclude that the stress created by the Flat Bed grader was stronger than those created by the other two graders. The Inside/Out grader, in turn, elicited a stronger stress than the Rotary grader. Spencer et al. (1992) found that the simulated grading of oysters by shaking them in air for 2 minutes at a monthly interval had a large effect on their growth and survival, especially when the oysters were kept out of water overnight.

The measurement of catecholamine levels has provided a useful insight into the stress effects of grading on Pacific oysters and the differences in stress caused by three graders commonly used by oyster farmers in South Australia. The establishment of a stress measurement tool is the first and critical step in the production of guidelines for low stress practices. It is anticipated that the optimal operation settings for each type of graders could be established by using information on both stress responses of the animals and the efficiency and accuracy of the grader. Comparison of the optimal settings between graders could also provide information for further improvement of grading technology for the oyster industry.

A10.6. Acknowledgements

This work was partially supported by the Fisheries Research and Development Corporation. The authors are grateful to Mr Gary Zippel of Zippel's Enterprises Pty Ltd for providing the oysters and one of the graders and to Mr Jeff Holmes and Mr Geoff Hammat for providing the other two graders used in this study.

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APPENDIX 11: PRELIMINARY STUDY ON THE EFFECTS OF DIFFERENT GRADING METHODS ON LYSOSOMAL MEMBRANE STABILITY IN PACIFIC OYSTERS, *CRASSOSTREA GIGAS* (THUNBERG), USING THE NEUTRAL RED RETENTION ASSAY

A11.1. Abstract

The neutral red retention (NRR) assay was used to evaluate lysosomal membrane integrity in the hemolymph of Pacific oysters (*Crassostrea gigas)* graded by three types of graders commonly used in SA – the Inside/Out, Rotary and Flat Bed graders. The Inside/Out grader was observed to have a significant effect on NRR time (P = 0.013), whilst the rotary grader had a close to significant effect (P = 0.052), suggesting that both these graders elicited a stress response in oysters.

A11.2. Introduction

Determination of best farming practices to optimise oyster performances on farms is one of major outcomes anticipated from this project. To achieve this, information on 1) the accuracy; 2) the efficiency; 3) the costs, 4) the mechanical damage and 5) the stress impacts of each practice would be required. Data on the first four components could be obtained from oyster growers or by conducting a standardised experiment across farms using different farming methodologies. The biggest challenge to the project is the development of a tool(s) that can be used to compare the levels of stress created by farming practices so that different practices or different techniques used for the same practice can be compared between farms. At the start of the project it was anticipated that when a method to prevent the rapid degeneration of catecholamines in oyster blood was developed the catecholamine assay used by Lacoste et al. (2001) to monitor oyster stress levels would be able to be applied in the field. Stress caused by grading was the first farming practice investigated. The results from the first two field experiments showed that the catecholamine test could detect a stress response in oysters graded by different graders. However, a power analysis on the data from these field experiments indicated that to differentiate the levels of stress created by different graders would require approximatly 75 replicate measurements. This would need at least 5 staff skillful at oyster blood collection and catecholamine extraction to work on oyster farms for 25 days, which is likely to be beyond the capacity of any project.

At the same time work was being conducted to assess the potential of catecholamines as a stress measurement tool, a Master's student from the Dalian Fisheries University, China, was investigating the merits of another stress test on Pacific oysters known as the neutral red retention assay (NRR). Results from his experiments

showed logical responses when the animals were exposed to the stressors assessed (for details refer to Chapters 10 and 11 of this report) (Zhang et al., 2006; Zhang & Li, 2006). In addition, this test is relatively simple and can be applied easily on farms using a microscope with a light source that is suitable for observing red particles. The aim of this study was to evaluate its suitability as a stress indicator by assessing stress responses in oysters graded by different graders.

A11.3. Methods

A11.3.1. Experimental animals and mechanical Graders

The Pacific oysters used in this experiment were from the same batch of oysters used in the experiments conducted in Chapter 13 "The effect of different grading equipment on stress levels in Pacific oysters, *Crassostrea gigas* (Thunberg)". The three types of graders (Inside/Out, Rotary and Flat Bed) and the operational settings used in those experiments were also applied in this study (for details refer to Chapter 14).

A11.3.2. Mechanical grading experiment

Each morning the oysters required for this study were collected from a farmer's lease prior to emersion. After being taken out of the baskets the oysters were immediately immersed in six 40 L plastic tubs pre-filled with seawater from the lease. They were then transferred back to a shed where the air temperature was controlled by an air conditioner. The time from when the oysters were taken out of the water on the lease until they reached the shed was approximately 30 minutes each day and the transportations were completed prior to sunrise. The water temperatures in the tubs were about 18.5°C during the grading experimental periods.

At approximately 9 am 30 oysters were randomly taken from the plastic tubs (5 individuals per tub) and used in the first experiment with the first grader. The same procedure was repeated with the 2nd and 3rd graders. All grading experiments were completed before 12 pm each day. The order in which the three graders were used was rotated during the three days the experiment was conducted to account for any potential effects caused by differences in the holding period in the plastic tubs. The graders were owned by commercial oysters farmers and kept in different sheds. The sheds were approximately 2 minutes drive apart. The oysters were transported to the different sheds in water in plastic tubs by car. Prior to each grading, a sample of blood was taken from 7 randomly selected oysters to use as a control. Within 10 minutes of the completion of each grading blood was sampled from another 7 randomly selected oysters. Each sample was collected within 1 min after opening the oyster. Blood samples with low hemocyte counts were not used for subsequent analysis.

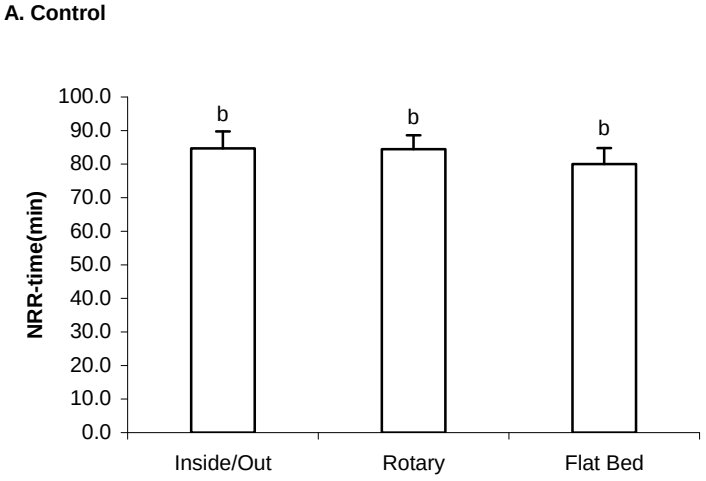
A11.3.3. Neutral red retention assay and statistical analysis

The methods used in this study for neutral red retention assay procedures and statistical analyses are the same as those described in Chapter 10 "Effects of Water Temperature and Air Exposure on the Lysosomal Membrane Stability of Hemocytes in Pacific Oysters, *Crassostrea gigas* (Thunberg)" (Zhang et al., 2006).

A11.4. Results

During the three-day experimental period no significant differences in NRR of control oysters were found between different graders on the same day or between days for the same grader ($P > 0.05$). As such, pooled data from the 3 days were used to analyse the effects of different graders on NRR times. The average NRR times in the control oysters for the three days was 83 min (Fig. A11.1A). After grading the NRR time was significantly reduced in the oysters graded by the Inside Out grader ($P = 0.013$) and nearly significantly reduced in those graded by the Rotary grader ($P = 0.052$). The difference in NRR time between the oysters graded by the Flat Bed grader and the corresponding control was not significant ($P = 0.839$).

A significant difference in NRR times was found among the oysters that had been graded by different graders ($P = 0.015$). The Flat Bed grader resulted in a significantly higher NRR time than the Rotary grader ($P = 0.04$) and the Inside/Out grader ($P = 0.03$) (Fig. A11.1B) indicating they experienced less stress. The Rotary grader and the Inside/Out grader produced similar levels of stress ($P = 0.698$). The NRR time in oysters graded by the Flat Bed grader was significantly lower on the second day compared to the other two days ($P = 0.001$).



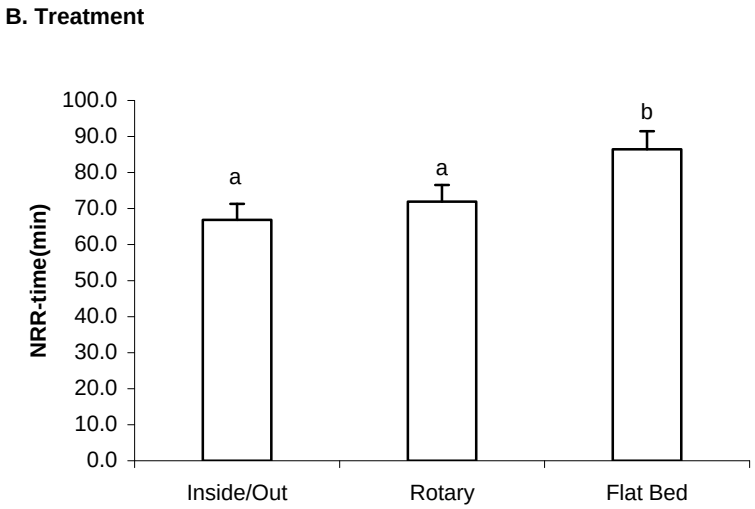


Fig. A11.1. Comparison of NRR times between oysters graded by different graders and between control oysters. Bars represent the mean + S.E., n > 16. Common letters denote no significant difference in NRR time. A: Control; B: Treatments.

A11.5. Discussion

In unstressed cells lysosomes accumulate and retain neutral red dye for extended periods of time. In stressed cells the neutral red dye flows into the cytosol much faster. In mussels, Harding et al. (2004) found that lysosomal membrane stability was associated with different post-harvest processing activities such as washing, declumping and storage practices. They have successfully applied the NRR assay to determine if various conditions to which cultured mussels had been exposed would induce a stress response at the sub cellular level. In the current experiment the NRR times in the controls were 83 min, which is slightly lower than the values reported by Zhang et al in 2006 (about 100 min) using similar water temperatures. The discrepancy between these two studies could be caused by the fact that oysters used in the study by Zhang et al (2006) had been acclimated in a consistent environment for 7 days prior to being used in the experiments, while those used in the current study had been removed from an intertidal lease and then placed in 40 L tubs of seawater. Collection from the intertidal lease and transport back to the farmer's shed is likely to have caused the oysters stress, thus explaining the higher NRR times of the control oysters in this study compared with those in Zhang et al. (2006).

After grading, the NRR times in the oysters graded by the Inside/Out grader were significantly shorter than in the control oysters whilst the NRR time in oysters graded by the Rotary grader were close to being significantly shorter than in the control oysters. These observations agree with the finding by Zhang & Li

(2006) that 1.5 min and 3 min of simulated grading also significantly reduced NRR times in the same species. Zhang & Li (2006) also found that 3 min of grading had a significantly greater effect on the NRR time in oysters than 1.5 min. In the current study although the grading duration of the Inside/Out grader (1.5 min) was shorter than that of the Rotary grader (3 min), the stress created by the former was slightly higher than that of the later based on NRR times. The inner layer of the Inside/Out grader is 74 cm in its diameter while the Rotary grader is 30 cm. According to the operational settings used in this study the distances the oysters travelled during the grading period would be 35 m and 25 m in the Inside/Out and the Rotary graders respectively. If the times the oysters rolled per unit distance were the same in both graders the oysters' rolling times and rolling speeds would be much higher in the Inside/Out grader than those in the Rotary grader. Our study using circulating noradrenaline levels as a stress indicator to differentiate the stress responses in oysters graded by different graders also showed that the Inside/Out grader induced stronger stress on oysters than the Rotary grader (Qu et al, submitted for publication). A Study by Lacoste et al. (2001) suggested that when Pacific oysters were subjected to rotational treatment in water their circulating catecholamine levels could reflect both treatment intensity (100 rpm or 300 rpm) and duration (1 min, 15 min or 60 min).

The NRR time in oysters graded by the Flat Bed grader was significantly higher than that in oysters graded by the Rotary and the Inside/Out grader but not significantly different from the NRR in the control oysters, suggesting that this was the least stressful grading method. However, this interpretation should be treated with caution because significant difference in post-grading NRR times between days were found in the oysters graded by this grader. The NRR time on the second day was significantly lower than on the other two days although the NRR times in the controls were similar during the three days ($P = 0.30$). The reason for this difference is not clear, although blood collection on the second day occurred between 5 and 10 min post-grading while on the other days blood collections occurred immediately after grading. A delayed time period might be required by the oysters to fully express their stress response after shaking for 30 sec with the Flat Bed grader. Further investigation on the NRR response pattern in oysters subjected to shaking stresses is required. The experiment conducted by Qu et al (submitted for publication) with catecholamine tests showed that the highest stress response for all three graders occurred at 5 min post grading and that the Flat Bed grader induced the highest stress response of all three.

The results from this study indicate that graders commonly used by farmers can affect lysosomal membrane stability significantly. It is, therefore, reasonable to assume that by using the NRR assay as a stress response indicator, various farming practices could be assessed and improved to minimize their stress effects. This could in turn help improve oyster performances on farms.

A11.6. References

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APPENDIX 12: BACKGROUND CATECHOLAMINE LEVELS IN FAMILY LINES OF PACIFIC OYSTERS *CRASSOSTREA GIGAS* (THUNBERG) GROWN IN SOUTH AUSTRALIA

A12.1. Abstract

In South Australia it has been reported that certain commercially produced family lines have a higher mortality level when compared to normal farm stock. In this preliminary study background catecholamine levels were used to determine if there are any differences between oyster lines currently being evaluated in South Australia and whether they correlate with oyster performance (growth rate, mortality etc.). The results showed differences in background noradrenaline and dopamine levels between 9 family lines were not significant (P = 0.21 and 0.07 respectively). However, a large amount of variation was found within some lines, suggesting that the sample size (3 replicates per line) used in this experiment might have been too small to differentiate the lines. Proposed stress challenge tests designed to compare the stress responses between family lines were cancelled due to early termination of the project.

A12.2. Introduction

In South Australia some oyster farmers have decided to grow family lines selectively bred for one or more commercially important traits through an oyster breeding program organised by the Australian Seafood Industries Pty Ltd (ASI). In the Pacific Oyster Farming Survey some farmers reported higher mortalities in selectively bred family lines than their normal stock, suggesting that susceptibility to mortality might have a genetic basis. Higher mortality in these lines has also generated interest in including survival traits in the existing breeding program and in developing a technique that could assess this trait under sublethal levels.

Catecholamine appears to play an important role in the physiological status of molluscs (Lacoste et al., 2001b) and have both stimulatory and inhibitory actions on immune functions in aquatic animals (Montpetit and Perry, 1999). Catecholamines consist of three chemicals - adrenaline, noradrenaline and dopamine. When vertebrates are stressed the catecholamines are released into their circulation system. As a result their heart beats stronger and faster, blood pressure rises, more blood flows to the brain and muscles, the liver releases stores of energy as a sugar (glucose) the body can readily use, the rate of breathing increases and airways widen, and digestion activity slows. These actions direct more oxygen and fuel to the organs most active in responding to stress, mainly the brain, heart, and skeletal muscles (Montpetit and Perry, 1999). The aim of this study was to determine; differences in background catecholamine levels between some family lines currently

being evaluated in South Australia, differences in stress responses between these family lines and correlations between these differences (if any) and observed performance. The experiments using phenoloxidase assay with similar aim were conducted simultaneously by Mr Manning as part of his Honours project at the Flinders University, Adelaide, South Australia. Details refer to his thesis in Appendix 3: "The Role of Phenoloxidase in Stress Response of Pacific Oyster, *Crassostrea gigas*".

A12.3. Methods

A12.3.1. Family lines oysters

The Pacific oyster family lines used in this experiment were provided by the ASI and obtained from a farm in Smoky Bay, South Australia. The oysters were transported to SARDI Aquatic Sciences Centre in a refrigerated container (5°C). On arrival the oysters were cleaned and placed into a tank on a flow-through system (15°C), where they were acclimated for 20 days prior to the experiment. During this period, the oysters were fed microalgae produced by a continuous bag culture system.

The 9 family lines used in this experiment were bred at the same time, managed using the same practices and maintained in the same environment. They were identified as A, B, C, D, E, F, G, H and I. Data collected by the ASI showed that family lines A, B and C were fast growing lines whilst D, E and F were slow growing lines. Family lines G and H were high survival lines and lines B, F and I were low survival lines. The control consisted of standard farm stock of the same age from the same farm.

A12.3.2. Catecholamine analysis

Three replicates were sampled from each of the 9 family lines and the control. For each replicate a pooled blood sample from 3 oysters (0.2mL/individual) was collected and kept on ice. The blood was sampled from the pericardial cavity using 1 mL syringes with 29-gauge needles. All oysters were opened within 30 sec, and their hemolymph collected within 30 sec after opening.

The extraction of catecholamine was conducted immediately after the completion of sampling. The methods described by Li and Vandepeer (2004) for catecholamine extraction and HPLC analysis were used in this study. For details refer to Chapter 14 "The effect of different grading equipment on stress levels in Pacific oysters, *Crassostrea gigas* (Thunberg)" (Qu et al. submitted for publication).

A12.3.3. Statistical analysis

All data was analysed by one-way ANOVA following testing for normality and homogeneity (SPSS 13.0 software). A probability level of $P < 0.05$ was considered statistically significant.

A12.4. Results

A12.4.1. Catecholamine levels in different family lines

No significant difference ($P = 0.21$) in the noradrenaline levels was found between the family lines tested (Fig. A12.1), although the levels in lines B and D appeared to be slightly higher than in the other lines (2.69 ± 1.94 and 2.34 ± 1.50 ng/mL respectively). Both these lines also had a large amount of variation among their replicates. Family line G had the lowest level of noradrenaline out of all the families tested (0.86 ± 0.18 ng/mL).

As for noradrenaline, differences in the background dopamine levels between the family lines were not significant ($P = 0.07$; Fig. A12.2). The control appeared to have the highest level (0.42 ± 0.26 ng/mL). A large amount of variation was also observed among its replicates. Line G had the lowest level of dopamine (0.09 ± 0.01 ng/mL).

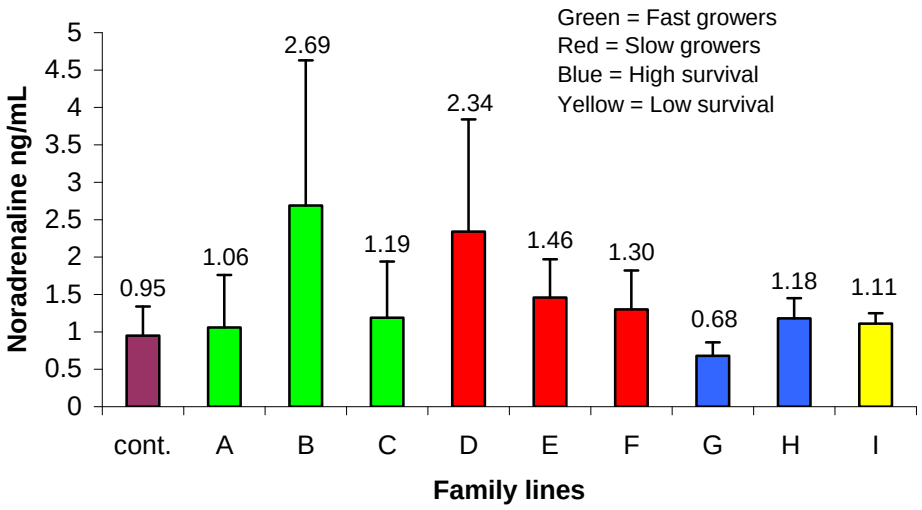

Fig. A12.1. The noradrenaline levels in the blood of Pacific oyster family lines. Data expressed as mean + standard error.

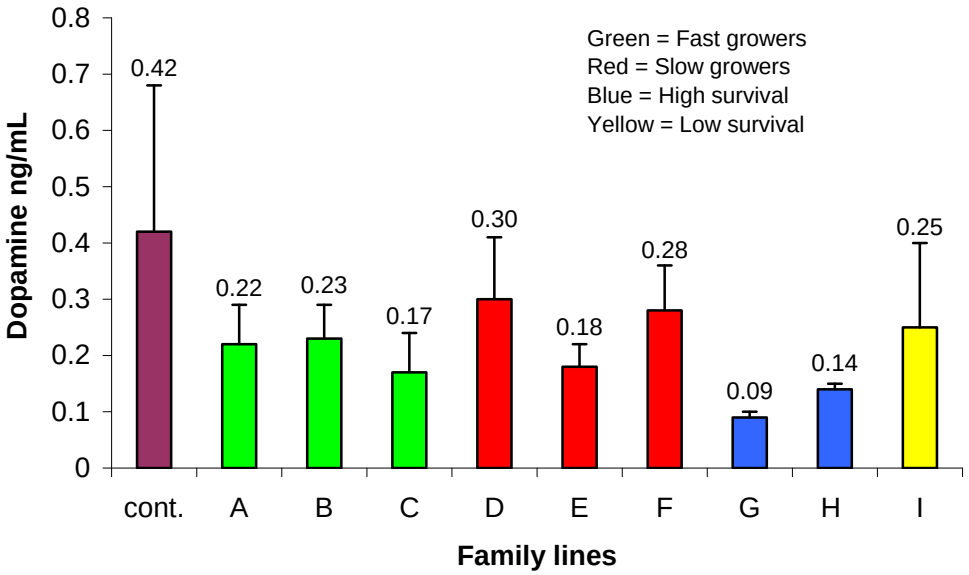

Fig. A12.2. The dopamine levels in different oyster family lines. Data expressed as mean + standard error.

A12.5. Discussion

In this study, no significant difference in the background noradrenaline or dopamine levels was found between the 9 family lines tested. The circulating noradrenaline concentrations fell within the ranges reported for the control oysters (1-4 ng/mL), while the dopamine concentrations were slightly lower than that in previous reports (0.4-0.8 ng/mL) (Lacoste et al., 2001b; Qu et al., submitted for publication). Family lines B and D showed slightly high background noradrenaline levels while the control showed the highest background dopamine level. The line G had the lowest background levels for both chemicals. The lines showing the highest background levels of catecholamines also had the highest variation among replicates. It should be noted that only three replicates were sampled from each family, with each replicate consisting of a pooled blood sample from 3 oysters. Power analysis shows that at least 24 replicates would be required to differentiate among families. Therefore, the results from this preliminary study on family lines should be treated with caution.

Due to the lack of significant difference in background catecholamine levels between the family lines used in this study the correlations between the catecholamine levels and specific traits were not analysed. However, results from the studies by Lacoste et al revealed that a 30-ng/g injection of noradrenaline increased both mortality and accumulation of *Vibrio splendidus* in juvenile Pacific oysters (2001a) and noradrenaline could inhibit hemocyte phagocytosis *in vitro* at concentrations ranging between 0.1 and 10μg, reducing the immune

capabilities of Pacific oysters and increasing the level of vulnerability to pathogens (2001c). These results suggest that higher catecholamine levels could be associated with lower survival. Family G was noted to have the lowest levels of both noradrenaline and dopamine when compared to the other families, and the trait associated with this family was high survival (ASI). A study with larger sample sizes is required to further investigate this. In addition, it is anticipated that challenge tests to compare catecholamine production over time following a stress event would further improve the power of this analysis. Challenge test experiments were originally proposed as part of this study but were cancelled due to early termination of the project.

It is recommended that further work be required to determine if relationships exist between catecholamine levels and specific traits by using larger sample sizes and families or family lines having distinct trait expressions. If correlations could be established, catecholamines could then be used as a selection criterion in the oyster selective breeding program.

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APPENDIX 13

The Role of Phenoloxidase in Stress Response of Pacific Oyster, *Crassostrea gigas* (Thunberg)

By Andrew Manning

Supervisors
Dr John Carragher (SARDI)
Dr Ken Sanderson (Flinders University of S.A.)

Thesis submitted in fulfilment of a Bachelor of Technology (Aquaculture) with honours, at the Flinders University of South Australia
08/11/2004

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any University; and to the best of my knowledge does not contain any material previously published by another person except where due reference is made in the text.

Andrew Manning

November 8 2004

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Abstract

Organisms are constantly challenged or threatened by a variety of physical, environmental or biological perturbations commonly known as stressors. To maintain homeostasis, living organisms coordinate a series of physiological events that collectively constitute the stress response. Fundamentally, the same stress response mechanisms are present in both vertebrates and invertebrates. Stress responses can result in short-term deleterious consequences for non-essential functions such as growth and reproduction, by diverting energy toward essential functions, such as the immune system, which ultimately benefits the organism. Thus, stress responses can be measured at a number of levels – primary (neuroendocrine), secondary (metabolism), tertiary (consequential functional systems) and quaternary (death, or reproductive death).

Pacific oysters (*Crassostrea gigas*) have become a lucrative aquaculture sector in South Australia and constitute an area of economic importance in regional settings. Stress is unavoidable in an aquaculture context. Oysters have to be handled a number of times during growout and after harvest. Similarly, they are exposed to rapid and extreme changes in their environment. The impacts of these stressors on growth and survival of oysters is of great importance to oyster aquaculturists.

This study was sought to determine the reliability of measuring hemolymph phenoloxidase activity as a potential indicator of a tertiary stress response (the enzyme phenoloxidase reportedly having a role in immune function in invertebrates, including molluscs) in Pacific oysters (*C. gigas*). Two previously published methods (using different substrates – L-DOPA and HQ) for measuring phenoloxidase activity in oysters were used. Oysters were experimentally challenged with commonly encountered physical (grading & rumbling) and environmental (emersion, salinity & nutrition) stressors associated with commercial oyster culture.

The results from this study were that:

1. The L-DOPA assay used does not appear to measure levels of hemolymph phenoloxidase enzyme activity in Pacific oysters (*C. gigas*)
2. The HQ assay used does not appear to measure monophenolase activity of the phenoloxidase enzyme in the hemolymph Pacific oysters (*C. gigas*)
3. Both assays show the existence of significant levels of non-enzymatic phenoloxidase-like activity in the hemolymph of Pacific oysters (*C. gigas*)
4. Using these published spectrophotometric techniques the measurement of hemolymph phenoloxidase activity in Pacific oysters (*C. gigas*) does not provide a reliable indicator of stress

Further work is most likely required to develop and validate assays for specific phenoloxidase enzyme activity in the Pacific oyster (*C. gigas*). However, using established assays to measure other components of immune function (eg lysosomes) in *C. gigas* might reveal the interaction occurring between stress and immune function in this commercially important species.

Acknowledgements

John Carragher (SARDI)

Ken Sanderson (Flinders University)

Xiaoxu Li (SARDI)

Meegan Vanderpeer (SARDI)

Gary and Bruce Zippel from Zippels Oyster Leases

Suzanne Bennett (SARDI)

Yi Qu (SARDI)

Kriston Bott (SARDI)

Kathleen Soole (Flinders University)

Marine Biology Study Group (Flinders University)

Commonly Used Abbreviations

- PO: Phenoloxidase
- proPO: Prophenoloxidase
- L-DOPA: L-3, 4-dihydroxyphenylalanine
- HQ/MBTH: HQ/MBTH/FSW blank
- MBTH: 3-methyl-2-benzothiazolinone hydrazone
- HQ: 4-Methoxyphenol, 99%
- Oyster: Pacific oyster, *Crassostrea gigas*
- T: Tropolone
- FSW: Filtered salt water
- MFTW: Millipore Filtered Tap Water
- Abs: Absorbance
- ppt: parts per thousand
- @ 6 hours: assayed spectrophotometrically after a period of six hours
- SARDI: South Australian Research and Development Institute

Chapter 1: Introduction

The Pacific Oyster Industry in South Australia

The Pacific oyster *Crassostrea gigas* (Thunberg) was considered a farming option by the CSIRO when attempts to farm native mud oysters (*Ostrea angasi*) and the Sydney rock oyster (*Saccostrea commercialis*) were only partially successful (Olsen 1994). In 1968 the Minister for Agriculture examined and passed a proposal from the Director of Fisheries allowing the Japanese endemic Pacific oyster to be introduced to South Australia from disease free Tasmanian stock. The adult Pacific oysters acclimatised and grew well in South Australian waters and quickly developed into a lucrative sector of the state aquaculture industry. The majority of oyster farming in South Australia occurs on the West Coast of Eyre Peninsula ranging from Coffin Bay to Ceduna, as well as at Cowell and some offshore areas at Yorke Peninsula and Kangaroo Island (***Figure 1.1***). The introduction of Pacific oysters has seen significant expansion in production and feasible farming areas in South Australia since their arrival. At present there are fourteen classified oyster-growing areas in the state, which together total 27 harvesting areas between them (Lee 2002). In 2001/2002 the number of Pacific oysters sold from South Australia leases was 3,464,087 dozen equating to $13,303,114 (SARDI 2003). This production figure indicates that the oyster industry contributes an important part of South Australia's aquaculture revenue.

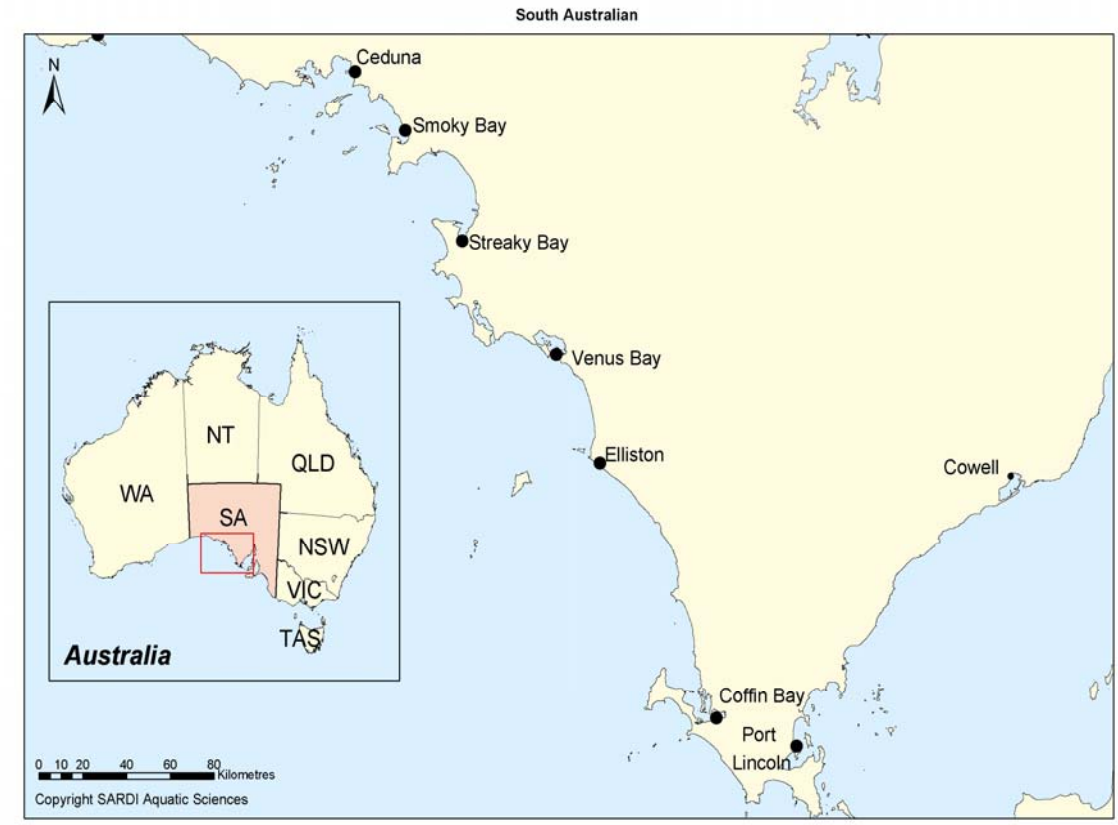


Figure 1.1: A map of the Eyre Peninsula indicating where the majority of Pacific oyster farms are situated in South Australia (Doonan 2004)

Line culture is recognised as the most common culture technique employed by oyster farmers in South Australia. Oysters are maintained in plastic baskets, referred to as units, and suspended by

rubber slings on a system of wooden racks that lie parallel to the soft substratum below. The advantages of rack culture systems include relatively cheap construction and maintenance costs, faster growth and easy access. Oyster spat is supplied to South Australian oyster growers by several hatcheries located in Tasmania and one small producing hatchery in South Australia. Harvested oysters are raised from spat (diameter 10mm) and are placed in plastic baskets within the intertidal zone in high numbers and are positioned along the wooden racks forming parallel rows. As spat increase in size their numbers are proportionately reduced and the basket mesh increases in size proportionate to the spat size, effectively promoting improved growth rates due to increasing availability of water flow, and thus food to the oysters. On average, oysters are graded between 1-6 times per year, according to size and categorized into their corresponding basket sizes where they remain for a period of 1-3 years, prior to harvesting and subsequent sale to local and interstate markets.

Stress and Stress Response in Organisms

Stress affects individual organisms and populations at all levels of organization ranging from biochemical changes to changes in community structure (Barton & Dwyer 1997). All living organisms survive by maintaining a complex dynamic equilibrium, known as homeostasis (Chrousos & Gold 1992). Homeostasis can be simply defined as the organism's ability to maintain a relatively constant internal environment, despite external fluctuations. Stressors are any external agent or stimulus that challenges the homeostatic control of an organism or threatens its survival (Colombo *et al* 1990). Factors that can threaten the homeostatic power of an organism include adverse physical or environmental events. As a result of stress-inducing conditions, organisms elicit a range of complex and coordinated physiological and behavioural changes that are collectively termed the 'stress response' (Lacoste *et al* 2001b; Lacoste *et al* 2002; Malham *et al* 2003). The stress response in all organisms is a fundamental process aimed at maintaining homeostasis, and is presumed to result in an increased chance of survival. Stress elicited from external sources is described as being either acute or chronic in nature. The stress response system is perceived as being adaptive for short term or acute stressors in which energy is directed away from non-essential functions and towards the 'fight or flight' stress response (Barton & Dwyer 1997). Chronic stress is caused by an extended duration of stress that inevitably causes drastic changes in the physiological state of the organism, thereby threatening its survival if it is unsuccessful in overcoming or avoiding the stressful stimuli (Barton & Iwama 1991).

The balanced state required for a successful adaptation to stress is controlled by counteracting/re-establishing forces, or adaptational responses, consisting of a repertoire of physiological and behavioural reactions that function to counteract the effects of the stress in an attempt to re-establish homeostasis (Chrousos & Gold 1992). During stressful stimuli, organisms divert bioenergetic resources away from non-essential functions, such as reproduction and growth, and redirect resources towards processes that aid to combat, adapt and overcome the stressful stimuli (Lacoste *et al* 2002). For example, respiration, glycolysis or lipolysis are normally increased to promote locomotion, behavioural or physiological responses (Lacoste *et al* 2002). In addition to these biochemical and physiological responses, the effects of the stress-response on the immune system are also diverse and mainly depend upon the animal species of choice, the nature of the stressor, the immune parameter under review and the physiological status of the animal at the specific time (Lacoste *et al* 2002).

Much attention has been directed towards researching the effects of stressful practices through the development of stress indicators, as a measure of stress response of animals in aquaculture. The majority of research has focused on the vertebrate stress response model by Barton & Peter (1982),

Barton & Iwama (1991) and Barton & Zitzow (1995), which examined the role of stress response in teleost fish. Stress response in an organism is described as being either primary, secondary, tertiary or quaternary in response. In teleost fish, primary responses to stressors are neuro-endocrine changes that are dominated by the activation of the sympatho-chromaffin system and the hypothalamo-pituitary-intererenal axis (Colombo *et al* 1990). The sympatho-chromaffin system acts on target cells by eliciting the release of cortisol and three catecholamines: adrenalin (A), noradrenalin (NA), and dopamine (DA) (Ottaviani & Franachesi 1996). Different proportions of these catecholamines are secreted depending upon the species of choice and its physiological status (Colombo *et al* 1990). Secondary responses in teleost fish are physiological consequences resulting from these primary neuro-endocrine changes in response to the secretion of glucocorticoids and catecholamines (Colombo *et al* 1990; Lacoste *et al* 2001b). Such secondary responses include modifications in intermediate metabolism, cardiovascular and respiratory changes, osmoregulatory alterations and immunosuppression (Colombo *et al* 1990). Finally, tertiary responses elicited by teleost fish affect the organism at the whole-animal level, which include changes in behaviour, food conversion efficiency, growth rate, reproductive success and mortality (Colombo *et al* 1990).

Determination of an organism's response to stress is measured by changes in levels of primary and secondary stress indicators. Fish appear to respond to stress in a manner that reflects both the severity and duration of the stressor (Strange *et al* 1978). As a result, mild transitory stressors evoke a short-lived response, whereas a severe and repeatedly applied stressor evokes an extended response of higher magnitude (Barton & Dwyer 1997). Such repeatedly elicited stressors have thus been shown by Barton *et al* (1986) and Maule *et al* (1988) to be cumulative and be directly correlated with the height of the response to stress. Severe and repeated stressors in fish have been shown to stimulate a tertiary stress response, thereby demonstrating changes in physiological state that are detrimental at the whole animal level (Barton & Iwama 1991).

Recent studies involving the application of physical and environmental perturbations (Lacoste *et al* 2001a, b; Malham *et al* 2003) have shown that molluscan species possess a primitive form of the vertebrate stress response model. In bivalve molluscs, this neuroendocrine or catecholaminergic response to stress involves circulating catecholamines, including dopamine (DA) and noradrenaline (NA), and neuropeptides such as adrenocorticotropic hormone (Lacoste *et al* 2001b; Lacoste *et al* 2002). It appears that several messengers, processing enzymes and controlled hormonal cascades contribute to the stress response in Pacific oysters (Lacoste *et al* 2001a). With the discovery of a correlation between several stress response indicators that exist in both teleost fish and bivalve molluscs, brings the possibility to link and apply known aspects of vertebrate stress response model to invertebrates, namely *C. gigas*. Established knowledge of the stress response system in vertebrates effectively allows researchers to understand how stress impacts immunological functions and resultant resistance to disease. However, in bivalve molluscs, information explaining how stress impacts immune function is required for comparative immunology purposes, because a number of pathogens pose the threat of exploiting stress-induced immunodeficiencies in stressed animals, thereby infecting and/or accumulating in their target organism (Lacoste *et al* 2002).

Stress Related Immunosuppression in Pacific Oysters

In an aquacultural context, disease is regarded as a threat to the economical performance of the cultured species (Colombo *et al* 1990). Studies on organisms from a variety of different phyla have demonstrated a clear link that exists between stress and increased mortality, supposedly due to a reduced disease resistance (Lee *et al* 1996; Malham *et al* 2003). Stress in teleost fish promotes immunosuppressive effects that increase the susceptibility of the animal to disease (Lacoste *et al* 2002). Evidence exists in teleost fish that stress induced elevation in circulating cortisol levels

renders fish more susceptible to pathogens commonly encountered in aquaculture systems (Colombo *et al* 1990). Such stress induced lowering of fish tolerance to pathogens can be explained by the immunosuppressive effects of cortisol, which is thought to interfere with the ability of lymphocytes to produce antibodies (Ellsaesser & Clem 1987).

Control of diseases in bivalve molluscs remains a serious challenge to both domestic and international shellfish industries (***Table 1.1***). Throughout their lifecycle, farmed oysters are repeatedly faced with stressful situations that require physiological adaptations to survive (Lacoste *et al* 2001b). Physical stressors associated with oyster farming include transport, handling, sorting, grading and associated physical damage resulting from poor handling practices. Environmental stressors that cultured oysters commonly encounter include poor water quality, emersion, salinity and temperature changes, food shortage, noxious chemicals, pathogens, predators and isospecific competitors. Both physical and environmental stressors are believed to cause an increase in oyster mortality due to a reduced resistance to disease, but currently little is known about the mechanisms that link physiological stress and disease resistance in molluscs. Environmental perturbations, such as sudden changes in salinity and emersion, have been shown to modulate immunocompetence in molluscan species (Fisher 1988). Physical perturbations have also been found to cause a transient state of stress in Pacific oysters (Lacoste *et al* 2002; Malham *et al* 2003), which effectively reduces the organisms capacity to combat the advances of infectious pathogens. *In vitro* investigations carried out by Lacoste *et al* (2002) revealed that noradrenaline, the principle catecholamine released into the haemolymph in response to stress, evokes an inhibitory effect on oyster immune defence functions. Currently, certain haemocyte immune defence functions including reactive oxygen intermediate production (Anderson 1994), phagocytosis (Canesi *et al* 2002) and antimicrobial peptide secretion (Mitta *et al* 1999, 2000) involved in host defence response are downregulated. It is suspected that during these times of immunosuppression pathogens opportunistically colonise the oyster at the height of immunocompetence. Results from Lacoste *et al* (2002) support the notion that, as in the vertebrate species, stress response can exert immunosuppressive effects on oyster immune functions and may explain why stress and the outbreak of disease are often linked in shellfish culture.

Pathogen	Disease	Oyster Species	Effect	Reference
Martellia sydneyi	QX disease	*S. glomerata*	Up to 98% mortality	Newton et al 2004
Maretllia refringens	Digestive gland (or Aber) disease	*C. gigas*	Mortalities up to 90%	Sindermann 1990
Perkinus marinus	Dermo Disease	*C. virginica*	Severe emaciation, mortalities up to 100%	Sindermann 1990
Haplosporidium nelsoni	MSX	*C. virginica*	Emaciation, reduced condition, mortalities up to 100%	Sindermann 1990
Haplosporidium costale	Seaside disease	*C. virginica*	Emaciation, discolouration, mortalities up to 40%	Sindermann 1990
Mikrocytos roughleyi	Winter Mortality disease	*S. glomerata*	Hihg mortalities	Newton et al 2004
Nocardia	Pacific oyster nocardiosa (PON)	*C. gigas*	Connective tissue degeneration, high mortalities	Paillard et al 1994
Vibrio bacterial sp.	Vibriosis	*C. virginica*	Up to 100% mortality	Sindermann 1990
□□ proteobacteria (CVSP)	Juvenile oyster disease (JOD)	*C. virginica*	Mortalities up to 90%	Boettcher et al 2000
Herpes-like virus	-	*C. gigas*	Feeding stopped followed by 60-100% mortality	Hine et al 1992
Bucephalus cuculus	-	*C. virginica*	Infection of gonad and digestive gland	Hopkins 1957
Gymnophalloides tokiensis	-	*C. gigas*	Growth retardation and reproductive inhibition	Sakaguchi 1967

Table 1.1: Common diseases that have been associated with commercial *Crassostrea* species culture.

Stress Indicator Criteria for Pacific Oysters

Stress associated with the culture of Pacific oysters is an unavoidable factor that occurs on a regular basis in the oyster industry. Such stress will never be completely abolished as techniques employed by farmers rely on their cost effectiveness, but efforts are being made to reduce their effects on the organism. Current research is attempting to develop new methods of measuring the changes in concentration and activity of certain chemicals produced in response to stressful stimuli. Such chemicals are termed 'stress indicators', which aim to elucidate the physiological condition of the organism when confronted with threatening agents. Oliver and Fisher (1999) defined an indicator as a sign or signal that relays a complex message, potentially from multiple sources, in a simplified and useful manner. These indicators provide a quantitative method for the determination of an organism's physiological state and may also enable the diagnosis of certain multiple acute, chronic or synergistic stresses (Oliver & Fisher 1999).

Stress indicators provide valuable mechanistic insights into sublethal physiological functions that will eventually elucidate the complex links that exist between stress, stress response and resistance to disease (Werner *et al* 2003). A successful stress indicator must be relevant to the type of stress applied and provide quantitative information that satisfies the specific objectives of the research (Oliver & Fisher 1999). A current challenge in Pacific oyster stress indicator research is being able to effectively elucidate reliable indicators, understand how they respond to specific stimuli and to what degree they effect an organism's physiological status when exposed to stressful stimuli. Measurements being made on bivalve haemocytes are considered attractive candidates for stress response indicators (Oliver & Fisher 1999). Haemocytes are regarded as the main cellular effectors elicited by oysters when confronted with stress inducing situations and are involved in inflammation, wound repair and encapsulation defence functions (Mitta *et al* 1999). Haemocyte activity and participation in various physiological functions supports the likelihood of measuring varying responses to stressful stimuli that in turn, provide a valid reflection on immunological changes occurring in an organism (Oliver & Fisher 1999). In recent years enzymatic activity has also been studied as an immune capacity indicator in many bivalve species (Xue & Renault 2000). Researchers are investigating the possibility of measuring specific enzymes that circulate in molluscan haemolymph and haemocytes as an indicator of stress. Such enzymes could allow farmers to effectively monitor stress in culture stock, and enable the identification of immunosuppressive farming practices to actively reduce the frequency of stress related oyster mortalities. The oxidative enzyme phenoloxidase is an enzyme that has been proposed as a possible molluscan stress indicator in cultured Pacific oysters.

The Role of Phenoloxidase in Pacific Oysters

Phenoloxidase (PO) (monophenol, dihydroxyphenylalanine: oxidoreductase; EC 1.14.18.1), also known as tyrosinase, is a multi-functional binuclear copper-cluster-containing enzyme from the oxidoreductase class that is ubiquitously present in biological systems (Pye 1974; Asokan *et al* 1997; Soderhall & Cerenius 1998; Dicko *et al* 2002). PO is a catalase enzyme, and is generally described as an enzyme which catalyses only catalytic reactions or which catalyses both catalytic and peroxidatic reactions (Coles & Pipe 1994). PO can be distinguished from related catechol oxidase, oxidase laccase and peroxidase enzymes by its substrate specificity and the type and number of catalytic coppers present on its active site (Dicko *et al* 2002). PO has been proposed as a component of internal defence and immunological recognition in invertebrates, including bivalve molluscs (Lanz *et al* 1993; Asokan *et al* 1997; Deaton *et al* 1999). PO is found in the mantle tissue of bivalve molluscs and is known primarily for its involvement in shell growth and repair mechanisms (Deaton *et al* 1999). Interest in PO as a defence enzyme has greatly increased since the discovery of its presence in

the haemolymph and haemocytes of many molluscan species (Coles & Pipe 1994; Deaton *et al* 1999). In higher invertebrates, PO is involved in sclerotization, wound healing and melanization of foreign bodies as well being involved in non-self recognition in host immune defence reactions (Coles & Pipe 1994; Asokan *et al* 1997). The potential exists for researchers to measure circulating PO levels and determine its specific role in immune defence mechanisms, which possibly may constitute an indication of stress in *C. gigas*.

Prophenoloxidase-Activating System

The terminal component of the prophenoloxidase-activating system is phenoloxidase, which is present as an inactive zymogen (proPO) (*o*-diphenol:O_2 oxidoreductase E.C. 1.14.18.1) in both bivalve haemolymph and haemocytes (Asokan *et al* 1997; Soderhall *et al* 1997). Asokan *et al* (1998) described the proPO activating system as a component of an immediate non-inducible system which functions in the recognition of foreignness and host immune defence. Soderhall and Cerenius (1998) have since purified the inactive monomer proPO, which has a molecular mass of 70-80kDa, prior to proteolytic activation, and a mass of 60-70kDa in an activated state. Activation of the prophenoloxidase system (***Figure 1.2***) is triggered by small amounts of microbial cell wall components including B-1,3-glucans (carbohydrates in fungal cell walls), peptidoglycans and lipopolysaccharides (LPS) from gram negative bacteria, which are involved in a complex cascade of native serine proteases among other factors (Asokan *et al* 1997; Asokan *et al* 1998; Sung *et al* 1998). Once the peptidoglycan recognition proteins or B-1,3-glucan recognition proteins bind to their appropriate elicitor, the proPO system becomes biologically active (Maramas *et al* 1996). Once the proPO system is triggered, it then produces immunologically active substances that include opsonins, cytotoxic molecules and specific signals for inducing different types of haemocyte-mediated host defence reactions (Asokan *et al* 1998). In summary, the process of proPO activation utilises a specific and highly sensitive activating enzyme, which initiates a cascade of serine proteases that results in proteolytic cleavage and system activation (Coles & Pipe 1994).

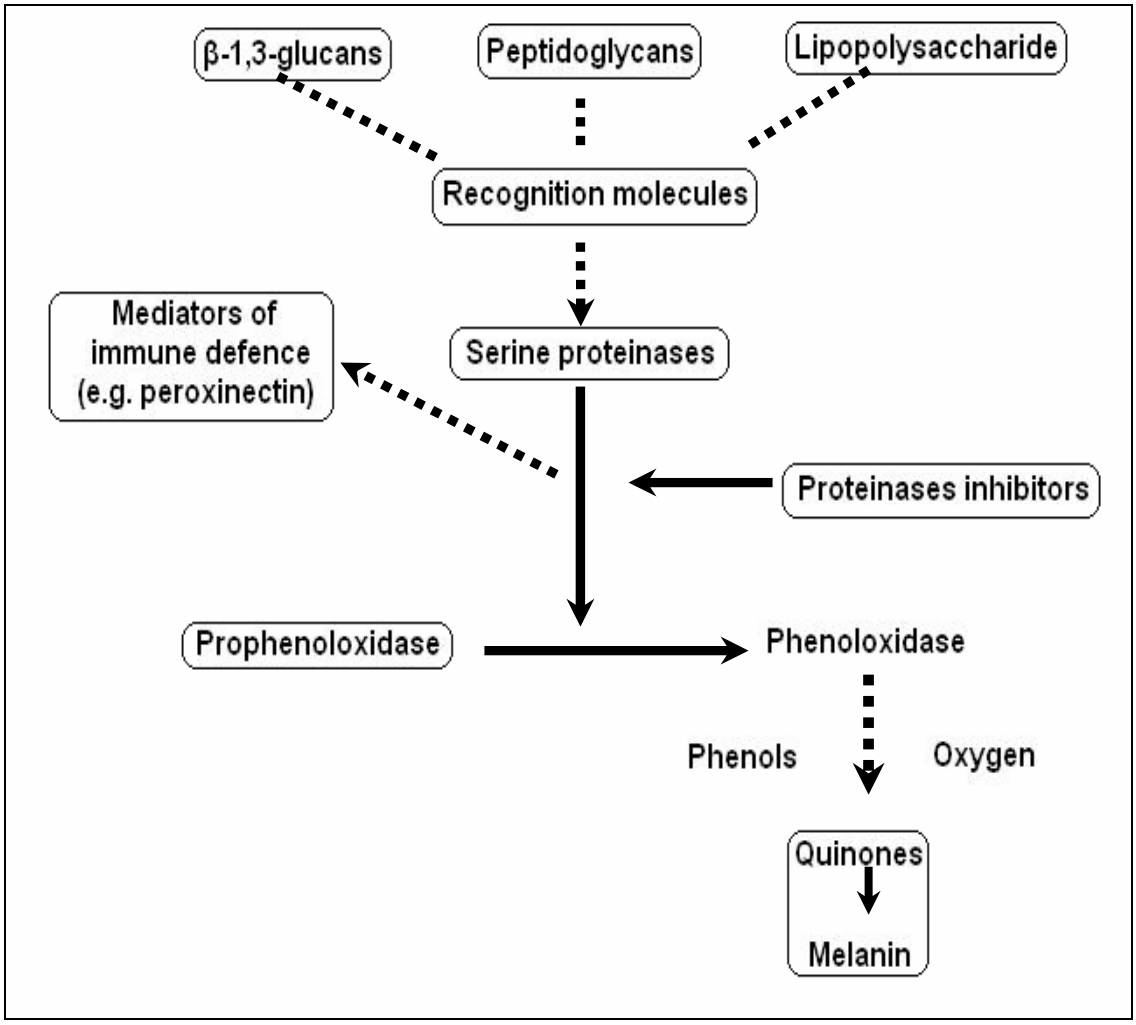


Figure 1.2: The process involved in prophenoloxidase (proPO) activation in invertebrates (Soderhall & Cerenius 1998)

The PO cascade in many invertebrates represents an important component of many invertebrate defence responses. Recognition components involved in the PO cascade can identify a variety of potentially pathogenic organisms and activate PO via the proteolysis of zymogen, proPO (Soderhall & Cerenius 1998; Newton *et al* 2004). The active form of proPO, phenoloxidase, which is a redox enzyme, catalyses two successive reactions in the presence of molecular oxygen (Espin *et al* 1997; Sung *et al* 1998; Lee & Soderhall 2002). The first reaction is the hydroxylation of substrates such as tyrosine to *o*-diphenols (monophenolase activity) followed by the oxidation of *o*-diphenols to reddish-pink *o*-quinones (diphenolase activity) (Soderhall & Cerenius 1998; Sritunyalucksana & Soderhall 2000; Lee & Soderhall 2002; Peters & Raftos 2003). *O*-quinone formation leads to the production of indole derivatives that are the cause of early stages of enzymatic browning (Girelli *et al* 2004). The *o*-quinones and indole derivatives produced then undergo nonenzymatic polymerisation and represent an important initial step in the biochemical cascade of spontaneous melanin biosynthesis (Soderhall & Cerenius 1998; Sung *et al* 1998; Lee & Soderhall 2002). These successive biochemical processes involved in melanization are demonstrated in ***Figure 1.3***.

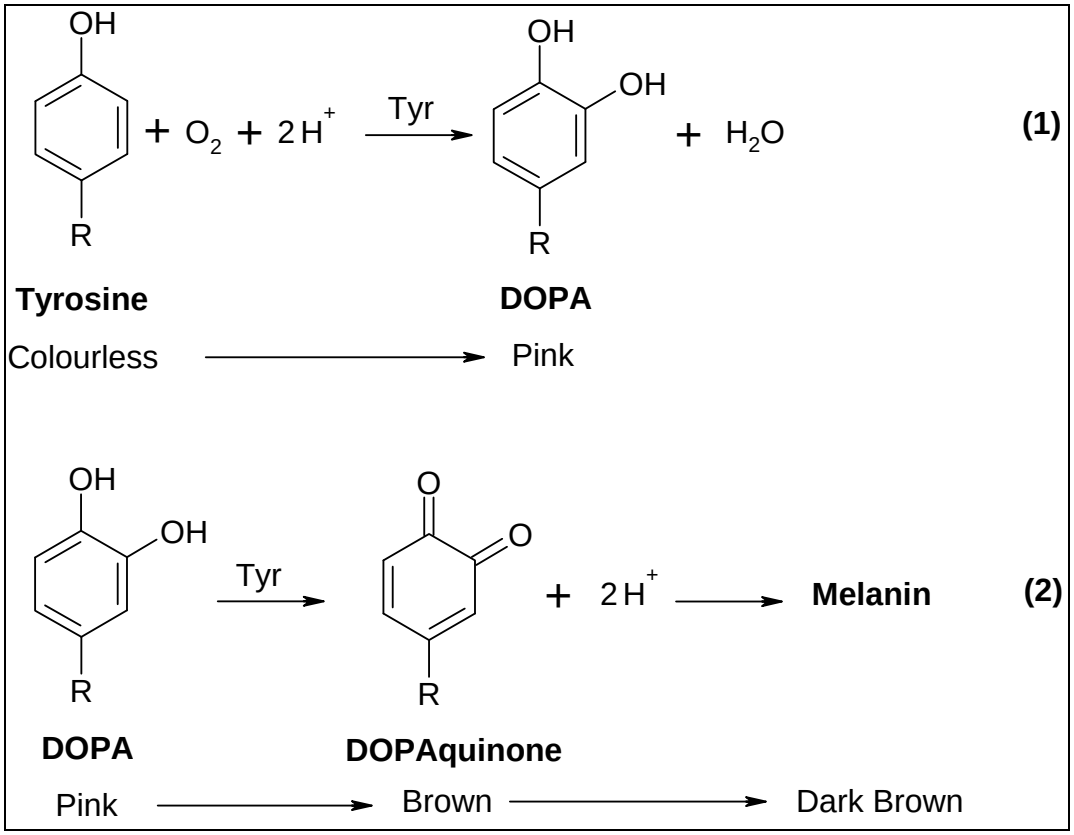


Figure 1.3: The biochemical processes involved in the ortho-hydroxylation of **(1)** tyrosine to L-DOPA (Monophenolase activity) and the oxidation of **(2)** L-DOPA to Dopaquinone (Diphenolase activity) catalysed by phenoloxidase. The Dopaquinone product then non-enzymatically polymerises to form melanin (Solomon *et al* 1996)

Melanization is widely considered a major innate defence mechanism in invertebrates that acts on pathogens and damaged tissues (Cerenius & Soderhall 2004). Various precursors to melanin biosynthesis demonstrate bactericidal effects that are involved with host immune defence (Coles & Pipe 1994). Such compounds include trihydroxyphenols, reactive oxygen intermediates and *o*-quinones, which react with numerous amino acids, proteins and various thiol and amino groups to yield cytotoxic and cytolytic effects (Pye 1974; Soderhall & Cerenius 1998). These intermediate compounds have the capacity to bind to foreign surface nucleophiles forming cross-linking complexes that effectively immobilise the pathogen by sequestering the microbe within a melanotic capsule (Nappi & Vass 1993). Melanin itself acts as a cation exchange polymer to protect tissues against oxidizing and reducing conditions, trapping free radicals and exerting highly toxic substances (Nappi & Vass 1993). Both melanin and *o*-quinones, produced from PO activity, have also been found to be highly reactive and toxic to microorganisms through the inhibition of bacterial enzymes (Sung *et al* 1998; Cerenius & Soderhall 2004).

In invertebrates, injuries or the presence of foreign microorganisms or parasites mediate melanin deposition around the injured tissue or foreign substance (Aspan & Soderhall 1991; Cerenius & Soderhall 2004). As a result, the pathogenic agent is blackened in the host haemolymph in a process called melanization, which acts to prevent and retard pathogen growth. However, the proPO system has to be regulated to avoid the potential deleterious effects of biosynthetic intermediates of cytotoxic activity (Lee & Soderhall 2002). PO has long been considered the principle enzyme

involved in the formation of melanin (Nappi & Vass 1993), however several other enzymes have also been reported in the synthesis of melanin (***Table 1.2***)

Enzyme	Substrate	Prosthetic Group	Reference
Tyrosinase EC 1.14.18.1	monphenols *o*-diphenols	Cu	Nappi & Vass 1993
Peroxidase EC 1.11.1.7	monphenols *o*-diphenols p-diphenols aminophenols	Heme	Nappi & Vass 1993
Catechol oxidase EC 1.10.3.1	*o*-diphenols	Cu	Shelby *et al* 2000
Laccase EC 1.10.3.2	*o*-diphenols p-diphenols aminophenols	Cu	Nappi & Vass 1993
Dopachrome tautomerase EC 5.3.2.3	dopachrome	Fe	Shelby *et al* 2000

Table 1.2: Various invertebrate enzymes that have been demonstrated to catabolize phenols to melanin (Nappi & Vass 1993)

Overall, intermediate compounds produced as a result of the proPO cascade clearly participate in defensive responses including; facilitating phagocytosis, nodule formation, wound healing, cytotoxicity and melanotic encapsulation of foreign agents (Peters & Raftos 2003; Newton *et al* 2004). It is widely recognised and acknowledged that melanization is a major innate defence system in invertebrates (Sritunyalucksana & Soderhall 2000), including bivalve molluscs such as *C. gigas*, therefore the potential for PO to serve as an indictor of stress is promising.

Measuring phenoloxidase activity

In previous research (Lanz *et al* 1993; Coles & Pipe 1994; Asokan *et al* 1997), enzyme assays used for the determination of PO activity in bivalve molluscs have primarily used L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate for the catalytic reaction. As mentioned earlier, PO catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). As a result, Espin *et al* (1995; 1997; 1998a, b) developed a new method for the determination of monophenolase activity of phenoloxidase. Monophenolase activity distinguishes phenoloxidase from other phenol-oxidizing enzymes, such as laccase (oxidizes only diphenols) and peroxidase (oxidizes catechols to quinones) (Maramas *et al* 1996).

This improved methodology is based on a coupling reaction occurring between 3-methyl-2-benzothiazolinone hydrazone (MBTH) and the quinone product of the oxidation of the substrate 4-Methoxyphenol (HQ) in the presence of phenoloxidase (Espin *et al* 1995). MBTH is a strong chromogenic nucleophile through its amino group, which attacks enzyme-generated *o*-quinones (Espin *et al* 1998a). The MBTH nucleophile traps the enzyme-generated *o*-quinones to render a stable MBTH-quinone adduct with high molar absorptivity (Espin *et al* 1997; 1998a). This method allows quantification of aminechrome formation (Espin *et al* 1995) via a pink colour change, which is measured spectrophotometrically (***Figure 2*** (**1**)). The stability of the MBTH-quinone adduct makes

this a reliable and suitable assay, reported as being a highly sensitive, reliable and precise method for determining monophenolase activity of phenoloxidase (***Figure 1.4***) (Rodriguez-Lopez *et al* 1994; Espin *et al* 1995; 1998a, b).

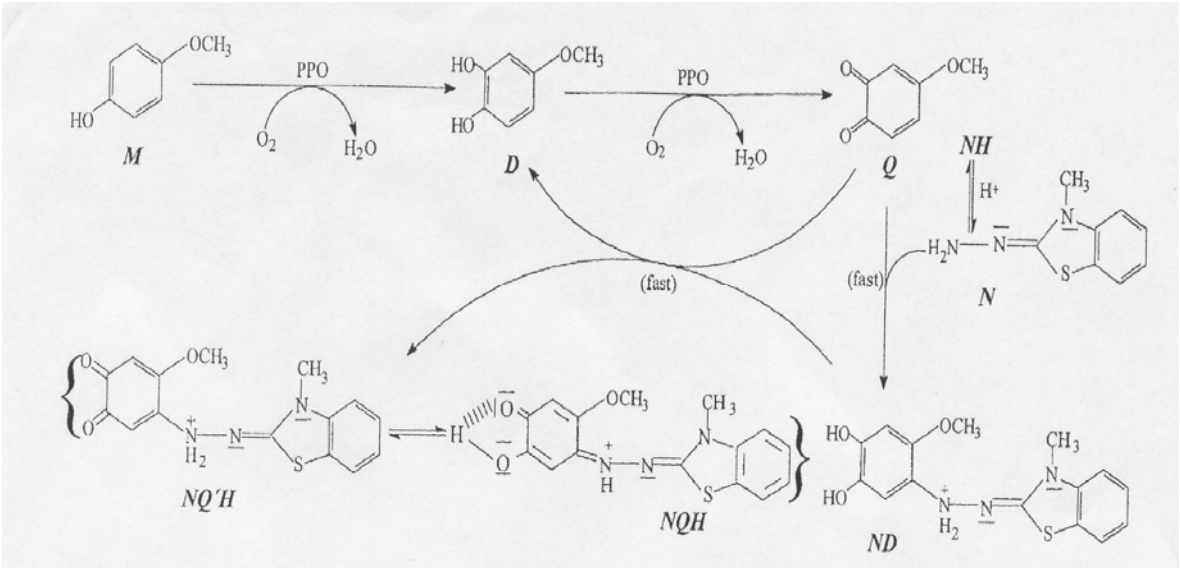


Figure 1.4: Proposed mechanism explaining the oxidation of 4-Methoxyphenol (HQ) by phenoloxidase using 3-methyl-2-benzothiazolinone hydrazone (MBTH) as a coupling reagent. *M*, monophenol (HQ); *D*, *o*-diphenol (3,4-dihydroxy anisole); *Q*, *o*-methoxy-quinone; *N*, nucleophile (MBTH); *NH*, protonated nucleophile; *ND*, nucleophile-diphenol adduct (MBTH-3,4dihydroxy anisole adduct); *NQ'H*, yellow protonated *o*-quinone in tautomery with *NHQ*, red protonated semi *p*-quinoneimine (Espin *et al* 1998b)

Significance of Study

Pacific oyster culture in South Australia comprises an important aquaculture sector that provides a reliable source of high quality seafood that is sold and distributed nation wide. The South Australian Pacific oyster industry is endeavouring to increase production from an established domestic market to supply international export markets with high quality oysters. However, increasing production in oyster aquaculture has also been accompanied with an increase in disease related epidemics that have significantly affected production and threaten the economic performance of the industry. Stress resulting from physical and environmental perturbations is recognised as being the primary cause for the spread of disease. Studies on various phyla have demonstrated a clear link existing between stress and increased mortality, supposedly due to a decreased resistance to disease (Malham *et al* 2003). Such stressful physical and environmental induced stressors associated with oyster culture pose a serious threat to the value of the oyster industry due to increased stock mortality through outbreaks of infectious disease.

Pacific oysters were selected for this study as they are a bivalve of great economic importance to South Australian aquaculture. Any resultant findings or recommendations concerning physical and environmental perturbations may have implications for increasing oyster production. Determination of the role of the prophenoloxidase activating system in oyster stress response will provide a novel insight into both immunological function and adaptative response of the Pacific oyster when subjected to adverse conditions. Such insight will allow for the quantitative identification of problematic stress inducing practices associated with present day culture techniques of *C. gigas*. To

date no previous research has explored the possibility of measuring haemolymph phenoloxidase activity as a potential indicator of stress.

In marine bivalves quantitative information on the role and mode of action of the stress response system remains largely unexplored and fragmentary. This thesis is primarily dedicated to addressing the issue of stress and stress-inducing practices commonly associated with commercial Pacific oyster (*Crassostrea gigas*) farming in South Australia. Determination of stress response is addressed by documentation of biochemical analysis of fluctuating haemolymph phenoloxidase (PO) activity when challenged by physical and environmental forces known as stressors. Overall, it is hoped that this research will provide valuable information concerning the role of phenoloxidase in host stress response and its potential role as an immunological indicator of stress. Furthermore, this research will also provide a greater understanding of the role that the prophenoloxidase activating system plays in internal defence mechanisms and disease resistance in farmed bivalve molluscs.

Chapter 2: General Methodology

2.1. Experimental Animals

Specimens of Pacific oysters (*Crassostrea gigas*) used in this investigation came from leases owned by Zippel's Oysters that are located in Smoky Bay on the West Coast of South Australia (***Figure 1.1***). All individual oysters ranged in size from 60-90mm shell length, weighed between 60-120 grams and were between 2-2.5 years of age unless specified otherwise. Oysters were harvested and sent to by refrigerated road transport to 600L acclimation tanks located at SARDI Aquatic Science Centre. All animals were maintained in a flow through system in 600L seawater tanks with continuous aeration until used for various experiments. Culture water was maintained at 37-38ppt; 15°C±1°C. Three 600L tanks containing oysters were fed a 1000L mix (2-3 million cells/mL) of two Golden algae species: *Pavlova lutheri* and *Isochrysis* species daily. Upon arrival all oysters remained in the recirculating tanks undisturbed for an acclimation period of at least two weeks to ensure that they were at basal phenoloxidase levels prior to experimental work. All oysters that were used in the following experiments spent no longer than eight weeks in the flow through system prior to experimental work.

2.2 Collection of Oyster Hemolymph

Oyster shells were shucked using an oyster knife inserted near the animal's adductor muscle sinus and 0.4-0.5mL of hemolymph was removed from the heart (***Figure 2.1***) using a sterile 29-gauge Ultra Fine Insulin needle (0.33mm x 12.7mm) into a 1mL disposable syringe. One millilitre syringes were used for sampling as they were appropriately matched to the range of 0.4-0.5mL hemolymph volumes removed from the sampled oysters. Stringent care was taken not to contaminate hemolymph samples with surrounding tissues and fluids during sampling. All syringes used for individual hemolymph samples were used only once and then discarded to prevent sample contamination. Hemolymph samples were then immediately transferred to 1.5mL microcentrifuge tubes and held on ice. Individual hemolymph samples were centrifuged in a S415C Eppendorf microcentrifuge for 2 minutes at 2990 rpm or pooled volumes > 2mL were transferred to 10mL polypropylene test tubes and centrifuged in an EBA 12 Hettich Zentrifugen ultracentrifuge for 5 minutes at 5000 rpm. The haemocyte supernatant was then removed using a 1mL Pasteur pipette and samples were again labelled and either assayed within two hours of sampling or frozen individually or as a pooled sample at -18^{0}C. Each sampled oyster had hemolymph collected only once and then the oyster was discarded following hemolymph freezing or analysis.

Figure 2.1: Hemolymph sampling from the oysters heart using a 29-gauge Ultra Fine Insulin needle

2.3 Spectrophotometric Assay of Phenoloxidase (PO) Activity

2.3.1 Spectrophotometric Assay using L-3, 4-dihydroxyphenylalanine (L-DOPA) as a Substrate

The protocol used in all of the experiments was modified from methods of Gollas-Galvan *et al* (1997) and Deaton *et al* (1999). Phenoloxidase activity in oyster hemolymph was assayed spectrophotometrically at a wavelength of 490nm to quantify the formation of dopachrome from L-3, 4-dihydroxyphenylalanine (L-DOPA) (Asokan *et al* 1998;Deaton *et al* 1999;Peters and Raftos 2003). This reaction catalysed by phenoloxidase intends to measure a dark brown colour change in the reaction mixture (***Figure 1.3*** (***1*** & ***2***)).

All L-3, 4-dihydroxyphenylalanine (L-DOPA: Sigma-Aldrich; D9628-25G) stock solutions were mixed with filtered salt water (FSW; 0.45 μm filtered) at a final concentration of 4 mg/mL. Ranges of preliminary hemolymph concentrations (5-100% v/v) were used to determine the most suitable hemolymph dilution to use for the assay. All hemolymph dilutions used for this assay were at a dilution factor of 1:10 (10% v/v) mixed with filtered salt water. In certain cases, the phenoloxidase-specific inhibitor, tropolone (T: Sigma-Aldrich; T7387-250MG) was used at concentrations of 1, 0.5 and 0.1 mM to determine if the assay was detecting activity of enzymes other than phenoloxidase.

Two types of spectrophotometers were used for measuring phenoloxidase activity. The assay using the Biochrom Ultraspec II spectrophotometer used 1mL cuvettes that each contained 500μL of the diluted hemolymph sample with 500μL of the L-DOPA substrate solution, unless stated otherwise. Due to the smaller wells used in the Labsystems Multiskan EX 96-well Microplate final volumes of 200μL were assayed that contained 100μL of diluted hemolymph and 100μL of the L-DOPA substrate solution, unless stated otherwise. Temperature during the assay remained at room temperature (18^0C).

Control wells containing 100µL of L-DOPA substrate solution and 100µL of FSW were included in each assay plate. Duplicate cuvette wells were analysed for each individual oyster and the absorbance data measured from the spectrophotometer was adjusted by subtracting the absorbance values from wells containing L-DOPA/FSW alone. Preliminary studies using 100µL of diluted hemolymph and 100µL of FSW as control wells proved to be unnecessary due to no change in background absorbance over the six-hour incubation period. Due to the slow initial rate of phenoloxidase activity, enzyme reactions were incubated for 6 hours at room temperature to measure the absorbance associated with the oxidation of the substrate and the formation of melanin, unless stated otherwise. Enzyme activity for all assays is expressed as absorbance, specific absorbance and percent increase in absorbance at 490nm over the L-DOPA/FSW blanks.

2.3.2 Spectrophotometric Assay using 4-Methoxyphenol (HQ) as a Substrate

Monophenolase activity from Pacific oyster hemolymph was determined spectrophotometrically at a wavelength of 490nm by using 4-Methoxyphenol (HQ: Sigma-Aldrich; M18655-100G) and 3-methyl-2-benzothiazolinone hydrazone (MBTH) as previously described by (Rodriguez-Lopez *et al* 1994;Espin *et al* 1995; 1998a, b). This method measures the direct formation of amine chrome (Espin *et al* 1995) that produces a pink colour change (***Figure 1.3*** (***1***)). This assay method is reported as being highly sensitive, reliable and precise (Rodriguez-Lopez *et al* 1994; Espin *et al* 1995; 1998a,b). A range of preliminary hemolymph concentrations (10-100% v/v) were used to determine the most suitable dilution to use for the assay. All hemolymph dilutions used for this assay were at a dilution factor of 1:2 (50% v/v) mixed with FSW. Stock solutions of the phenolic 4-Methoxyphenol substrate solutions were prepared in FSW at a final concentration of 2.5 mg/mL. MBTH was added to the stock substrate solution at a final concentration of 1mM. Both HQ and MBTH were not water-soluble therefore 0.5mL and 1mL volumes of 100% ethanol respectively were used to dissolve the compounds. Upon dissolving, both MBTH and HQ were topped up with FSW to reach the required final concentration of HQ substrate/MBTH-quinone adduct for assaying. In certain cases, the phenoloxidase-specific inhibitor, tropolone (T: Sigma-Aldrich; T7387-250MG) was added as a control at concentrations of 2, 1, 0.5 and 0.1 mM to determine if the assay was detecting activity of enzymes other than phenoloxidase. Assay volumes and temperature conditions used for the Biochrom Ultraspec II spectrophotometer and the Labsystems Multiskan EX 96-well Microplate were identical to those used in the L-DOPA assay (**2.3.1**).

Control wells containing 100µL of HQ/MBTH substrate solution and 100µL of FSW were included in each assay plate. Duplicate cuvette wells were analysed for each individual oyster and the absorbance data measured from the spectrophotometer was adjusted by subtracting the absorbance values from wells containing HQ/MBTH/FSW alone. Preliminary studies using 100µL of diluted hemolymph and 100µL of FSW as control wells proved to be unnecessary due to no change in background absorbance over the incubation period. Due to the slow initial rate of monophenolase activity of phenoloxidase activity, enzyme reactions were incubated normally for 6 hours at room temperature to measure the absorbance associated with the oxidation of the substrate, unless stated otherwise. Enzyme activity for all assays is expressed as specific absorbance and percent increase in absorbance at 490nm over the HQ/MBTH/FSW blanks, unless specified otherwise.

2.4 Preparation of Oyster Hemolymph for Native PAGE

Oysters were removed from the 600L acclimation tanks and left to air dry for twenty minutes allowing for any additional saltwater to drain from their mantle prior to hemolymph removal. Each

oyster had 1mL of hemolymph removed and transferred directly into 1.5mL microcentrifuge tubes. Hemolymph was then centrifuged for 5 minutes at 3000rpm at 4°C in an Eppendorf microcentrifuge to increase haemocyte concentration. Following centrifugation, 500µL of haemocyte supernatant was pipetted out and transferred to a microcentrifuge tube and held as the haemocyte supernatant sample with all remaining hemolymph resuspended and centrifuged for a further 3 minutes at 3000rpm. Once centrifugation was complete a further 250µL of supernatant was removed and discarded, the remaining 250µL had haemocyte supernatant resuspended in 50:1 2% v/v Nonident-P40 (NP-40; Fluka) in 10mM Tris-HCL (pH 7.0) (Bezemer 2004). Haemocyte lysate samples were centrifuged at 6000rpm for 20 seconds to remove cellular debris prior to use for Native PAGE.

2.4.1 Native Polyacrylamide Gel Electrophoresis (Native PAGE)

The protocol used in this experiment was refined from the methods of Newton *et al* (2004). Different isoforms of the enzyme phenoloxidase were determined electrophotoretically using Gradipore Native PAGE precast gels in a Bio-rad Mini-Protean II vertical slab gel system. Forty microliters of the haemocyte lysate were mixed with ten microliters of premixed native PAGE sample buffer (0.35M Tris-HCL, 10% v/v glycerol, pH 6.8) prior to direct addition to 8% precast Gels. Gels were then electrophoresed in running buffer (0.03M Tris-HCL, 0.187M Glycine in distilled water, pH 8.3) for 3 hours at 110v at 4°C. Following the completion of electrophoresis the precast gels were stained with 4mg/mL L-3, 4-dihydroxyphenylalanine (L-DOPA; Sigma Aldrich) mixed in FSW and 20mM 4-Methoxyphenol (HQ; Sigma Aldrich) in FSW containing the chromogenic nucleophile 5mM 3-methyl-2-benzothiazolinone hydrazone (MBTH; Sigma Aldrich). Both HQ and MBTH were not water-soluble therefore 1mL and 0.5mL volumes of 100% ethanol respectively, were used to dissolve the compounds prior to reaching the final substrate volumes. Precast gels were stained over night and then rinsed with distilled water, transferred to plastic sleeves and scanned to determine the presence of any resolved protein band(s) in the precast gels.

2.5 Hemolymph Heating

Oysters were removed from the 600L acclimation tanks and left to air dry for twenty minutes allowing for any additional saltwater to drain from their mantle prior to hemolymph removal. Six oysters had 500µL of hemolymph removed and added to a polypropylene test tube that comprised of the pooled sample. The sample was then split up into two separate 1.5mL microcentrifuges and labelled heated and unheated. Water was brought to the boil and the heated sample was added and boiled for 15 minutes at ~100°C. The unheated sample was left undisturbed. Both samples were then centrifuged for 2 minutes at 2990 rpm prior to assaying.

2.6 Statistical Analysis

All data was processed and analysed using SPSS computer software version 11. Homogeneity of normality was determined using graphical methods (scatter plot, probability plot and histogram of residuals) to satisfy the assumptions of normality involved with ANOVA. Where appropriate, data was transformed using square root transformation ($\sqrt{x} = x^{1/2}$) and reciprocal transformation ($1/x$). Both one and two-way analysis of variances (ANOVA) were used to determine significant differences ($p < 0.05$) that existed between levels of phenoloxidase activity. Bonferroni *post hoc* multiple comparison and pairwise comparison analysis were used to determine significant differences that existed with respect to phenoloxidase. The Bonferroni *post hoc* analysis is more likely to reject a

type 1 error (accepting a positive) than the ANOVA. As a result, the Bonferroni analysis is a more conservative statistic comparison. Factor analysis was conducted to determine the associations between the various forms of induced stress on resultant hemolymph phenoloxidase activity. The Null Hypothesis (H_0) was accepted when the P-value was > 0.05, and rejected when < 0.05.

Preliminary Methodology

2.6 L-DOPA Assay

2.6.1 Dilution Series Experiment

This preliminary experiment was a simple dilution series of hemolymph to aid in determining what concentrations of hemolymph were required for the L-DOPA assay. Hemolymph used was part of a pooled sample of twelve individual oysters. This dilution series allowed for the determination of what volume of hemolymph would be required to be sampled from each of the oysters and volume required for assaying. Four initial dilution factors that were tested; Neat (1:1), 1:2, 1:4 and 1:8 (***Table 2.1)***.

Dilution	Hemolymph (µL)	FSW (µL)	L-DOPA (µL)
Neat	500	-	500
1:10	250	250	500
1:4	125	375	500
1:8	62.5	437.5	500

Table 2.1: Dilution series of Pacific oyster hemolymph (L-DOPA assay)

All of the four dilution series treatments and the L-DOPA/FSW control in ***Figure 2.2*** follow a general linear trend increasing in phenoloxidase activity over the 6-hour (360 min) time period. A distinguishable decrease in phenoloxidase activity is obtained with every successive dilution of hemolymph.

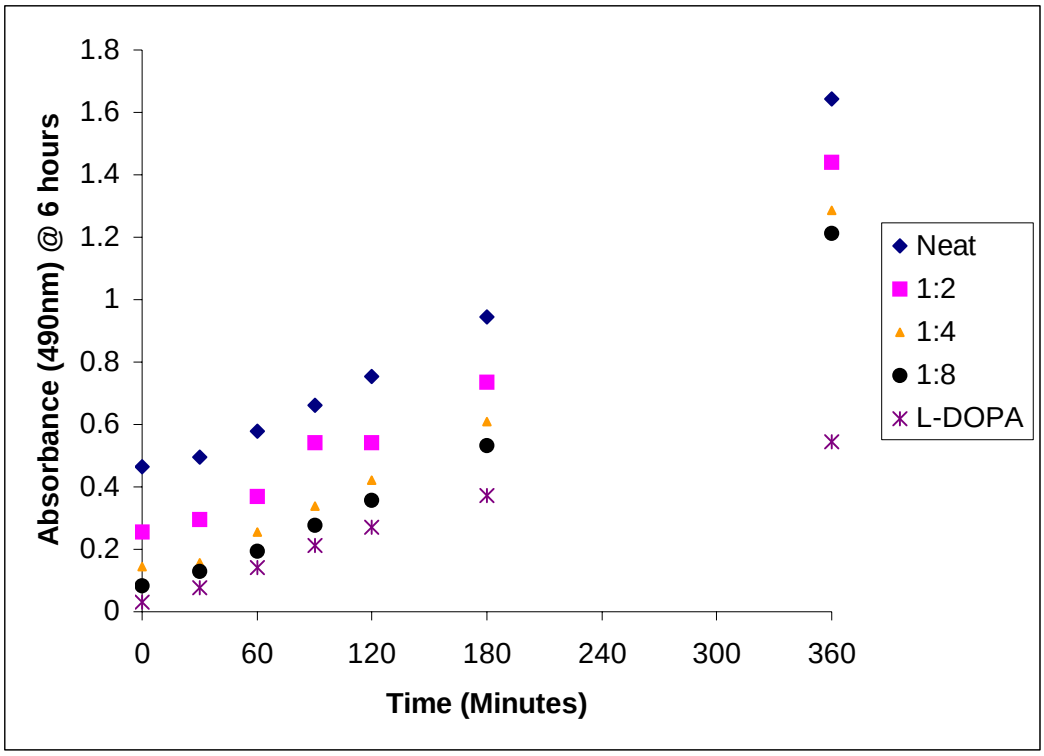


Figure 2.2: Phenoloxidase activity (L-DOPA assay) of hemolymph dilution series (raw absorbance values blanked in distilled water)

In order to determine the specific change in absorbance, both the L-DOPA/FSW blanks and initial hemolymph absorbance values were subtracted from the raw absorbance values (***Figure 2.3***). Phenoloxidase activity is slow up until the 180-minute time points, and then has an accelerated rate of reaction between the 180 and 360-minute time points. However, all four experimental dilutions; Neat, 1:2, 1:4 and 1:8 all showed very similar specific absorbance values at the 360-minute time point. Therefore, due to the inability to distinguish phenoloxidase activity between similar absorbance values this warranted another dilution experiment that included hemolymph dilutions greater than 1:8 to produce absorbance values value on the linear section of the dilution curve.

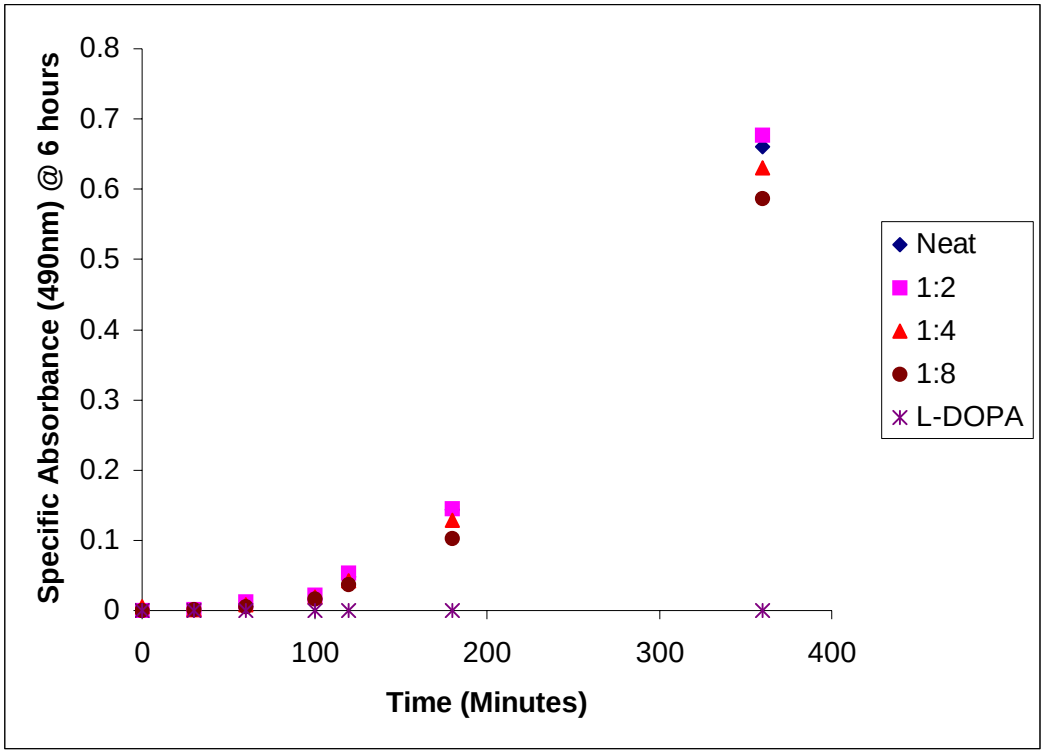


Figure 2.3: Phenoloxidase activity (L-DOPA assay) of hemolymph dilution series corrected for L-DOPA/FSW blanks and initial absorbance

2.6.2 Second Dilution Series Experiment

This second dilution series tested a further six hemolymph dilutions; 1:2, 1:2.5, 1:3.3, 1:5, 1:10 and 1:20 (***Table 2.2***) using hemolymph from the same pooled sample used in the first dilution experiment. This second dilution series was carried out to determine what concentration of hemolymph was required for assaying to produce observable differences in phenoloxidase activity between the different hemolymph dilutions.

Dilution	Hemolymph (µL)	FSW (µL)	L-DOPA (µL)
1:2	250	250	500
1:2.5	200	300	500
1:3.3	150	350	500
1:5	100	400	500
1:10	50	450	500
1:20	25	475	500

Table 2.2: Second dilution series of Pacific oyster hemolymph (L-DOPA assay)

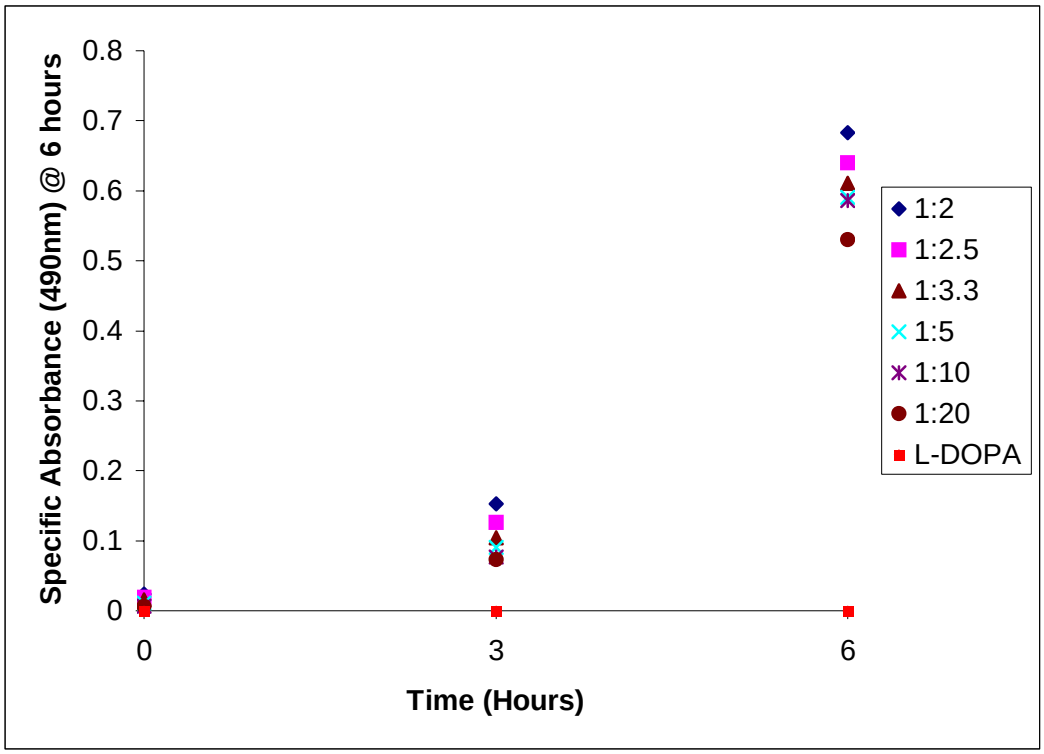


Figure 2.4: Phenoloxidase activity (L-DOPA assay) of the second dilution series corrected for L-DOPA/FSW blanks and initial absorbance

Enough observable difference in phenoloxidase activity existed between the 1:5/1:10 and 1:10/1:20 dilution series steps to use a 1:10 dilution of hemolymph for all further assays using L-DOPA as the substrate. The rate and observable patterns of absorbance change in this second dilution experiment was similar to that of the first dilution experiment in that an initial lag period of activity was seen up until the 3 hour time point, followed by a rapid increase in phenoloxidase activity up to the six hour time point.

2.6.3 L-DOPA Substrate Concentration Experiment

This experiment looked at varying L-DOPA substrate concentrations based upon previous research by Asokan *et al (*1997) and Peters & Raftos (2003), to decide what concentrations would be used for assaying. Four substrate concentrations were tested; 4, 2, 1 and 0.5mg/mL respectively. This experiment used the same hemolymph pool used at a 1:10 dilution used in previous preliminary experiments.

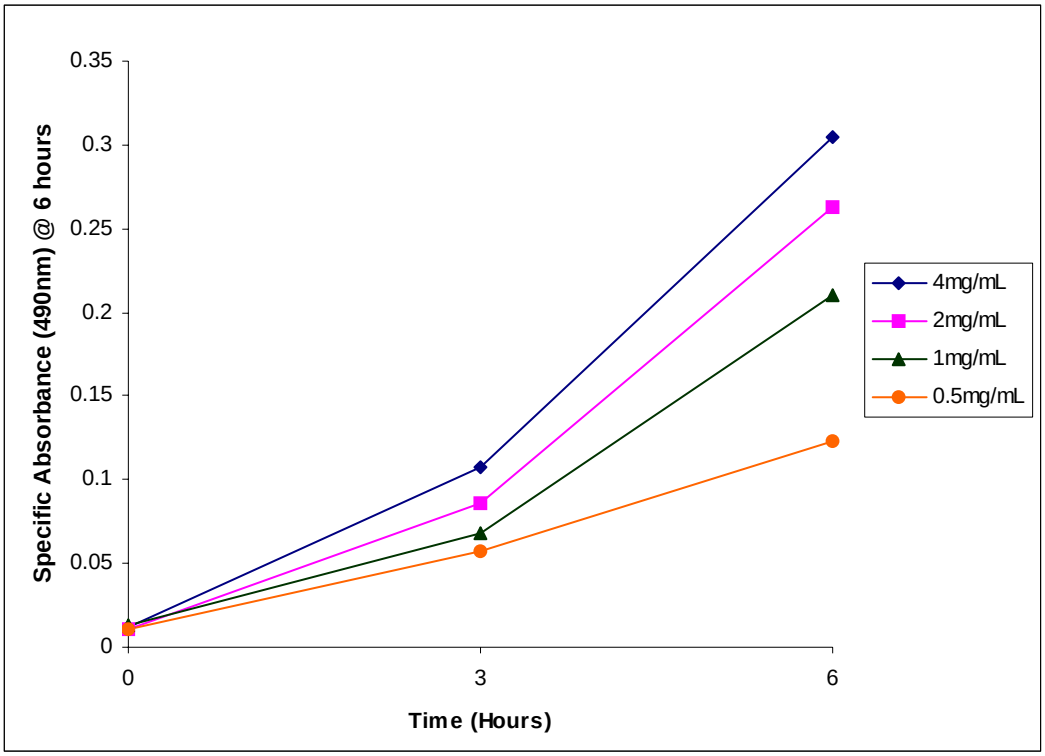


Figure 2.5: Phenoloxidase activity (L-DOPA assay) using different concentrations of substrate

An initial slow rate of reaction is observed in ***Figure 2.5***, followed by an accelerated rate of reaction observed between the 3 and 6-hour time points. All of the substrate concentrations follow a dose dependent response, in that the degree of enzyme activity is reliant on the increasing concentration of substrate. L-DOPA solution at 0.5mg/mL follows the initial slow rate of activity up until 3 hours, however a reduced rate of activity is observed from the 3 to 6 hour time point due to the limitation of available substrate. Due to the sudden increase in observable phenoloxidase activity recorded from 4, 2 and 1mg/mL treatments, it was decided that all assaying would be carried out using 4mg/mL L-DOPA stock solutions as this concentration recorded the highest measurable level of phenoloxidase activity.

2.6.4 Centrifugation Experiment

The aim of this experiment was to compare the effect of centrifugation versus no centrifugation of hemolymph samples prior to assaying. A 2mL volume of hemolymph was sampled, divided into equal volumes and transferred into two separate 1.5mL microcentrifuge tubes and either centrifuged for 2 minutes at 2990rpm or left undisturbed. Both centrifuged and non-centrifuged hemolymph was then diluted with FSW to Neat, 1:10, 1:100 and 1:1000. This experiment used hemolymph samples from a pooled sample used for previous experiments and was incubated for two hours (120min), rather than six hours (360 min), as was the case in previous dilution and substrate concentration experiments.

Figure 2.6 shows a clear difference in phenoloxidase activity between the centrifuged samples. All

four dilutions follow a linear trend of increasing phenoloxidase activity over time. The neat dilution for non-centrifuged hemolymph is excessively high when compared to the other three dilutions and the opposing centrifuged neat dilution from ***Figure 2.7***. The generalised reason for this abrupt increase in absorbance seen in ***Figure 2.7*** is a result from particles of tissue that were included in the hemolymph sample and remain in suspension during assaying producing higher than normal absorbance values. As a result from these findings from ***Figures 2.6*** and ***2.7***, all future hemolymph samples were centrifuged to produce hemolymph supernatants that are free of any suspended material, haemocytes and cellular debris that could lead to distortions in absorbance readings

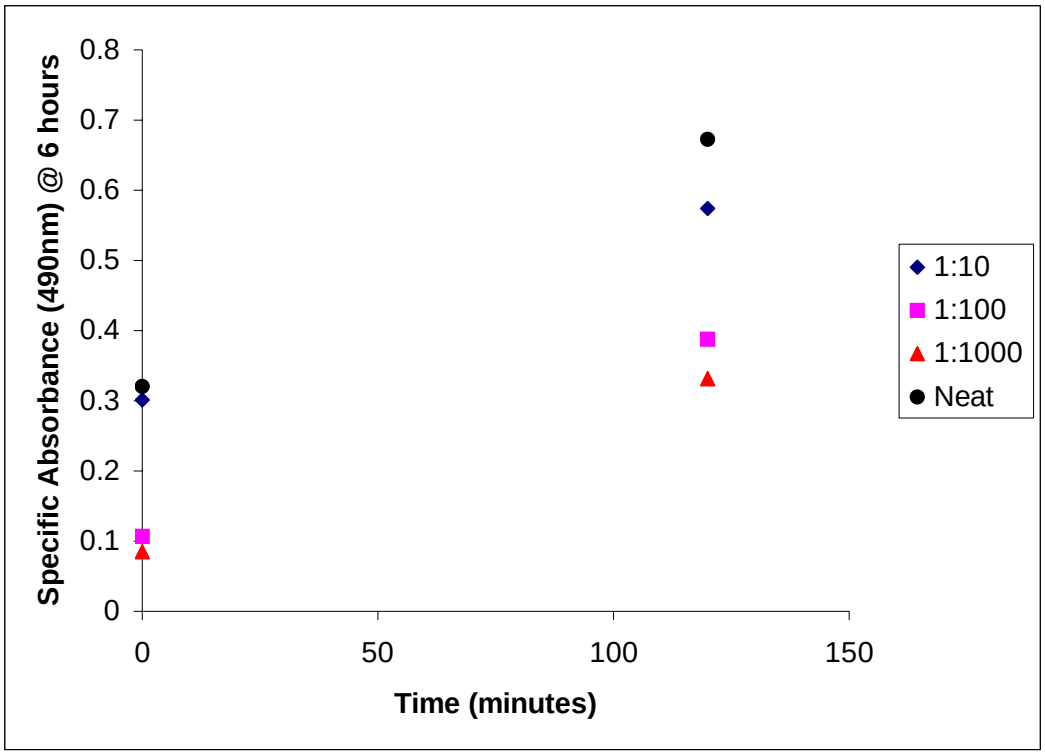


Figure 2.6: The effect of haemolymph centrifugation on phenoloxidase activity (L-DOPA assay) corrected for L-DOPA/FSW blanks and initial absorbance

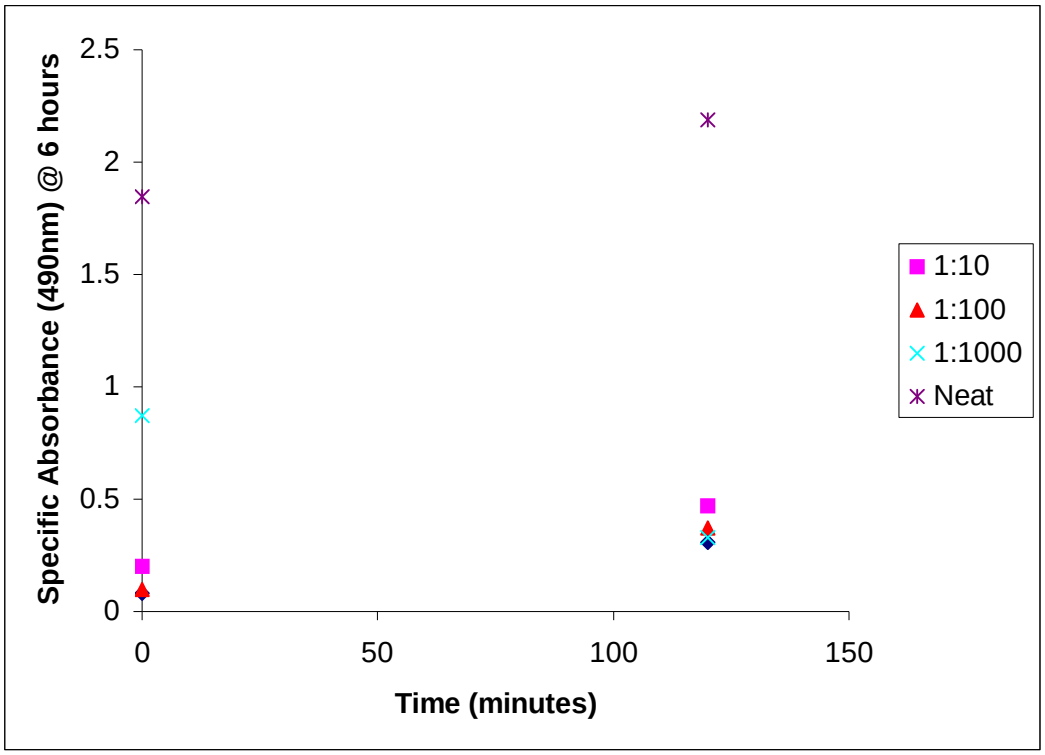


***Figure 2.7*:** The effect of non-centrifugation hemolymph on phenoloxidase activity (L-DOPA assay) corrected for L-DOPA/FSW blanks and initial absorbance

2.6.5 Hemolymph Storage Experiment

This experiment looked at the various methods used for the storage of hemolymph samples and their effects on phenoloxidase activity. Four storage methods that are commonly encountered when sampling oyster hemolymph that were used are:

1. Fr-Freezing (-18°C)
2. F/T-Daily Freeze/Thaw (-18°C to 20°C)
3. RT-Room Temperature (20°C)
4. Refrigeration (4°C)

Hemolymph was removed from a pooled sample and centrifuged at 5500 rpm for five minutes. Hemolymph supernatant was then removed and stored according to the specific treatment. Assays were conducted daily using a 1:10 hemolymph dilution with the addition of 4mg/mL L-DOPA substrate solution and incubated for six hours.

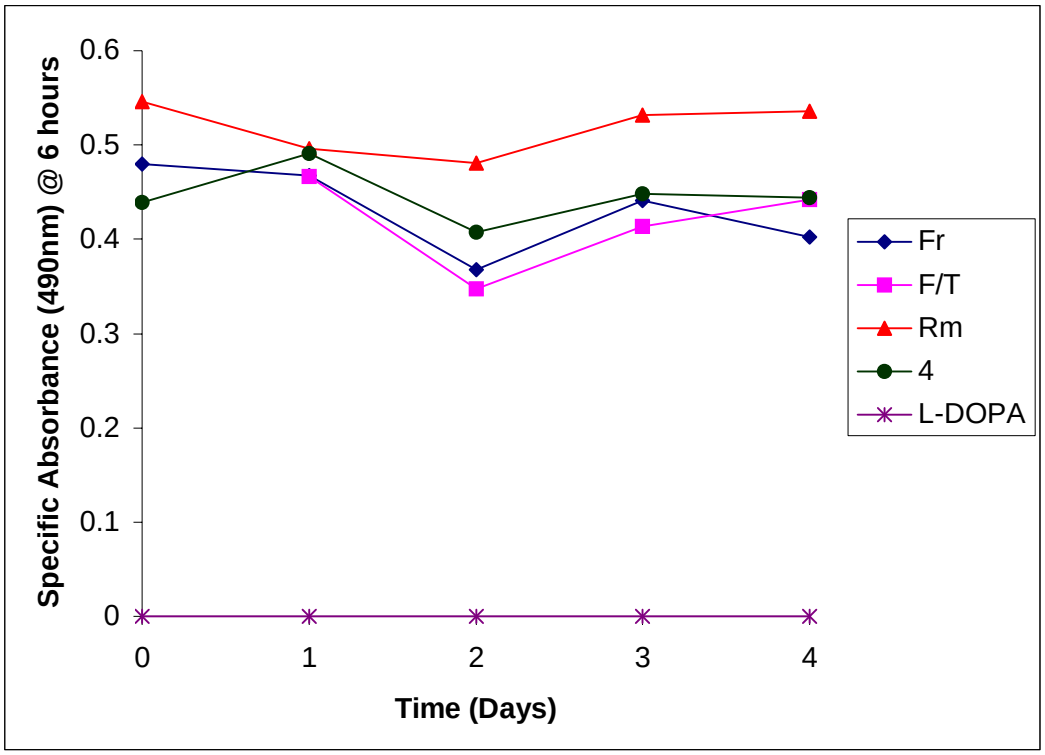


Figure 2.8: Phenoloxidase Activity (L-DOPA assay) from the four-hemolymph treatment storage methods

Phenoloxidase activity in all storage treatment conditions remained constant over the four-day experiment duration (***Figure 2.8***). No substantial difference in activity is observed between each of the storage treatments, suggesting that phenoloxidase activity is not affected by changing temperatures associated with the storage conditions.

2.7 HQ Assay

2.7.1 Dilution Series Experiment

This preliminary experiment was carried out to determine what concentrations of hemolymph were required to use for the HQ assay. This allowed for determining what volume of hemolymph would be required to be sampled from each of the oysters. The initial five dilution factors tested were Neat, 1:2, 1:4, 1:8 and 1:10 (***Table 2.3***).

Dilution	Hemolymph (µL)	FSW (µL)	HQ/MBTH (µL)
Neat	100	0	100
1: 2	50	50	100
1: 4	25	75	100
1: 8	12.5	87.5	100
1:10	10	90	100

Table 2.3: The five different hemolymph dilutions used for dilution determination

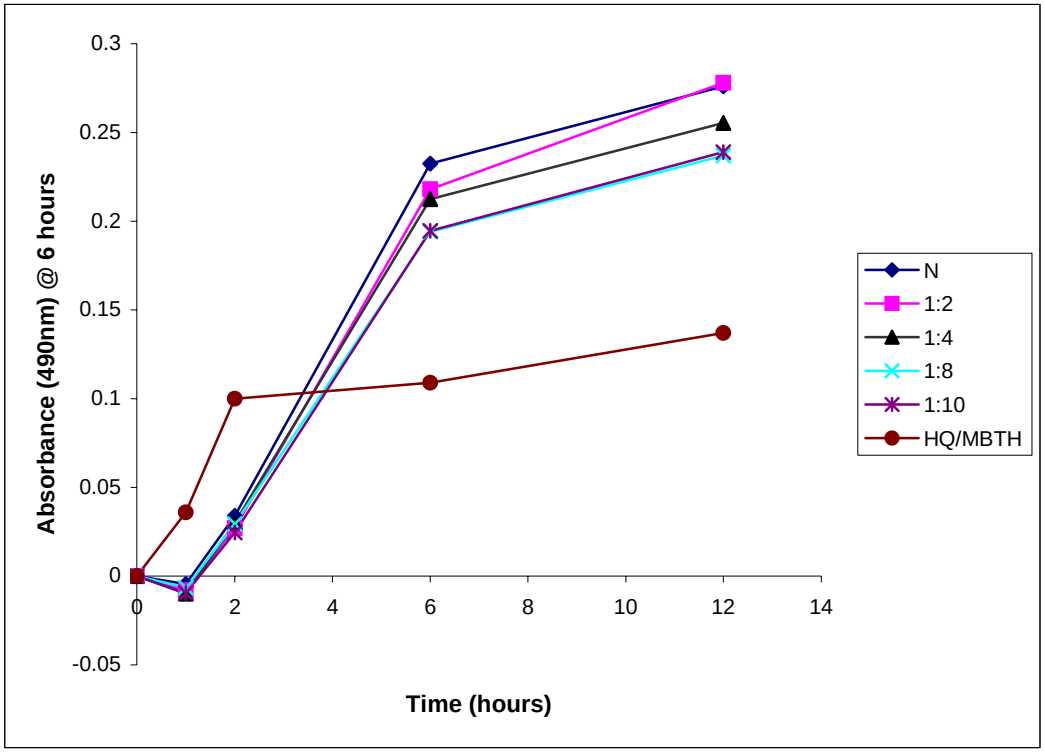


Figure 2.9: Hemolymph dilution series minus initial absorbance (HQ assay)

In order to determine the specific change in absorbance the HQ/MBTH blanks and initial absorbance were subtracted from the raw absorbance values (***Figure 2.9***). All of the various dilution series treatments show an initial decrease in absorbance up until two hours prior to an increase in absorbance up until the six-hour time point. Following the increase in absorbance, all of the dilutions follow a linear trend that has distinguishable steps between the dilution increments, allowing for simple quantification between the five dilutions. As a result from ***Figure 2.10***, it was decided that a 1:2 hemolymph dilution was used in all future HQ assays.

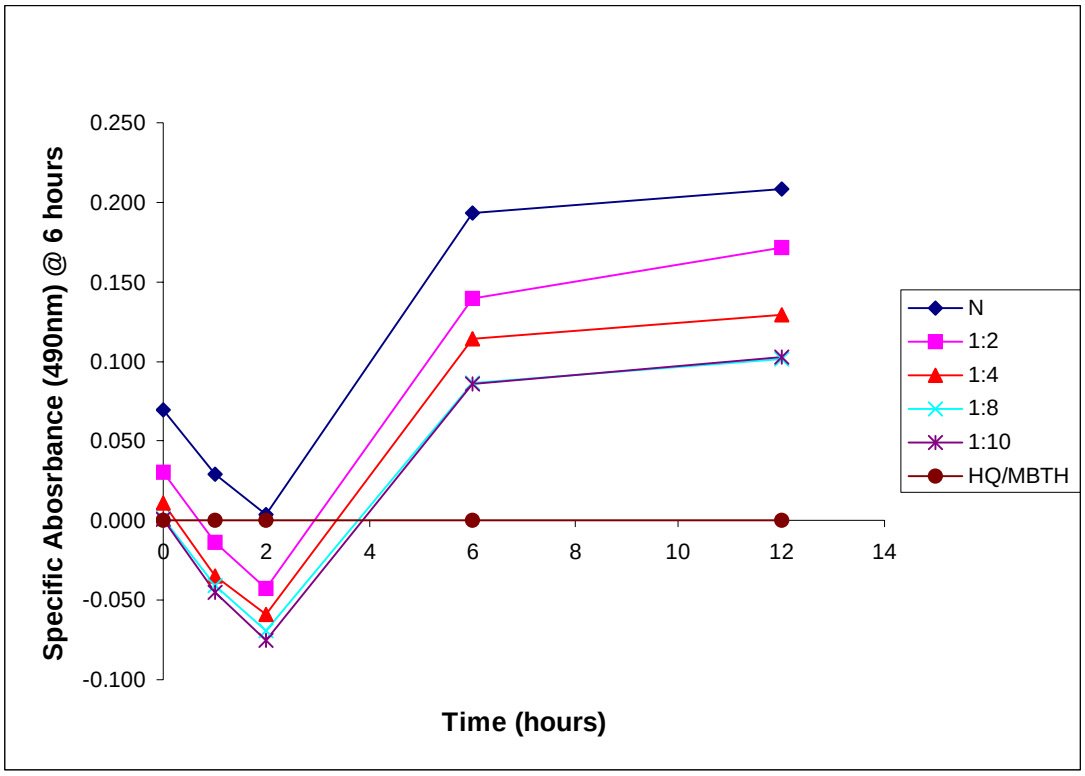


Figure 2.10. Phenoloxidase activity (HQ assay) of hemolymph dilution series

2.7.2 HQ Substrate Concentration Experiment

This experiment looked at varying HQ substrate concentrations based upon previous research by Espin *et al* (1998b) and Newton *et al* (2004). Four HQ substrate concentrations were tested; 10, 7.5, 5 and 2.5mg/mL, all included a concentration of 1mM MBTH (***Figure 2.11***). This experiment used 1:2 hemolymph dilutions that were removed from the pooled sample used in the previous HQ dilution experiment.

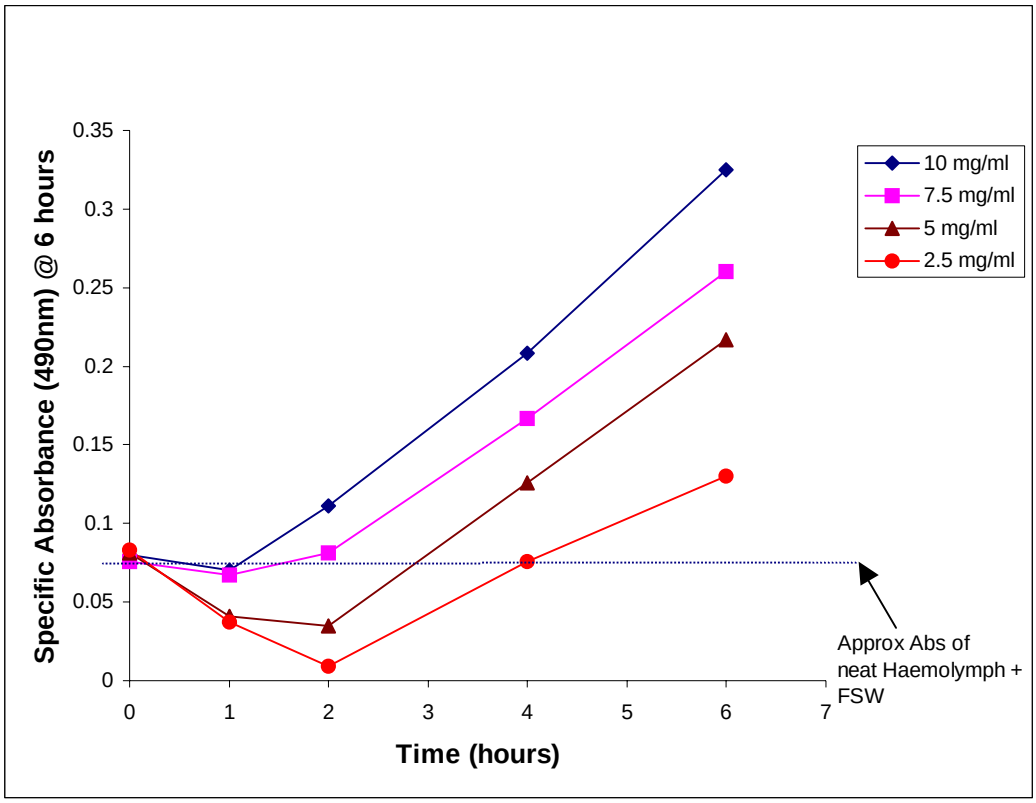


Figure 2.11: Phenoloxidase activity (HQ assay) using different concentrations of HQ substrate solution

Initially all of the HQ substrate concentration treatments (***Figure 2.11***) experienced a decrease in absorbance due to the subtraction of the HQ/MBTH blanks and initial absorbance subtracted from the raw absorbance values. By the four-hour time point all values followed a linear trend by increasing phenoloxidase activity with time. From the results in ***Figure 2.11*** and recommendations from previous literature (Newton *et al* 2004), it was decided that HQ substrate concentration would be set at 2.5mg/mL for future assays.

2.7.3 Centrifugation Experiment

The aim of this preliminary experiment was to compare the effect of centrifugation versus no centrifugation of hemolymph samples prior to being assayed. A 2mL volume of hemolymph was sampled, split and transferred into two separate 1.5mL microcentrifuge tubes and either centrifuged for 2 minutes at 2990rpm or left undisturbed. Both centrifuged and non-centrifuged hemolymph was then diluted down with FSW to the five corresponding dilution treatments; Neat, 1:2, 1:4, 1:8 and 1:10 respectively.

Figure 2.12 shows a clear difference in phenoloxidase activity between dilutions 1:10, 1:8 and 1:4 show little difference in phenoloxidase activity over the six-hour incubation. However, samples from ***Figure 2.13*** all have a higher initial absorbance at time 0 and final absorbance at time 6, when compared to the corresponding centrifuged dilution treatments in ***Figure 2.12***. The generalised reason for this abrupt increase in absorbance seen in ***Figure 2.13*** is a result from particles or tissue that have been sampled and remain in suspension during assaying. Hemolymph sampling can be contaminated by a layer of thin tissue that surrounds the oyster's heart or from a milky inclusion that is released from the oyster gonad that lies directly opposite the heart. Phenoloxidase activity is then seen to decrease in ***Figure 2.13*** over the six hour time course, indicating that the sampled suspended

material has fallen out of solution prior to repeat absorbance readings at time 6. As a result of these findings, all hemolymph samples were centrifuged to remove any suspended material, haemocytes and cellular debris and that could lead to distortions in absorbance readings prior to assaying and provide biased results for phenoloxidase activity determination.

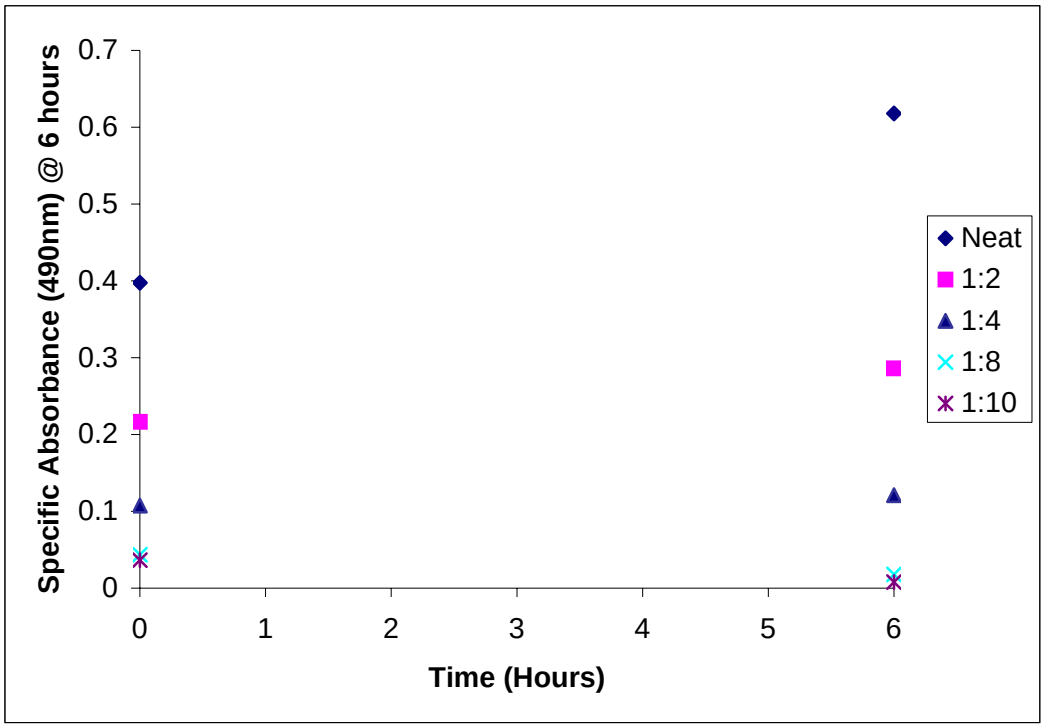


Figure 2.12: The effect of hemolymph centrifugation on phenoloxidase activity (HQ assay)

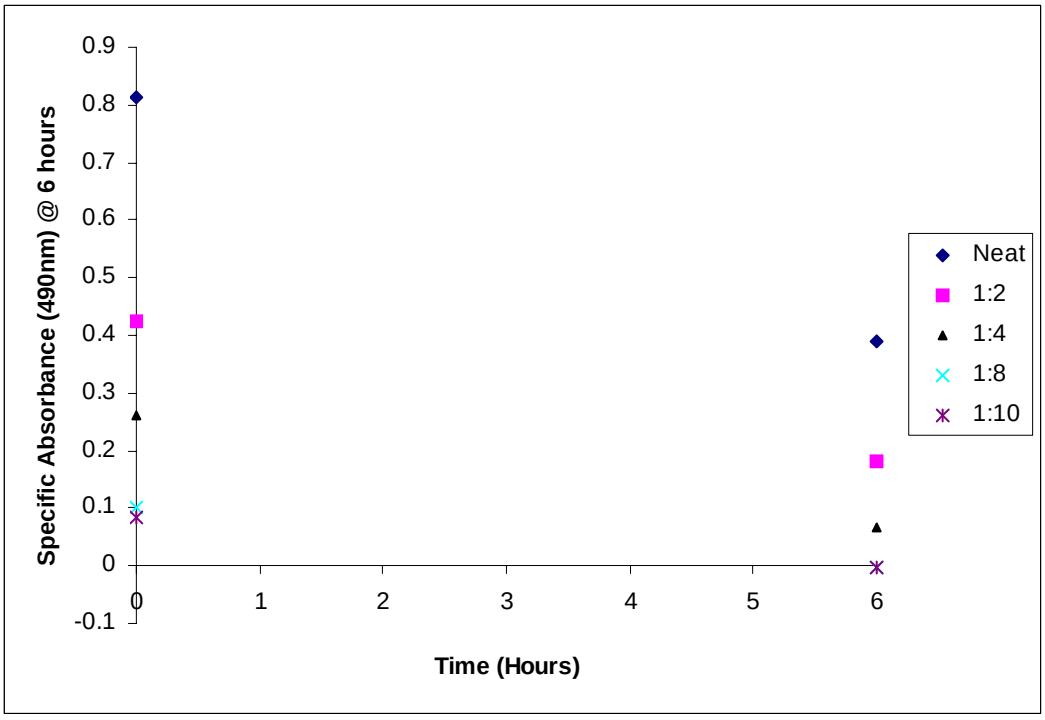


Figure 2.13: Effect of non-centrifugation of hemolymph on phenoloxidase activity (HQ assay)

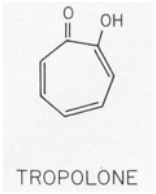
2.8 Tropolone Inhibition Experiment

2.8.1 Background

Phenoloxidase is well a documented enzyme that is found in both the hemolymph and haemocytes of many commercially important marine bivalve species, most importantly in *C. gigas*. However, phenoloxidase is accompanied by a variety of other oxidative enzymes that include catechol oxidases, laccases and peroxidases, which have led to a great deal of confusion when trying to measure specific phenoloxidase activity (Solomon *et al* 1996).

Previous studies focusing primarily on melanogenesis (Pye 1974; Cerenius & Soderhall 2004) demonstrate the importance of effectively differentiating between specific enzyme activity (Kahn & Andrawis 1985). Enzyme inhibitors are chemicals that selectively inhibit reactions involving enzymes. The use of the phenoloxidase specific-inhibitor, tropolone (2,4,6-cycloheptatriene-1-one, 2-hydroxy-2, 4,6-cycloheptatrien-one), has been well documented (Kahn 1985; Kahn & Andrawis 1985; Newton *et al* 2004) for inhibition of enzymatic activity. Tropolone is an effective copper chelator (at the enzymes active site), which binds to and forms a complex with the active site (***Figure 2.14***) (Kahn 1985; Kahn & Andrawis 1985). Kahn & Andrawis (1985) demonstrated that the effectiveness of tropolone as an inhibitor of the enzyme phenoloxidase depends directly upon the substrate; inhibition using tropolone as the inhibitor is highest with L-DOPA.

(**1**)



(**2**)

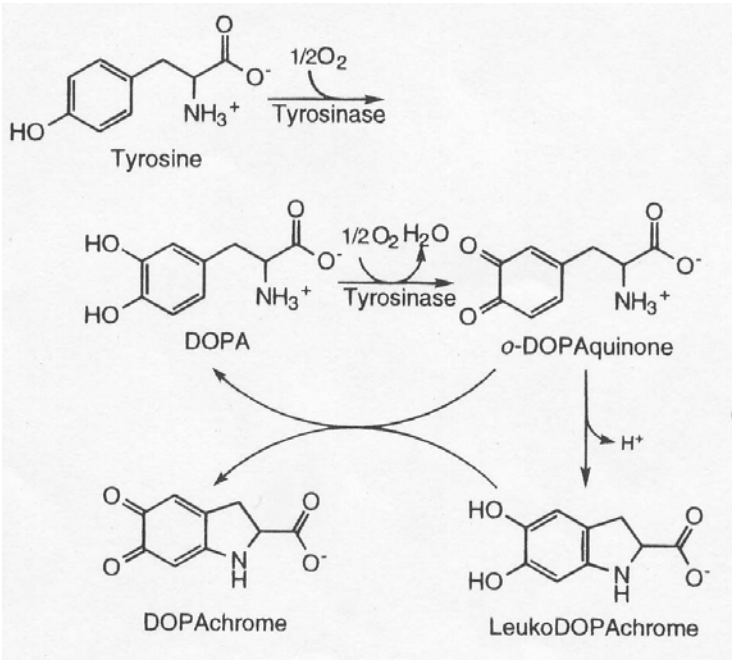


Figure 2.14: (1) The chemical structure that provides the ability of tropolone to effectively chelate metals and inhibit enzymatic activity and (2) the biochemical pathway of phenoloxidase inhibition (Kahn & Andrawis 1985; Solomon *et al* 1996).

Tropolone structure and specificity enables it successfully inhibit both mono- and *o*-diphenolase activity of phenoloxidase. The initial hydroxylation of substrates in the monophenolase reaction by phenoloxidase experiences an initial lag period (Kahn & Andrawis 1985). Previous research by Kahn & Andrawis (1985) described tropolone as slowing the rate of dopachrome formation following the

lag period, and thereby inhibiting the enzymatic action of phenoloxidase. Tropolone has also been successfully used to inhibit oxidation of the substrates in the *o*-diphenolase reaction by binding to copper present on the active site of phenoloxidase (Kahn & Andrawis 1985). Therefore, tropolone is regarded as the most effective inhibitor to specifically use when dealing with phenoloxidase.

2.8.2 L-DOPA assay

The inhibitory effect of tropolone was assessed using L-DOPA as a substrate at a concentration of 4mg/mL, both with (1:10 dilution) and without the addition of hemolymph. With the addition of 1, 0.5 and 0.1mM tropolone to the with hemolymph assay samples, activity was effectively reduced compared to the enzyme activity measured from the hemolymph without tropolone control sample (***Figure 2.15***). However, it is evident from this assay experiment that other enzymes or activities are present in the hemolymph. These other activities can effectively utilise and react with the L-DOPA substrate and contribute to proposed phenoloxidase activity that is measured in the hemolymph without tropolone sample. Tropolone inhibited both mono and *o*-diphenolase activities of phenoloxidase, whilst also reducing colour change associated with the oxidation of the L-DOPA/FSW/Tropolone samples observed in all 1, 0.5 and 0.1mM no hemolymph samples (***Figure 2.15***). This reduced level of autooxidation associated with the L-DOPA/FSW/Tropolone samples indicates that there is phenoloxidase-like activity in the treatment mixture, as tropolone is effectively reducing the rate of substrate autooxidation. No statistical analysis was conducted as each treatment consisted of one averaged duplicate sample.

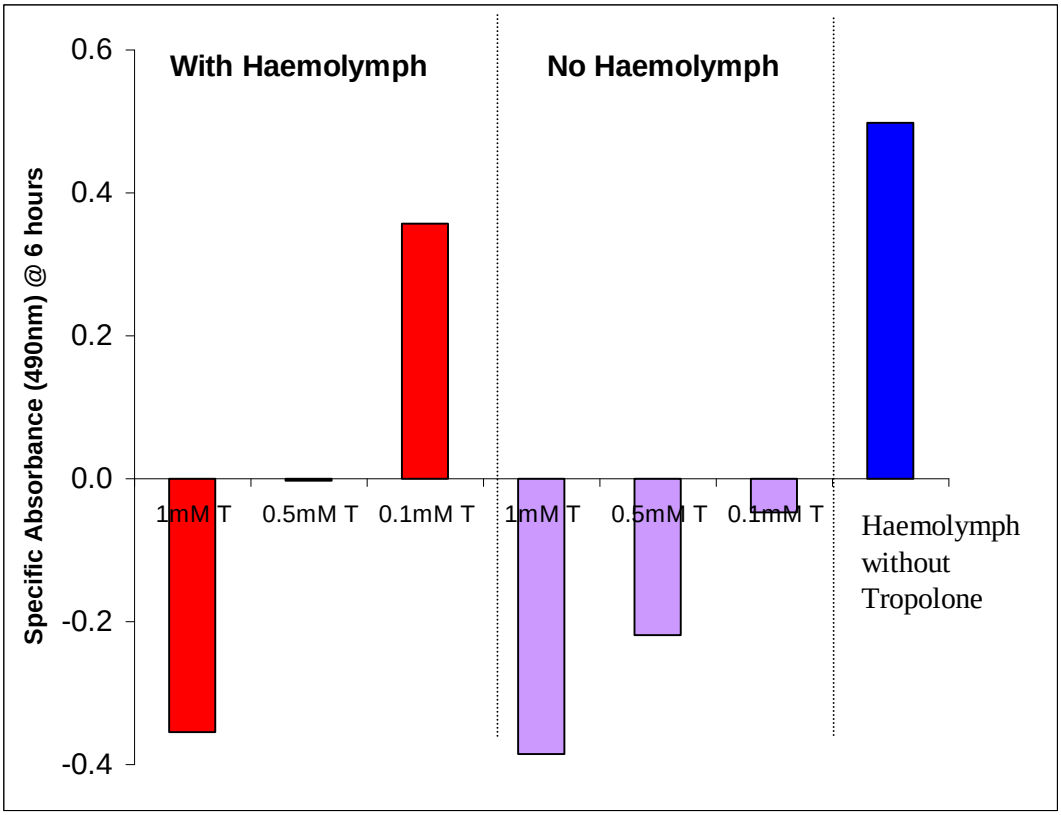


Figure 2.15: Mean inhibition of phenoloxidase (L-DOPA assay) by different concentrations of tropolone (T; 1, 0.5 and 0.1mM) with or without hemolymph. Mean values of duplicate corrected for L-DOPA/FSW control blanks.

2.8.3 HQ assay

The inhibitory effect of tropolone on the monophenolase activity of phenoloxidase was assessed using HQ/MBTH as a substrate adduct at a concentration of 2.5mg/mL and 1mM both with (1:2 dilution) and without the addition of hemolymph. With the addition of 2, 1, 0.5 and 0.1mM tropolone to the with hemolymph assay samples, activity was effectively reduced when compared to the enzyme activity measured from the hemolymph without tropolone control sample (***Figure 2.16***). Phenoloxidase activity has been inhibited through the addition of tropolone, however it is evident that again other enzymes or activities are present and are contributing to proposed phenoloxidase activity that is measured in the hemolymph without tropolone sample. These other activities are able to utilise and react with the HQ substrate and are effectively contributing to the overall measured monophenolase activity. Tropolone inhibited both monophenolase activity of phenoloxidase, whilst also reducing colour change associated with the oxidation of the HQ/MBTH/Tropolone samples observed in all 2, 1, 0.5 and 0.1mM no hemolymph samples (***Figure 2.16***). This reduced level of autooxidation associated with the HQ/MBTH/Tropolone samples indicates that there is phenoloxidase-like activity in the treatment mixture, as tropolone is effectively reducing the rate of substrate autooxidation. No statistical analysis was conducted as each treatment consisted of one averaged duplicate sample.

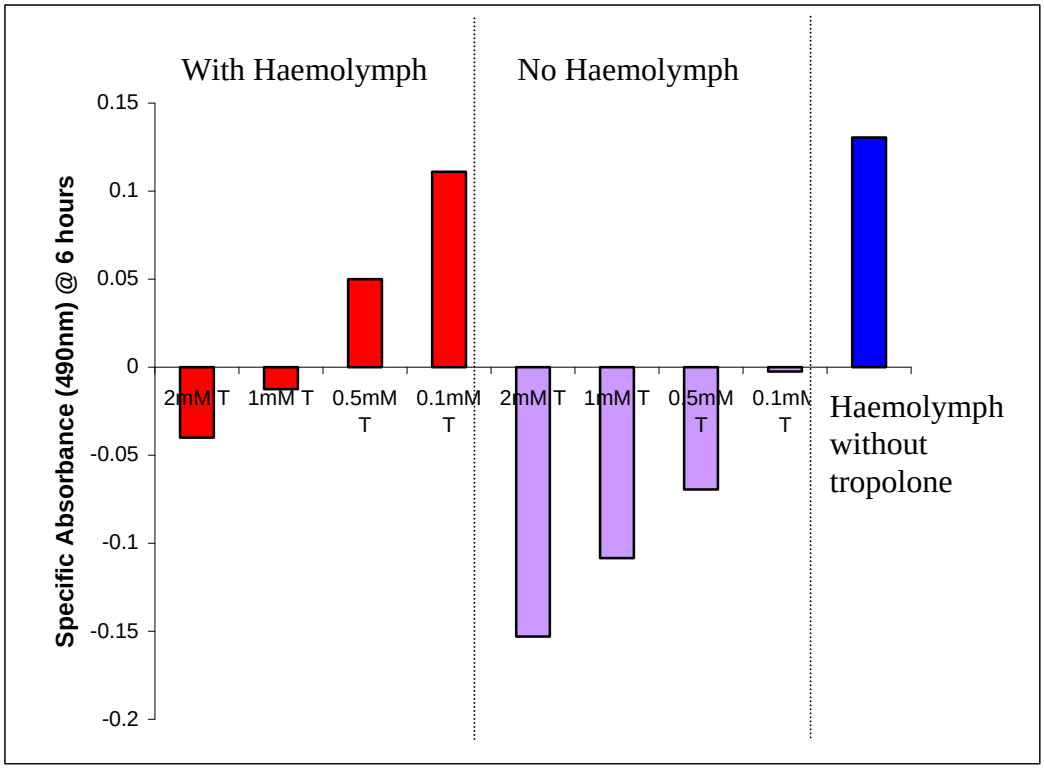


Figure 2.16: Mean inhibition of phenoloxidase (HQ assay) by different concentrations of tropolone (T; 2, 1, 0.5 and 0.1mM) with or without hemolymph. Mean values of duplicate corrected for HQ/MBTH control blanks.

2.9 Heat Treatment

Figure 2.17 shows the effect of heated and unheated hemolymph with and without the addition of tropolone on levels of phenoloxidase activity. Unheated and heated hemolymph with and without the addition of tropolone does not show any substantial difference in phenoloxidase activity between each dilution step tested. However, there is a difference between heated hemolymph with and without the addition of tropolone at a 1:10 dilution factor. No statistical analysis was conducted as each treatment consisted of one replicate.

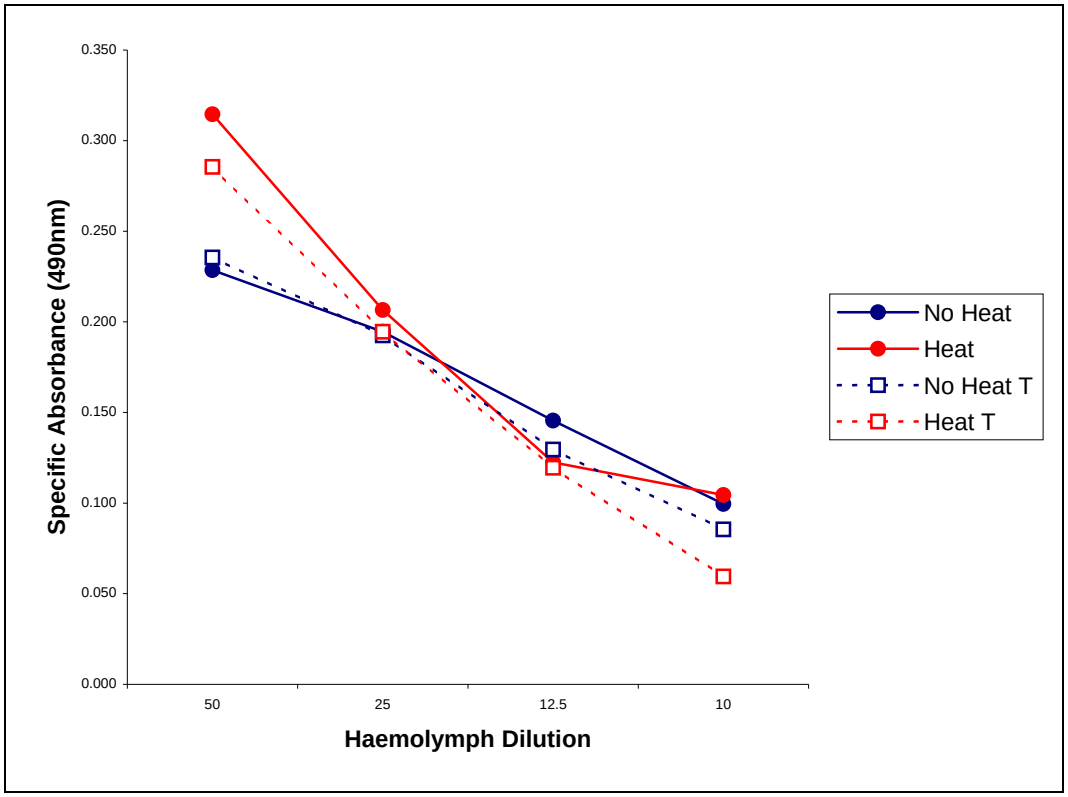


Figure 2.17: The effect of heated and unheated hemolymph with or without the addition of tropolone (T) on levels of phenoloxidase activity

2.10 Electrophoresis Experiment

2.10.1 Background

Native polyacrylamide gel electrophoresis (native PAGE) is regarded as the most highly resolving electrophoretic technique developed for separating protein(s) based upon their size and weight. Previous studies conducted by Nellaiappan & Vinayakam (1993) and Newton *et al* (2004) have demonstrated the reliability and effectiveness of using native PAGE for effectively discriminating between one or more isoforms of the enzyme phenoloxidase. To avoid confusion with assay measurements, native PAGE allows for the quantification of resolved protein band(s) determining whether multiple enzymes present in the hemolymph are capable of reacting with the substrate solution and produce individual resolved protein bands.

2.10.2 Results

Figure 2.18 show that there is protein present in the lysate supernatant that is being analysed for phenoloxidase activity (stained with bromophenol blue). The left lane 1 consists of proteins present in the haemocyte lysate, and the right lane (Lane 2) consists of proteins present in the hemolymph. There are several resolved protein bands present in both lanes of the gel stained with bromophenol blue, however it is difficult to determine the final number of distinct protein bands that are present, as several unresolved proteins are present near the base of lanes 1 and 2. It is clear from both lanes 1 and 2 that there are there are other enzymes, apart from phenoloxidase that are present in *C. gigas* hemolymph.

There are no visible resolved protein bands that appear in either the haemocyte lysate or hemolymph sample lanes on the gel that was stained with the HQ substrate.

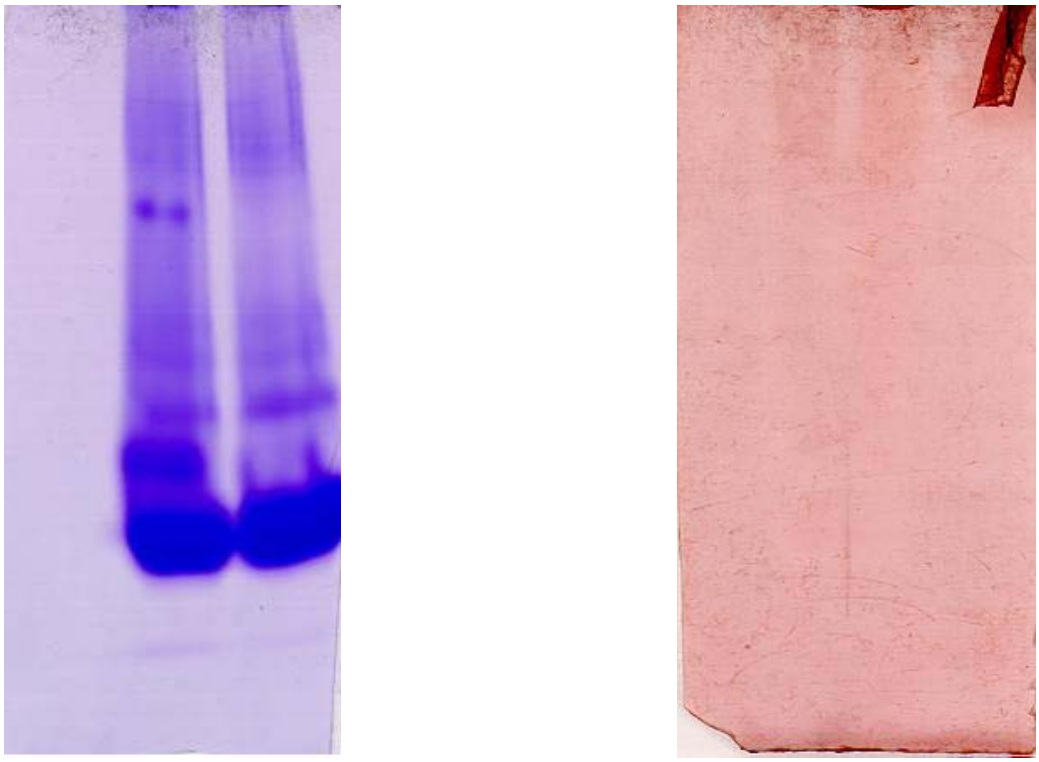


Figure 2.18: Native PAGE electrophoresis of Pacific oyster haemocyte lysate (left lane) and hemolymph (right lane). The gel on the left was stained with Bromophenol blue, that on the right was stained with HQ.

Specific Methodology

2.11 Experiment One: Mechanical Grading

2.11.1 Background

Mechanical grading is a technique utilised by oyster farmers to separate oysters in relation to size. As a result of increasing size, cultured oysters are subjected to repeated physical perturbations throughout their life cycles (Malham *et al* 2003). Three main types of mechanical graders; Rotary, Inside/Out and Flat Bed graders are used in commercial South Australian oyster farms. Each of these graders works on a system that uses different sized wire mesh, which effectively separates oysters according to size. Grading stress occurs on a regular basis, between 1-6 times per year, as oyster growth is not uniform. Once oysters are grouped according to size, they are then returned back to the offshore leases where they remain until further grading practices or subsequent harvesting for sale. Each successive grading practice involves transport, handling, sorting and emersion that further stress the oysters. Mechanical disturbances resulting from grading practices produces a transient state of stress in oysters (Lacoste *et al* 2002), leaving the potential for colonisation by pathogens due to a suppressed immune system.

2.11.2 Experiment Aims

- To determine what effects three commonly used mechanical graders have on levels of hemolymph phenoloxidase activity in Pacific oysters (*C. gigas*)
- Provide relevant/quantitative information to farmers resulting in improved commercial grading practices effectively reducing associated grading mortality

Figure 2.19: Rotary grader used for mechanical grading of Pacific oysters (*C. gigas*)

Figure 2.20: Mechanical grading of post harvest Pacific oysters (*C. gigas*) using the Rotary Grader

Figure 2.21: The Inside/Out grader used for mechanical grading of Pacific oysters (*C. gigas*)

Figure 2.22: Mechanical grading of post harvest Pacific oysters (*C. gigas*) using the Inside/Out Grader

Figure 2.23: Flat Bed Grader used for mechanical grading of Pacific oysters (*C. gigas*)

Figure 2.24. Mechanical grading of post harvest Pacific oysters (*C. gigas*) using the Flat Bed Grader

2.11.3 Hypotheses (H_A) to be Tested

- There will be significant difference in hemolymph phenoloxidase activity between the three mechanical graders
- Hemolymph phenoloxidase activity will provide a reliable means to measure stressful grading practices in Pacific oysters (*C. gigas*)

2.11.4 Null Hypotheses (H_0) to be Tested

- There will be no significant difference in hemolymph phenoloxidase activity between the three mechanical graders
- Hemolymph phenoloxidase activity will not provide a reliable means of stress response determination in Pacific oysters (*C. gigas*)

2.11.5 Experimental Animals used for Grading

Experimental oysters used in this grading experiment ranged between 70-90mm in shell length and were 3 years of age. Older and larger oysters were specifically chosen and used for experimentation because oyster farmers suggest they are more prone to stress when subjected to repeated mechanical grading practices. It is also suspected that stress in mechanical graders is proportionate to the numbers of oysters in the graders, therefore a fixed number of oysters were used in all of the mechanical grading treatments (200 oysters). Oysters were only sampled if they retained their original integrity and did not have damaged tissue or shells.

2.11.6 Determination of Grading Time

The three most commonly used graders in the oyster industry were used for this grading experiment; Inside/Out (A), Rotary (B) and Flat Bed (C) graders. Grading duration for each of the three graders differed between grader types. As a result of grading duration differences three trial runs were initially conducted before grading commenced for each grader type. This was conducted to determine the average oyster grading duration. Each of the three trial runs for each grader was timed using a stopwatch and recorded in minutes and seconds. These three repeated grading durations were measured from when the first oyster fell off the escalator belt and fell into the grader, until when the last oyster had emerged from the grader. Following completion of the three trial runs, an average grading time was quantified for each grader and was subsequently used as a set time for all the following grading experiments. The average grading duration used for the Rotary grader was 3 minutes; 1 minute 30 seconds for the Inside/out grader and 34 seconds for the Flat Bed grader.

2.11.7 Mechanical Grading Stressor

A short boat trip was taken on each of the mornings (16/3, 17/3 & 18/3) prior to the commencement of the grading experiments out to the farm leases to collect lease control (LC) samples. Lease control hemolymph samples consisted of four randomly picked oysters submerged at the time of arrival. Each oyster had 0.4-0.5mL of hemolymph removed then transferred into labelled 1.5mL microcentrifuge tubes and placed directly onto ice. Both air and water temperatures out on the lease and back in the grading shed were measured using a thermometer and recorded in degrees Celsius (°C). The appropriate number of submerged baskets required for the following grading experiments were collected and placed upon the barge and transferred back to the grading shed on shore. The time the oysters spent out of water until they reached the desired grading shed was recorded (approximately 30 minutes for each of the three days).

Upon arrival back to the first grading shed oysters were divided up into three equal grading treatment groups; 1, 2 and 3 respectively. At shed one, hemolymph samples were taken from four randomly selected oysters from group 1 and transferred individually to microcentrifuge tubes. These before grading (BG) hemolymph samples constitute a second set of control oysters to prevent any confounding results produced from the time spent out of water on the barge to the time of arrival back at the grading shed. The remaining oysters were then added to the designated mechanical grader. Upon completion of mechanical grading, oysters were separated into the appropriate three and six hour sampling time points and were transferred into two 40L plastic tubs where they remained in a shaded section of the shed until their subsequent sampling. At each of the three and six hour time points following cessation of grading, a further four randomly selected oysters from each container had hemolymph sampled, transferred to labelled 1.5mL microcentrifuge tubes and placed directly onto ice. All hemolymph samples were then taken directly from the grading sheds and placed directly into a freezer and frozen at -18°C. Following completion of grading at grader 1, oysters from groups 2 and 3 were transported to the second and third grading sheds and the previous method repeated again. This same process was repeated three times over three consecutive days (16/3, 17/3 & 18/3). On each day the order that the three graders were used was rotated (***Table 2.4***). Grader rotation was carried out in case there are any effects of the time taken from when the oysters are removed from the water to when they are graded on phenoloxidase levels, which could confound the effects produced from any one grader alone.

Day	Order of grader use		
	First	Second	Third
1 (16/3)	Inside/Out	Flat Bed	Rotary
2 (17/3)	Rotary	Inside/Out	Flat Bed
3 (18/3)	Flat Bed	Rotary	Inside/Out

Table 2.4: Three-day rotation of mechanical graders Rotary, Inside/Out and Flat Bed

2.12 Experiment Two: Mechanical Grading II

2.12.1 Background

Refer to **2.11.1**

2.12.2 Specific Methodology

Refer to **2.11.2**-**2.11.7**

2.12.3 Experiment Aims

- To determine what effects three commonly used mechanical graders have on levels of hemolymph phenoloxidase activity in Pacific oysters (*C. gigas*)
- Provide relevant/quantitative information to farmers resulting in improved commercial grading practices effectively reducing associated grading mortality

2.12.4 Hypotheses (H_A) to be Tested

- There will be significant difference in hemolymph phenoloxidase activity between the three mechanical graders
- Hemolymph phenoloxidase activity will provide a reliable means to measure stressful grading practices in Pacific oysters (*C. gigas*)

2.12.5 Null Hypotheses (H_0) to be Tested

- There will be no significant difference in hemolymph phenoloxidase activity between the three mechanical graders
- Hemolymph phenoloxidase activity will not provide a reliable means of stress response determination in Pacific oysters (*C. gigas*)

2.12.6 Mechanical Grading Stressor

This mechanical grading experiment was carried out under exactly the same conditions as the first mechanical grading experiment. However, this second grading experiment differed to the first in that it sampled hemolymph at five, ten and fifteen minutes following cessation of grading.

2.13 Experiment 3: Rumbling

2.13.1 Background

Physical stress is a commonly encountered problem imposed upon oysters in aquaculture. Physical stressors associated with oyster farming result from practices that aid in differentiating oysters according to size and restocking them in relation to basket biomass. Such stressors associated with oyster production include transport, handling, sorting, grading and associated physical damage resulting from poor handling practices. The rumbling stressor was suitable to use as it mimics what oysters experience during handling, transport and sorting procedures whilst in oyster farms.

2.13.2 Experiment Aims

- To determine the effect of a 3 minute rumbling stressor on levels of phenoloxidase activity in Pacific oysters (*C. gigas*)
- To determine the time it takes phenoloxidase to return back to baseline levels of phenoloxidase activity following cessation of rumbling

2.13.3 Hypotheses (H_A) to be Tested

- There will be significant hemolymph phenoloxidase activity when oysters are subjected to the rumbling stressor over the sampling period

2.13.4 Null Hypotheses (H_0) be Tested

- There will be no significant difference in hemolymph phenoloxidase activity when subjected to the rumbling stressor over the sampling time period

2.13.5 Experimental animals

Refer to **2.1**

2.13.6 Rumbling Stressor

The rumbling stressor consisted of removing 120 oysters from their tanks and physically shaking them by rolling them along a laboratory room floor for 3 minutes in a sealed 60L cylindrical tub

(240mm diameter; 440mm in length) at room temperature (18°C). Using a sealed tub aids as an efficient means of imitating a stressful stimulus that is capable of simple repetition in experimental conditions. Oysters were only sampled if they retained their original integrity and did not have damaged tissue or shells.

Following the completion of the rumbling stressor, oysters remained in the cylindrical tub with six randomly sampled oysters sampled at each of the following time points: 0, 5, 10, 15, 20, 30, 40, 50, 60, 2 hours, 3 hours and 6 hours following cessation of rumbling. Six control oysters were sampled directly out of the 15°C 600L acclimation tanks following the completion of sampling time points.

2.13.7 Collection of Oyster Hemolymph

Refer to **2.2.**

2.14 Experiment 4: Pacific Oyster Family Lines

2.14.1 Background

Pacific oyster (*C. gigas*) populations exhibit large amounts of genetic variation, of which much is inherited from one generation to the next. Current selective breeding programs seek to exploit this variation to produce new lines of oysters that are selected for one or more inherited trait that aim to optimise production. Almost all South Australian farmers grow what is termed a 'commercial line' of Pacific oyster that promotes one or more desirable trait. However, trials in South Australia and Tasmania are underway that involve the use of several genetically distinct lines of oysters in an attempt to improve growth and reduce stock mortality. Hatchery production of seed stock allows for genetic manipulation through selective breeding allowing the production of improved oysters lines that have one or more desired traits incorporated primarily to increase production, and or to reduce disease related stock mortality (Langdon *et al* 2003). Selective breeding programs can be easily facilitated in commercial aquaculture as culture methods allow for easy separation of family lines. However, current culture techniques leave oysters susceptible to adverse environmental conditions that can lead to a state of stress in *C. gigas* (Ward *et al* 2000).

Pacific oysters are cultured in the intertidal zone and as a result they become exposed through periodical emersion due to tidal variations. Periods of emersion in oysters cause them to close their shells to minimize desiccation in response to fluctuating tides. When the valves pull the shell closed, activity becomes depressed and results in the oyster respiring at lower levels (Akberali & Trueman 1985). Akberali & Trueman (1985) described marine bivalve exposure to air resulted in metabolic stress through an inability to successfully feed, limitation for gas and metabolite exchange and desiccation. The stressor consisted of emersion that is a daily occurrence on oyster leases due to the dynamic nature of fluctuating tidal movements. Emersion stress was deemed suitable for experimentation, as Pacific oysters are cultured on wooden lines that enable greater accessibility by workers, however in down sight oysters are left exposed at 30% of the time due to tidal movements, which is thought to result in stress.

2.14.2 Experiment Aim

- To determine whether any variation in hemolymph phenoloxidase activity exists between the five selected lines of Pacific oysters (*C. gigas*) when subjected to a 25-hour period of emersion.

2.14.3 Hypotheses (H_A) to be Tested

- There will be significant differences in hemolymph phenoloxidase activity between different genetic lines of Pacific Oysters (*C. gigas*).

2.14.4 Null Hypotheses (H_0) be Tested

- There will be no significant difference in hemolymph phenoloxidase activity between different genetic lines of Pacific oysters (*C. gigas*).

2.14.5 Experimental Lines of Pacific Oysters

Five family lines of Pacific oysters (*C. gigas*) were obtained from leases owned by Gary Zippel in Smoky Bay. The five family lines of oysters used for experimentation were termed A, B, C, D and E lines respectively. Due to commercial sensitivity involving the five selected lines of oysters, no additional information was made available. Family lines were sourced from Tasmanian hatcheries that provide spat to the majority of South Australian growers. All five lines of oysters ranged in size from 60-80mm shell length and were two years old. All five lines of oysters sampled had been cultured under the same environmental and husbandry conditions to eliminate any environmental effects on the oyster phenotype.

2.14.6 Sampling of Oyster Haemolymph

Four oysters from each of the five genetic lines had hemolymph sampled (0.4-0.5mL/oyster) out on the lease to provide basal phenoloxidase levels prior to removal from the leases and transportation back to shore. The time the oysters remained out of water once removed from the lease and transported back to the grading shed was recorded and included as part of the duration of the emersion stressor being tested. All five lines of oysters remained in their units undisturbed and were left emersed overnight for a period of 25 hours prior to subsequent hemolymph sampling.

2.15 Experiment 5: Salinity

2.15.1 Background

Pacific oysters are generally cultured in intertidal areas that are exposed to dynamic salinity regimes. As a result, oysters are often confronted with varying salinities that inevitably lead to a state of stress in the organism. Changes in salinity can be due to a variety of reasons including evaporation, localised flooding and/or in times of high precipitation. Generally bivalve's first response to fluctuating salinity is to retract or close their shells. As a result of shell closure, the oyster's tissue

becomes isolated from osmotic changes in the external medium (Hawkins & Hilbish 1992). Previous studies conducted by Higashi *et al* (1989) demonstrated that high and low salinity levels produced large and rapid alterations in stress indicating metabolites in the mollusk *Haliotis laevigata*. Molluscs are well known to exhibit responses to salinity stresses that are a combination of inorganic and organic ionic restoration of balance (Edwards 2003). Increasing and decreasing salinity levels elevate various ninhydrin-positive chemicals and amino acids in a variety of bivalve molluscs that inevitably lead to stress (Shumway *et al* 1977). Changes induced by fluctuating salinities on the physiological and behavioural condition of oysters is generally related to the organism trying to reestablish homeostasis to cope with the salinity change. Hawkins & Hilbish (1992) proposed that small and regular fluctuations in salinity constitute a stressful stimulus, compromising the organism's immune system and possibly leading to mortality. It is unusual for South Australian growing conditions to be subjected to low salinity levels, however hypersaline conditions have been reported.

2.15.2 Experiment Aims

- To determine whether varying water salinities effect levels of phenoloxidase activity in Pacific oysters (*C. gigas*)
- To determine if instant ocean synthetic sea salt has an effect on levels of phenoloxidase activity in Pacific oysters (*C. gigas*)

2.15.3 Hypotheses (H_A) to be Tested

- There will be a significant difference in hemolymph phenoloxidase activity between the five salinity treatments

2.15.4 Null Hypotheses (H_0) to be Tested

- There will be no significant differences in phenoloxidase activity between the five salinity treatments

2.15.5 Experimental Animals

Refer to **2.1.**

2.15.6 Salinity Stressor

Fifteen 100L plastic transportable containers were set up and used as experimental replicate tanks. Each of the five treatment groups; A, B, C, D and E respectively, consisted of three replicate tanks containing ten oysters per tank. Five different salinity treatments were tested (***Table 2.6***) each having six oysters randomly sampled over four consecutive days.

Treatment	Salinity (ppt)	Amount of Instant Ocean (g)	Source	Final Volume (L)
A	5	100	MFTW	20
B	15	300	MFTW	20
C1	38	700	MFTW	20
C2	38	-	Seawater	20
D	50	1000	MFTW	20

Table 2.6: Various salinity treatments used for the elicitation of salinity stress

The culture solution used for treatments A, B, C2 and D were made up to the specified salinity using instant ocean synthetic sea salt mixed with Millipore filtered tap water (MFTW; 0.22 μm filter). Each tank from the four Instant Ocean treatments had the specified amount of instant ocean powder (***Table 2.6***) mixed thoroughly into MFTW until no powder residue was visible. Full strength seawater (38ppt) used for treatment C1 came from the SARDI saltwater recirculation system. Two sets of control treatments were used, treatment C1 was made up to 38ppt using Instant Ocean, whilst treatment C2 used 38ppt recirculation seawater. The purpose of two control treatments was to determine if instant ocean powder had any effects on levels of phenoloxidase activity in oysters from treatments A, B, C2 and D. Water for each of the replicate tanks was left for an acclimation period of 24 hours and vigorously aerated prior to the addition of the ten experimental animals to ensure that water temperature (15°C) had adjusted to that of the air temperature (17.5°C). Both water and air temperature were measured daily using a thermometer to determine if the fluctuating water and air temperature had any effect on salinity levels of each tank. Oysters were transferred to treatment tanks when the water temperature had adjusted to room temperature. Oysters remained in each of the tanks for an acclimation period of six hours prior to the commencement of hemolymph sampling on Day 0. All additional hemolymph sampling occurred at a set time point 24 hours following sampling on day 0 over the four-day period. Salinity was checked and recorded daily using a refractometer and expressed as parts per thousand (ppt). Calibration of the refractometer with Millipore-filtered tap water occurred each day prior to the testing of each replicate tank. Salinity determination was repeated twice daily (morning/afternoon) producing an average value for each of the four days of experimentation. Salinity levels were tested and recorded daily to determine if abrupt changes in levels between the differing treatments had any effect on phenoloxidase activity, with the ability of confounding subsequent final results.

Each replicate tank received a 25% volume water exchange each day over the four days of experimentation. Exchanged water was prepared and aerated the previous day allowing for adjustment of water temperature to the air temperature, ensuring dissolved oxygen was high and instant ocean powder in treatments A, B, C1 and D was completely dissolved. Again salinity and temperature of this water was checked prior to water exchange to ensure it had the same characteristics as the treatment water it was exchanged with. Plastic removable lids were sealed on the plastic tanks over the duration of the four-day experiment to prevent any water evaporation that could inevitably lead to variation in salinities between replicates tanks.

2.15.7 Collection of Oyster Hemolymph

Refer to **2.2**.

2.16 Experiment 6: Nutrition

2.16.1 Background

In the wild bivalve molluscs feed on a variety of materials in suspension that include phytoplankton, bacteria and particulate organic material. Bivalves are non-selective filter feeders that feed by means of ciliated fleshy extrusions of their labial palps (Beninger *et al* 1991). Oysters filter water through their gills to remove suspended particles via an inhalant current that then transports and expels filtered water from the mantle cavity. Filter feeding is an essential process in maintaining metabolic action in Pacific oysters and food supply is considered the most important factor that affects growth in Pacific oysters (Brown 1988). By providing preferred and non-preferred microalgal species or no food at all this experiment aims to reduce oyster growth. By effectively reducing growth, it is proposed that the metabolic state of the organism is being challenged, leading to a state of stress in the organism.

2.16.2 Experiment Aims

- To determine if feeding preferred and non-preferred algae and starving oysters has any resultant effect on phenoloxidase activity in Pacific oysters
- To gauge the general health of oysters by using condition indices to determine whether oyster condition affects the levels of hemolymph phenoloxidase activity
- To determine the correlations that exist between feed preference, condition indices and hemolymph phenoloxidase activity responses

2.16.3 Hypotheses (H_A) to be Tested

- There will be a significant difference in hemolymph phenoloxidase activity between the three feeding treatments
- Hemolymph phenoloxidase activity will be significantly higher in oysters from the starvation treatment
- There will be a significant difference in oyster condition index between the three feeding treatments

2.16.4 Null Hypotheses (H_0) to be Tested

- There will be no significant difference in hemolymph phenoloxidase activity between the three feeding treatment groups
- Hemolymph phenoloxidase activity will not be significantly higher in oysters from the starvation treatment
- There will be no differences in condition indices between oysters from each of the three treatment groups

2.16.5 Tank Design

Three treatment groups (A, B & C) each comprising six 20L tanks that were set up on a continuously flowing saltwater recirculation system receiving vigorous aeration. The recirculation water was maintained at 37-38ppt; 15±1°C for the thirty day experiment duration.

Each of the 20L tanks contained 8 oysters, which were taken from the 600L acclimation tanks connected to the same recirculation system. Oysters were transferred to tanks for an acclimation period of 24 hours prior to the commencement of the experiment. All tanks were randomly placed along the recirculation system to prevent any confounding effect that may occur due to differences between tank placement. Six oysters were randomly sampled from each of the three treatments at a standardised time each day. Hemolymph sampling occurred on days: 0, 1, 3, 5, 7, 14, 21 and 28. One randomly chosen oyster from each of the eighteen tanks was sampled time point, resulting in six hemolymph samples taken for each treatment at each of the eight time points. Control oysters consisted of oysters sampled from the 600L acclimation tanks on Day 0 and all following oysters sampled from treatment A.

2.16.6 Microalgae Supplementation

Three different treatments (A, B & C) were used to determine the effect of feeding preferred (A), non-preferred (B) and no microalgae (C) to oysters in each of the treatments (***Table 2.7***). The preferred microalgae species of choice used was a mixture of two Golden algae species: *Pavlova lutheri* and *Isochrysis* sp. The non-preferred species of microalgae used was the Green algae species *Nannochloropsis atomus*. The final treatment group received no microalgae supplementation and were effectively starved for the four-week experiment duration. All replicate tanks of treatment A and B each received 500mL of concentrated microalgae measured with a graduated cylinder at a standardised time every two days for twenty-eight days. Immediately prior to the addition of algae to all replicate tanks in treatment A and B, flow through water was turned off for a period of three hours to allow adequate filtration by the oysters and ensure that the microalgae was not being flushed out of the system.

Treatment	Algae species	Amount fed every 2nd day (mL)
A	*Pavlova lutheri & Isochrysis*	500
B	*Nannochloropsis atomus*	500
C	Starvation treatment	0

Table 2.7: Different feeding treatments used for the experiment

2.16.7 Calculation of Condition Indexes (CI)

The Condition Index (CI), which is a value used to gauge the general health of Pacific oysters, was a protocol refined from the methods of Peters and Raftos (2003). The Condition Index of a bivalve mollusc is a numerical representation of the nutritive status or quality of its soft tissue at any particular time (Abbe & Albright 2003). The overall condition of the oyster is determined by the numerical figure produced that lies somewhere in between 1 (best condition) and 0 (worst condition). In this case condition indices were used to determine if oysters were affected by the

three treatments.

CI = wet tissue weight ÷ (weight prior to shucking – oven dried shell weight)

Oysters were removed from their appropriate treatment tanks, scrubbed with a plastic brush and air-dried for a period of one hour prior to weighing. Following weighing, oysters were shucked and had between 0.4-0.5mL of hemolymph removed from each individual oyster. Oyster soft tissue was then removed from the shell using a scalpel and the wet tissue weight was measured using a Mettler Toledo AE 240 scale and recorded. Hemolymph volume was then included in the total wet oyster weight recorded. Both shell and wet tissue weight was weighed to the nearest 0.001g. Oyster shells were dried in an oven set at 50°C for a period of 48 hours; the oysters were continually weighed until there was no measurable change in dried shell weight.

Chapter 3 Results

3.1 Experiment 1: Mechanical Grading

3.1.1 L-DOPA Assay

Figures 3.1, 3.*2* and 3.*3* show the mean level of phenoloxidase activity from elicited by the three types of mechanical graders tested. Lease control (LC) represents baseline control levels, before grading (BG) represents a second set of controls measuring the effect of time out of water prior to grading on phenoloxidase levels and 3 and 6 hour are sampling time points following cessation of grading. Both the Rotary (***Figure 3.1***) and Inside/Out (***Figure 3.2***) graders did not cause any substantial change in phenoloxidase levels from the basal control value (61.1±8.9%). Results from the two-way ANOVA (***Table 3.1***) reveal that there is no significant difference in levels of phenoloxidase activity between both the independent factors TIME ($P > 0.249$) or GRADER ($P > 0.105$) and there is significant interaction between TIME * GRADER ($P < 0.035$). Pairwise comparisons (***Table 3.2***) reveal that the flat bed grader had significantly higher levels of phenoloxidase activity than both the rotary ($P < 0.003$) and Inside/Out ($P < 0.001$) graders at six hours following the cessation of grading (***Figure 3.4***).

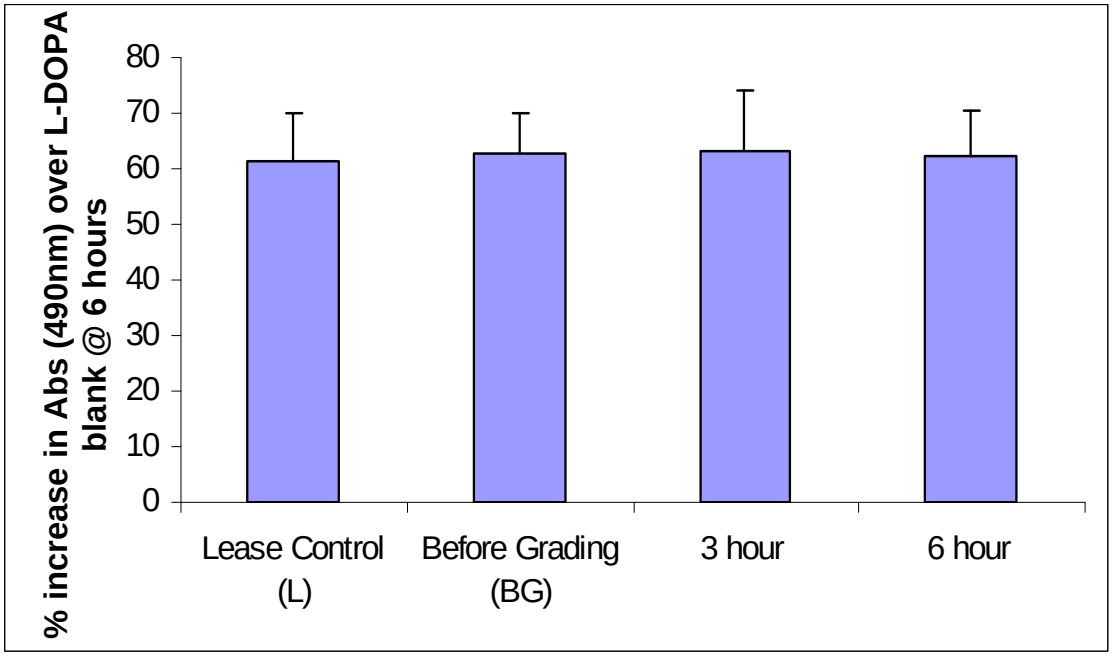


Figure 3.1: Mean (±SD) phenoloxidase activity (L-DOPA assay) (%) resulting from processing by a rotary grader (n = 4).

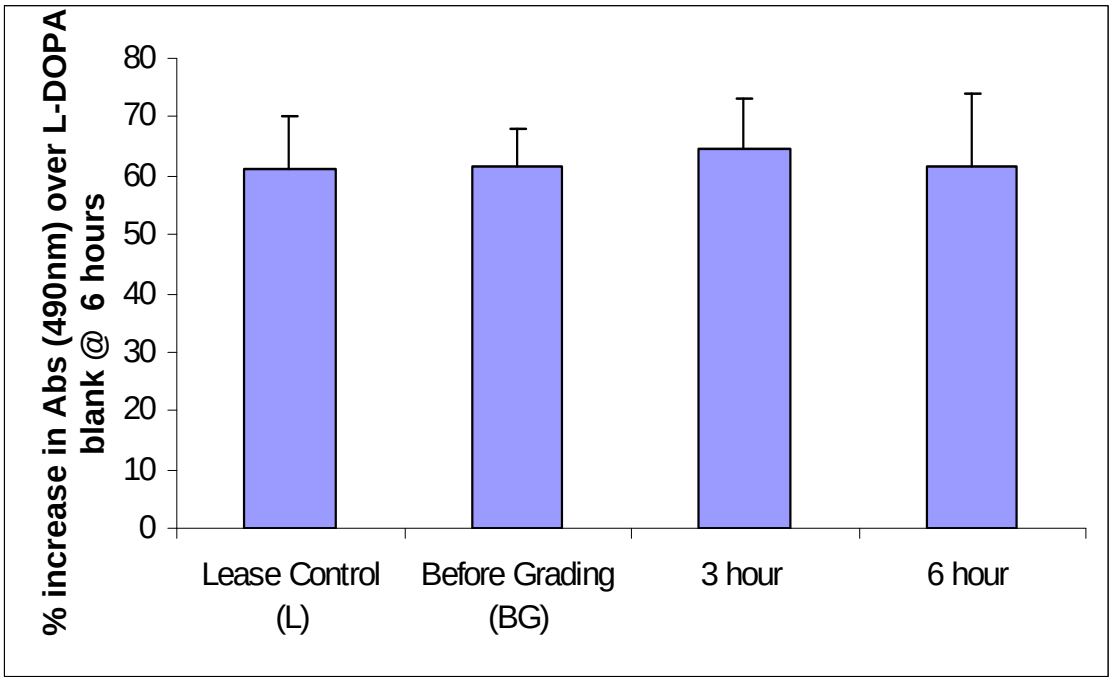


Figure 3.2: Mean (±SD) phenoloxidase activity (L-DOPA assay) (%) resulting from processing by a Inside/Out grader (n = 4)

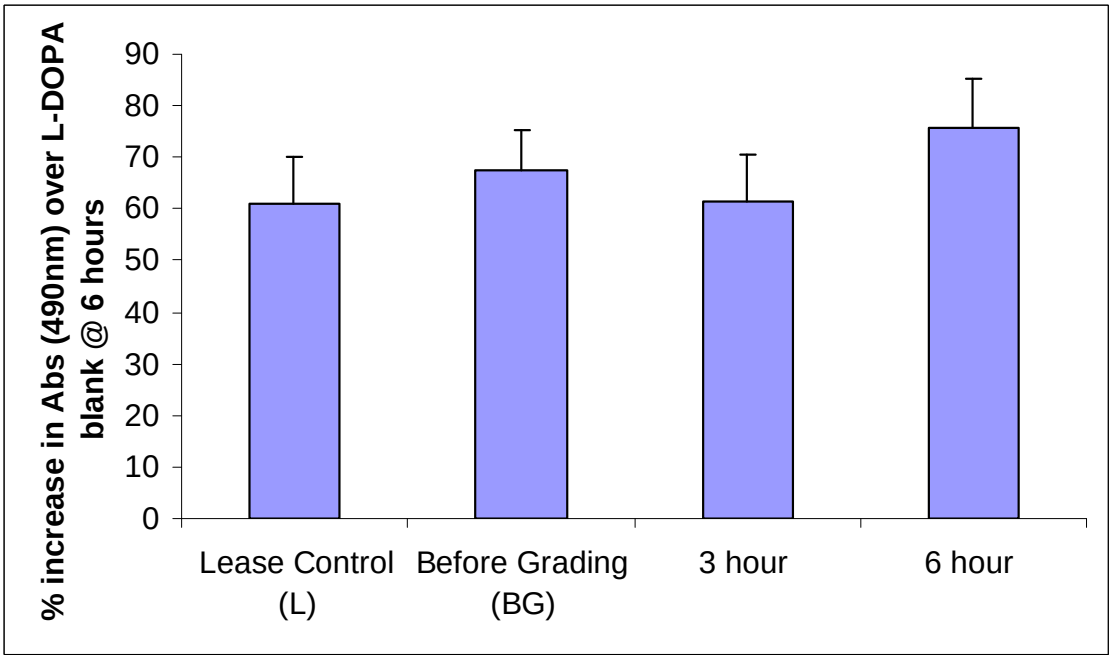


Figure 3.3: Mean (±SD) phenoloxidase activity (L-DOPA assay) (%) resulting from processing by a flat bed grader (n = 4)

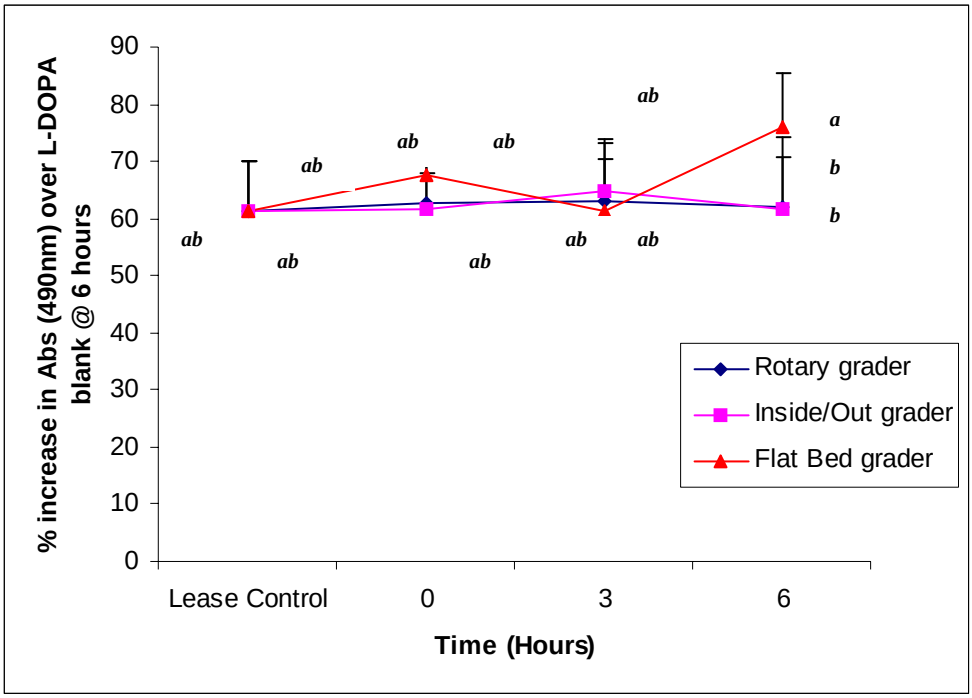


Figure 3.4: Mean (±SD) phenoloxidase activity (L-DOPA assay) (%) resulting from processing by a rotary, Inside/Out and flat bed grader (n = 4). Letters ***a*** and ***b*** denote significance between graders at a particular time.

Tests of Between-Subjects Effects

Dependent Variable: ACTIVITY

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2124.787(a)	11	193.162	2.323	.013
Intercept	473461.730	1	473461.730	5694.276	.000
TIME	347.691	3	115.897	1.394	.249
GRADER	382.406	2	191.203	2.300	.105
TIME * GRADER	1180.536	6	196.756	2.366	.035
Error	8730.430	105	83.147		
Total	496341.257	117			
Corrected Total	10855.218	116			

a R Squared = .196 (Adjusted R Squared = .111)

Table 3.1: Two-way analysis of variance (ANOVA) for mechanical grading experiment (L-DOPA assay)

Pairwise Comparisons

Dependent Variable: ACTIVITY

TIME	(I) GRADER	(J) GRADER	Mean Difference (I-J)	Std. Error	Sig.(a)	95% Confidence Interval for Difference (a)	
						Lower Bound	Upper Bound
6 hour	Flat bed	In/out	14.064(*)	3.806	.001	4.804	23.325
		Rotary	13.366(*)	3.904	.003	3.867	22.865

Table 3.2: Pairwise comparisons indicating significant difference ($P < 0.05$) in levels of phenoloxidase activity between the rotary, Inside/Out and flat bed graders (L-DOPA assay)

3.1.2 HQ Assay

The rotary (***Figure 3.5***), Inside/Out (***Figure 3.6***) and the flat bed (***Figure 3.7***) graders did not cause any substantial change in levels of phenoloxidase activity from the baseline control value (28.9 ± 20.5%) following cessation of grading. The two-way ANOVA (***Table 3.3***) revealed that there was no significant difference in levels of phenoloxidase activity between both the independent factors TIME ($P > 0.219$) and GRADER ($P > 0.656$) and the interaction between TIME * GRADER ($P > 0.619$) (***Figure 3.8***).

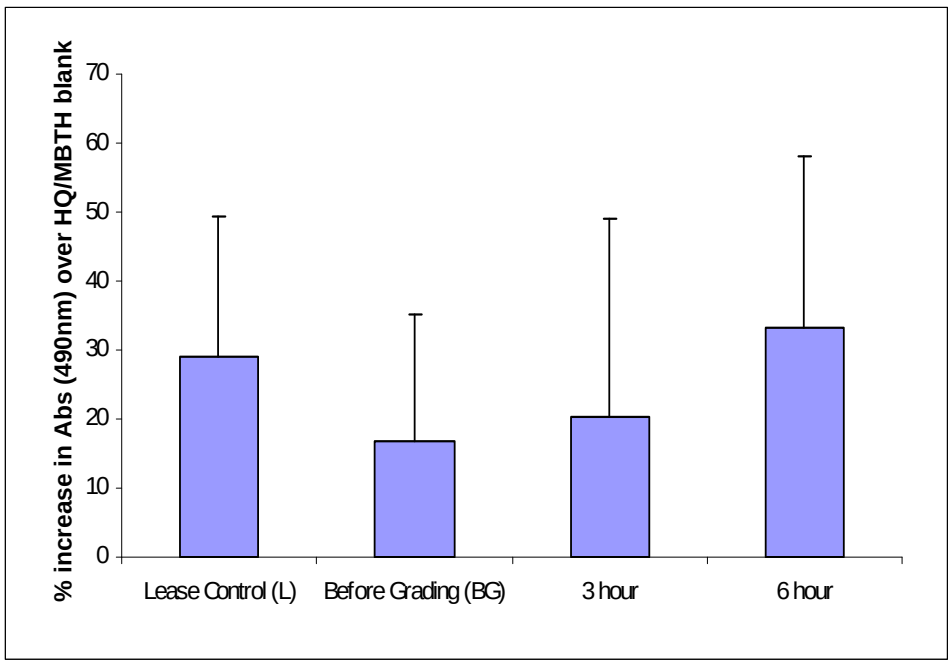


Figure 3.5: Mean (±SD) phenoloxidase activity (HQ assay) (%) resulting from processing by a rotary grader (n = 4).

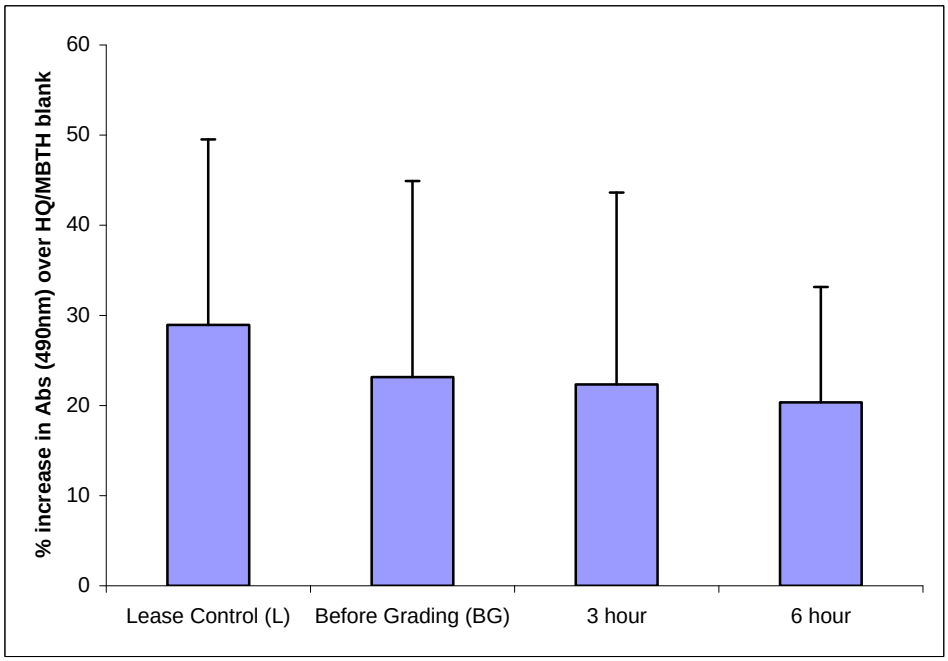


Figure 3.6: Mean (±SD) phenoloxidase activity (HQ assay) (%) resulting from processing by a Inside/Out grader (n = 4).

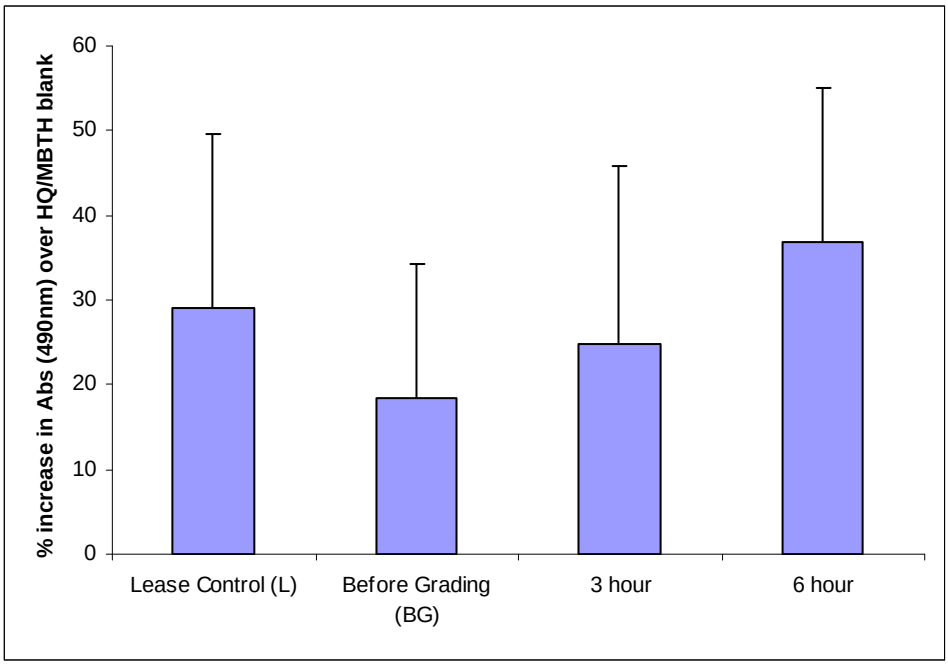


Figure 3.7: Mean (±SD) phenoloxidase activity (HQ assay) (%) resulting from processing by a flat bed grader (n = 4)

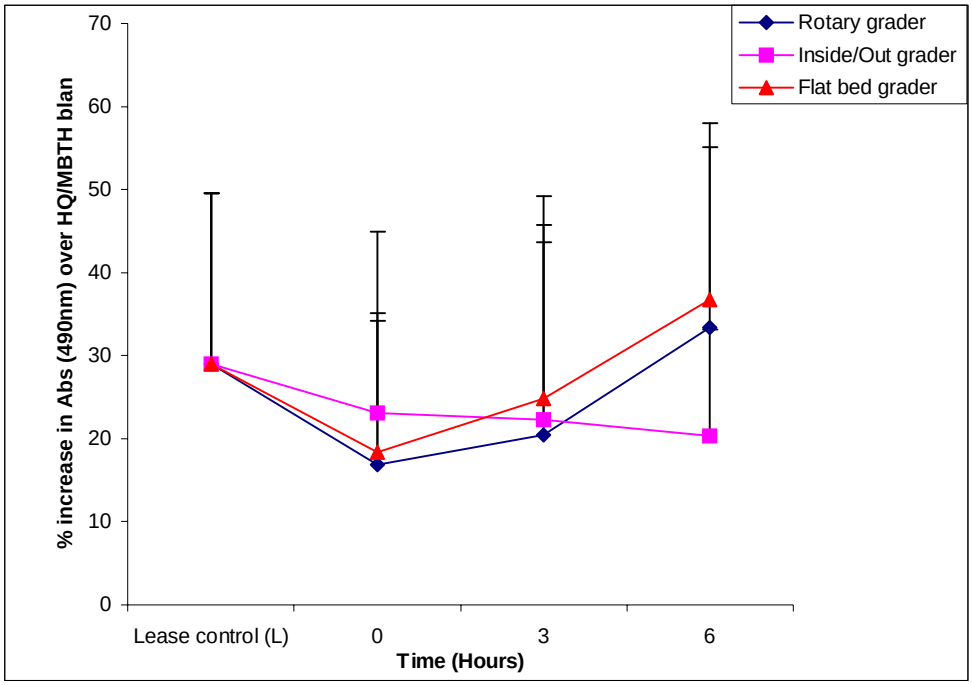


Figure 3.8: Mean (±SD) phenoloxidase activity (HQ assay) (%) resulting from processing by a rotary, Inside/Out and flat bed grader (n = 4).

Tests of Between-Subjects Effects

Dependent Variable: ACTIVITY

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4258.326(a)	11	387.121	.901	.542
Intercept	74500.635	1	74500.635	173.363	.000
TIME	1925.436	3	641.812	1.493	.219
GRADER	362.953	2	181.477	.422	.656
TIME * GRADER	1906.670	6	317.778	.739	.619
Error	55436.244	129	429.738		
Total	134561.868	141			
Corrected Total	59694.570	140			

a R Squared = .071 (Adjusted R Squared = -.008)

Table 3.3: Two-way analysis of variance (ANOVA) for mechanical grading experiment (HQ assay)

3.2 Experiment 2: Mechanical Grading 2

3.2.1 L-DOPA Assay

Figures 3.9, ***3.10*** and ***3.11*** show the mean level of phenoloxidase activity from elicited by the three types of mechanical graders tested. Lease control (LC) represents baseline control levels, before grading (BG) represents a second set of controls measuring the effect of time out of water prior to grading on phenoloxidase levels and 5, 10 and 15 minutes represent sampling times following cessation of rumbling. The rotary (***Figure 3.9***), Inside/Out (***Figure 3.10***) and flat bed (***Figure 3.11***) graders all follow a uniform trend in levels of phenoloxidase activity that deviates little from the baseline levels of phenoloxidase activity (55.78±10.7%). Results from the two-way ANOVA (***Table 3.4***) reveal that there is no significant difference in levels of phenoloxidase activity from the independent factor GRADER ($P > 0.057$) and there is a significant difference from both the independent factor TIME ($P < 0.000$) and the interaction between the factors TIME * GRADER ($P < 0.024$). Pairwise comparisons (***Table 3.5***) reveal that the flat bed grader had significantly higher levels of phenoloxidase activity compared to both the rotary grader ($P < 0.011$) at ten minutes following cessation of grading and the Inside/Out grader ($P < 0.005$) at fifteen minutes following cessation of grading (***Figure 3.12***).

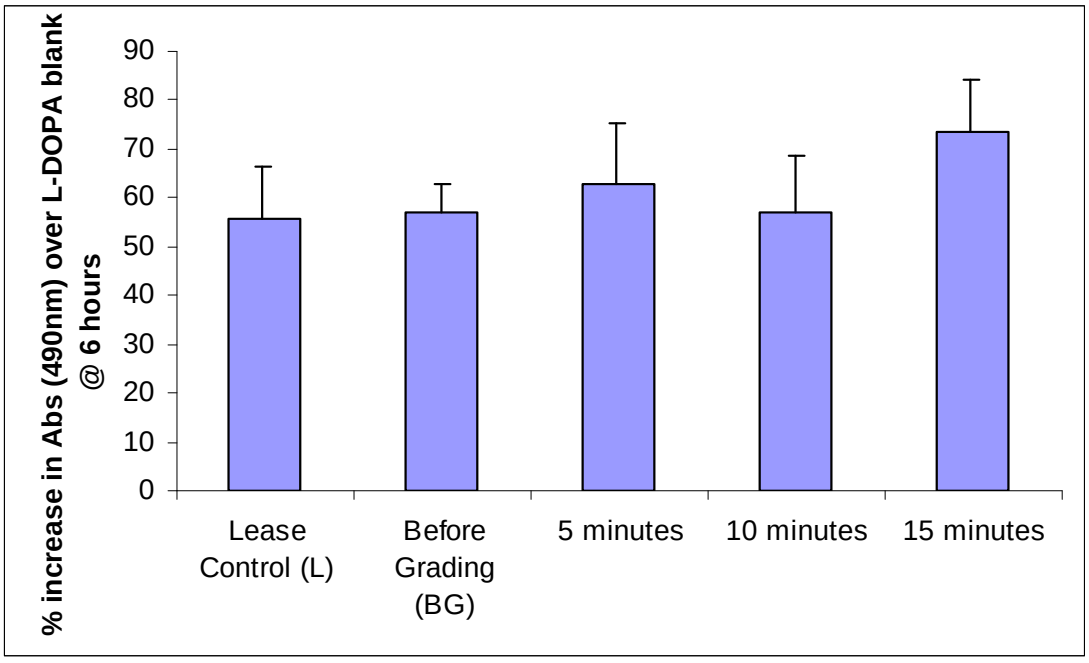


Figure 3.9: Mean (±SD) phenoloxidase activity (L-DOPA assay) (%) resulting from processing by a Rotary grader (n = 4)

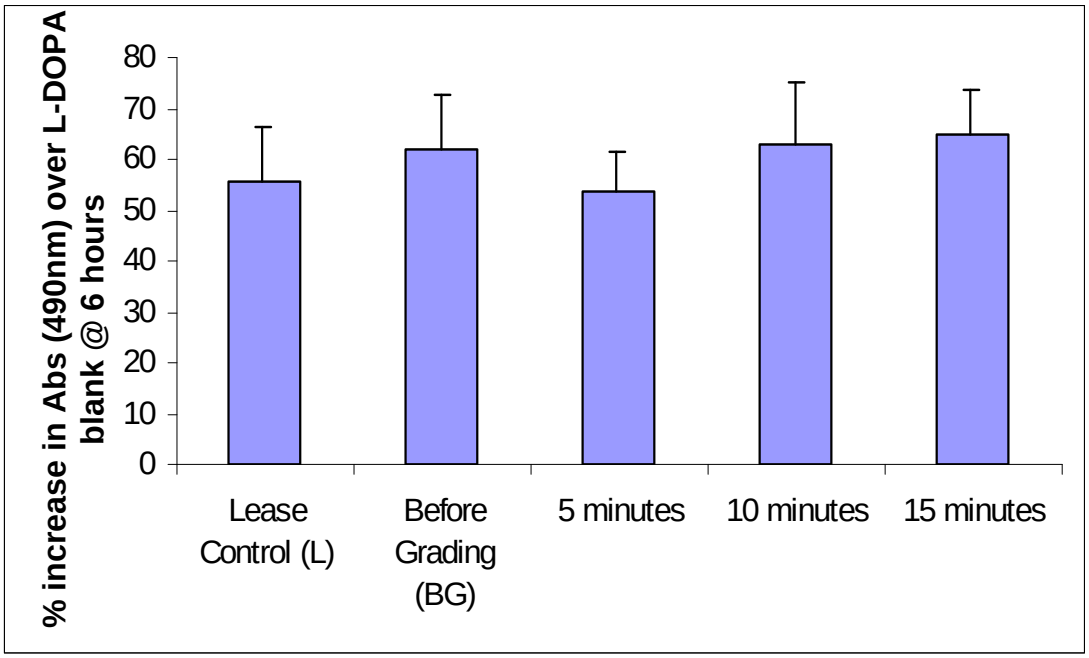


Figure 3.10: Mean (±SD) phenoloxidase activity (L-DOPA assay) (%) resulting from processing by a Inside/Out grader (n = 4)

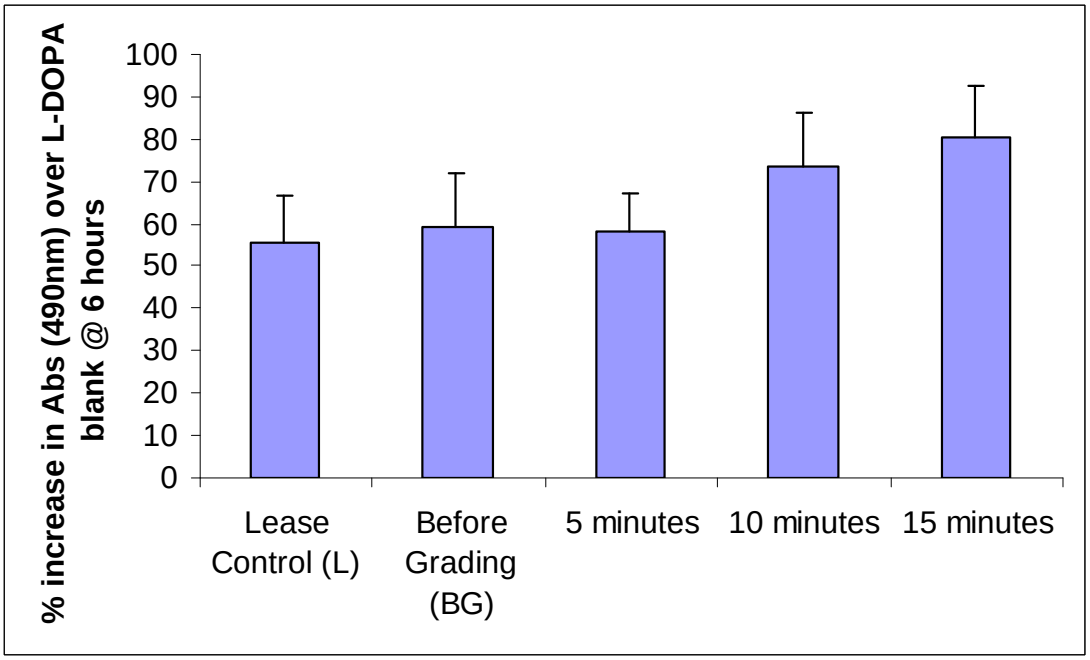


Figure 3.11: Mean (±SD) phenoloxidase activity (L-DOPA assay) (%) resulting from processing by a flat bed grader (n = 4)

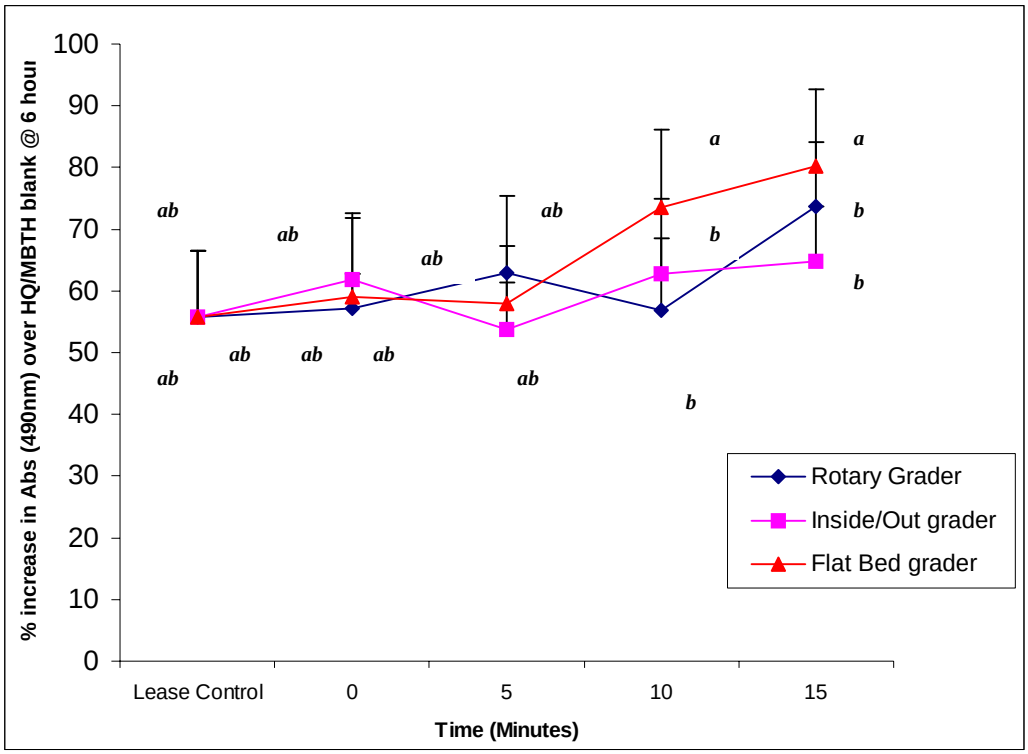


Figure 3.12: Mean (±SD) phenoloxidase activity (L-DOPA assay) (%) resulting from processing by a rotary, Inside/Out and flat bed grader (n = 4). Letters ***a*** and ***b*** denote significance between graders at a particular time.

Tests of Between-Subjects Effects

Dependent Variable: ACTIVITY

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8655.514(a)	14	618.251	5.092	.000
Intercept	554780.591	1	554780.591	4569.310	.000
TIME	4970.908	4	1242.727	10.235	.000
GRADER	712.954	2	356.477	2.936	.057
TIME * GRADER	2240.356	8	280.045	2.307	.024
Error	15783.889	130	121.415		
Total	609421.910	145			
Corrected Total	24439.403	144			

a R Squared = .354 (Adjusted R Squared = .285)

Table 3.4: Two-way analysis of variance (ANOVA) for mechanical grading experiment 2 (L-DOPA assay)

Pairwise Comparisons

Dependent Variable: ACTIVITY

TIME	(I) GRADER	(J) GRADER	Mean Difference (I-J)	Std. Error	Sig.(a)	95% Confidence Interval for Difference (a)	
						Lower Bound	Upper Bound
10 minutes	Flat Bed	Rotary	15.531(*)	5.240	.011	2.821	28.241
15 minutes	Flat Bed	In/Out	15.229(*)	4.718	.005	3.787	26.672

Table 3.5: Pairwise comparisons indicating significant differences ($P < 0.05$) in levels of phenoloxidase activity between rotary, Inside/out and flat bed graders (L-DOPA assay).

3.2.2 HQ Assay

The rotary (***Figure 3.13***), Inside/Out (***Figure 3.14***) and flat bed (***Figure 3.15***) graders all follow a uniform trend in levels of phenoloxidase activity that deviates little from the baseline level of activity (20.8±13.3%). Results from the two-way ANOVA (***Table 3.6***) reveal that there is no significant difference in levels of phenoloxidase activity between both the independent factors TIME ($P > 0.178$) and GRADER ($P > 0.759$) and between the interaction of TIME * GRADER ($P > 0.986$) (***Figure 3.16***).

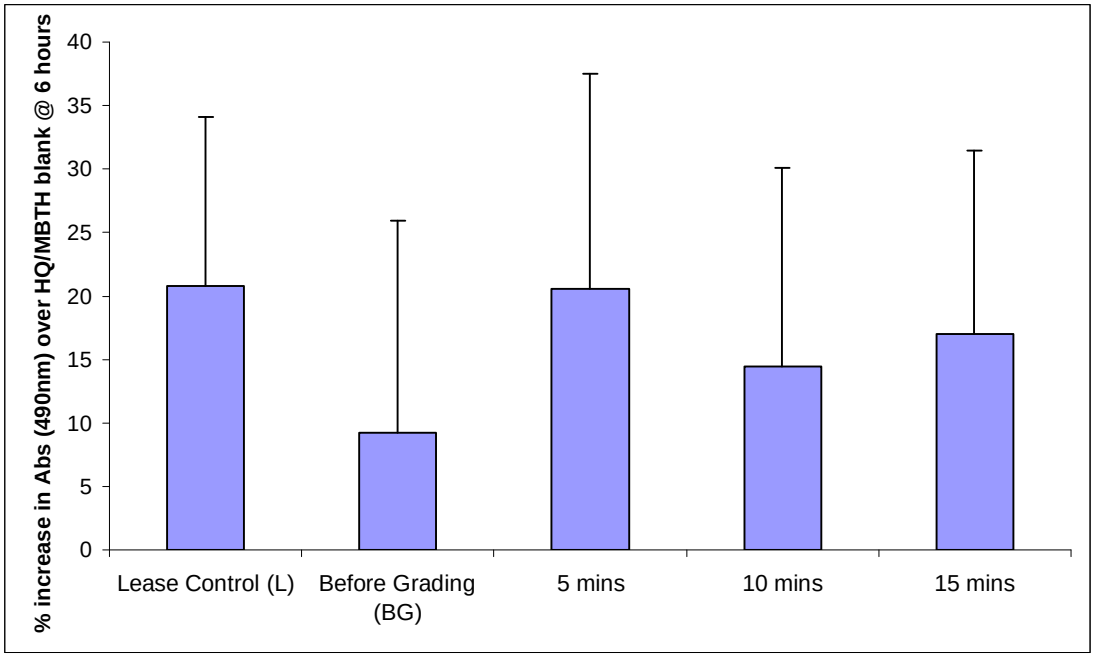


Figure 3.13: Mean (±SD) phenoloxidase activity (HQ assay) (%) resulting from processing by a Rotary grader (n = 4)

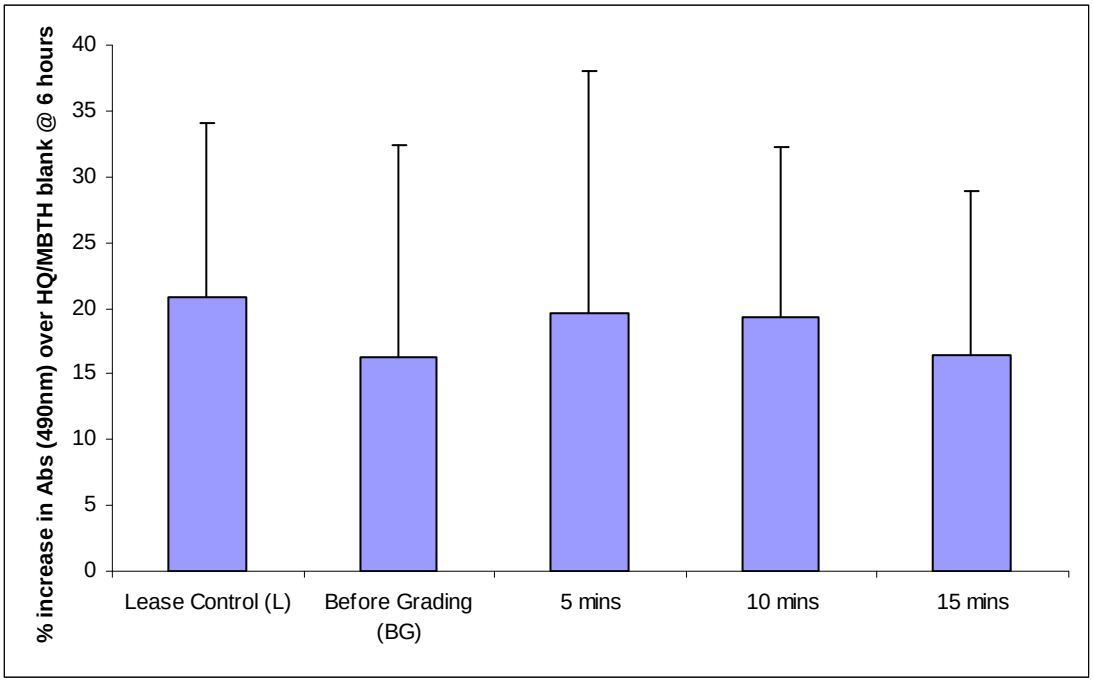


Figure 3.14: Mean (±SD) phenoloxidase activity (HQ assay) (%) resulting from processing by an Inside/Out grader (n = 4)

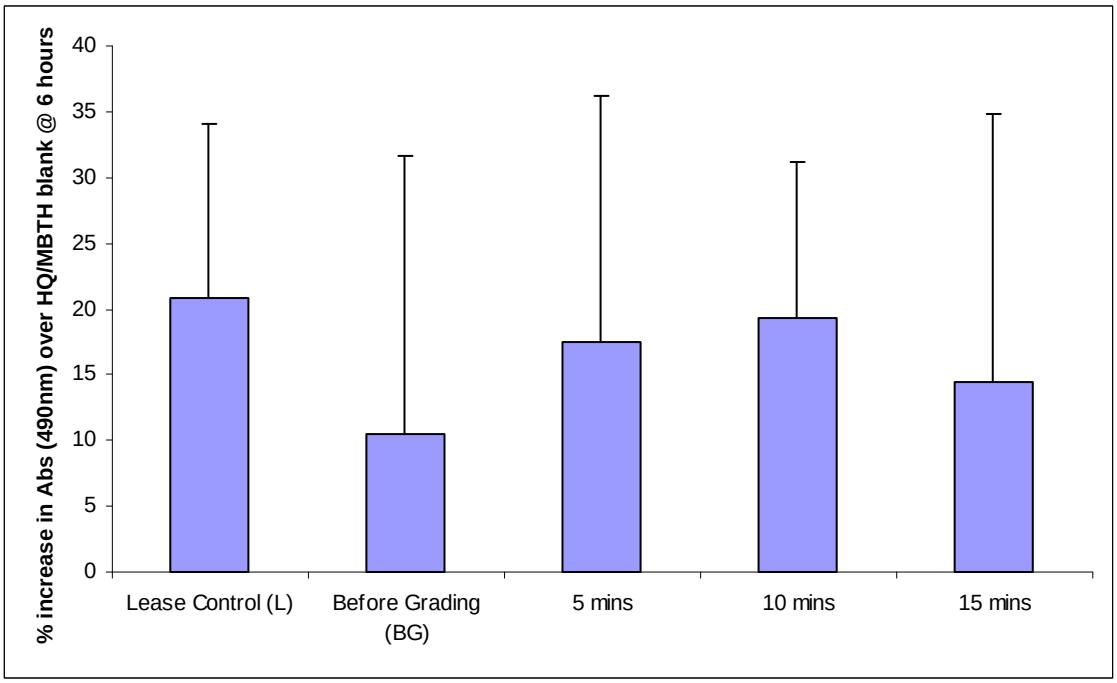


Figure 3.15: Mean (±SD) phenoloxidase activity (HQ assay) (%) resulting from processing by a flat bed grader (n = 4)

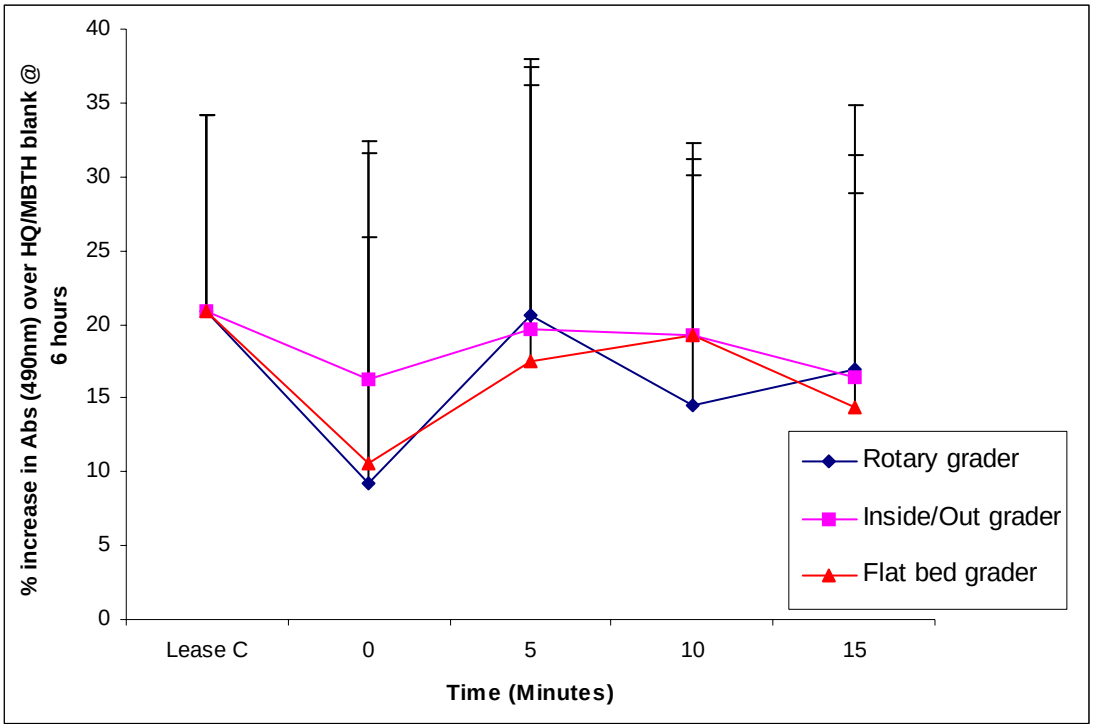


Figure 3.16: Mean (±SD) phenoloxidase activity (HQ assay) (%) resulting from processing by a rotary, Inside/Out and flat bed grader (n = 4).

Tests of Between-Subjects Effects

Dependent Variable: ACTIVITY

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2204.820(a)	14	157.487	.625	.841
Intercept	55390.013	1	55390.013	219.869	.000
TIME	1605.978	4	401.495	1.594	.178
GRADER	138.924	2	69.462	.276	.759
TIME * GRADER	451.668	8	56.458	.224	.986
Error	40559.629	161	251.923		
Total	97883.849	176			
Corrected Total	42764.449	175			

a R Squared = .052 (Adjusted R Squared = -.031)

Table 3.6: Two-way analysis of variance (ANOVA) for mechanical grading experiment 2 (HQ assay)

3.3 Experiment 3: Rumbling

3.3.1 HQ Assay

Levels of phenoloxidase activity in ***Figure 3.17*** follow a general trend of increasing levels of activity from the 15 minute time point up until the 60 minute time point, however the results of the one-way ANOVA (***Table 3.7***) indicate that there is no significant interaction occurring between phenoloxidase activity and the independent factor TIME ($P > 0.578$). As a result, all levels of phenoloxidase activity do not significantly differ from the baseline control level of activity (31.3±21.2%) for the 360-minute sampling duration. Standard deviations (±SD) recorded for levels of phenoloxidase activity are larger than have been measured in previous experiments, indicating that there is a large amount of variation existing within the sampling population.

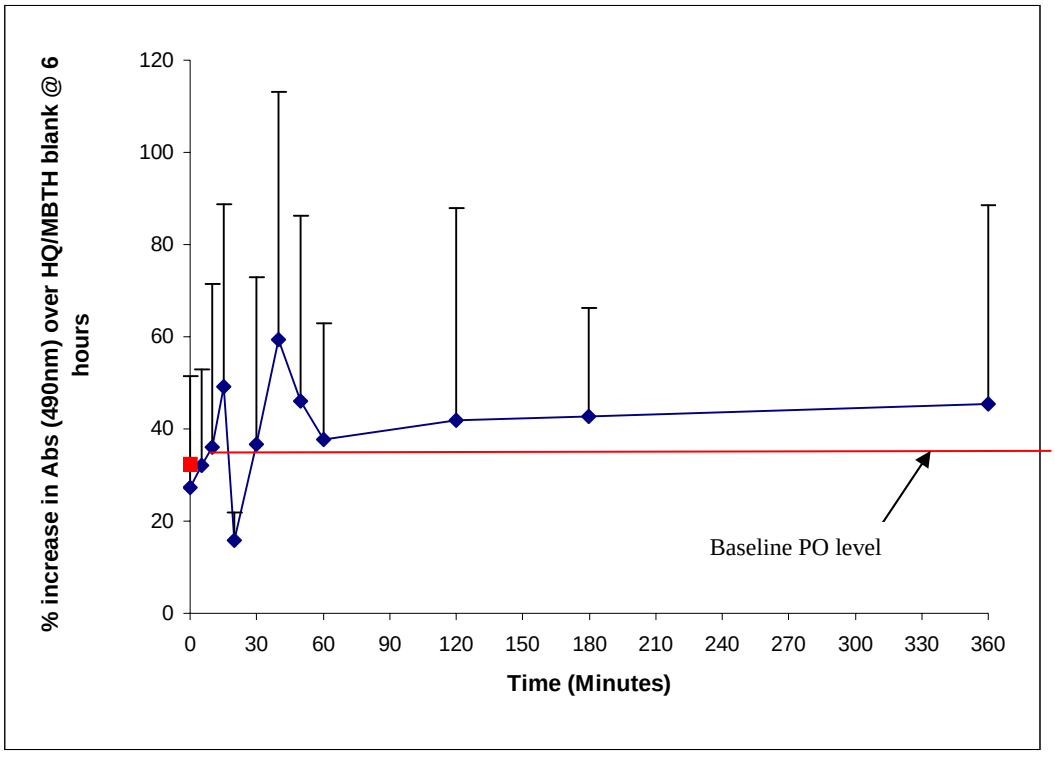


Figure 3.17: Mean (±SD) phenoloxidase activity (HQ assay) (%) of Pacific oysters (*C. gigas*) exposed to the three minute rumbling stressor (n = 6)

Tests of Between-Subjects Effects

Dependent Variable: RECIP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.010(a)	12	.001	.873	.578
Intercept	.136	1	.136	139.313	.000
TIME	.010	12	.001	.873	.578
Error	.054	55	.001		
Total	.202	68			
Corrected Total	.064	67			

a R Squared = .160 (Adjusted R Squared = -.023)

Table 3.7: One-way analysis of variance (ANOVA) for the rumbling experiment (HQ assay). Data was transformed using the reciprocal transformation function (1/x)

3.4 Experiment 4: Pacific Oyster Family Lines

3.4.1 L-DOPA Assay

Figure 3.18 shows the mean phenoloxidase activity from five family lines of Pacific oysters. Unstressed oysters represent baseline levels of phenoloxidase activity, whilst stressed represent oysters subjected to the 25-hour emersion stressor. Results from the two-way ANOVA (***Table 3.8***) indicate that there is no significant difference in levels of phenoloxidase activity between both the independent factors TIME ($P > 0.344$) and between the interaction of TIME * LINES ($P > 0.242$). However, the independent factor LINES ($P < 0.000$) does show significantly different levels of phenoloxidase activity. Bonferroni *post hoc* analysis (***Table 3.9***) reveals that line B has significantly lower levels of phenoloxidase activity compared to lines A ($P < 0.020$), D ($P < 0.000$) and E ($P < 0.000$) and line C has significantly lower levels of phenoloxidase activity compared to line E ($P < 0.021$).

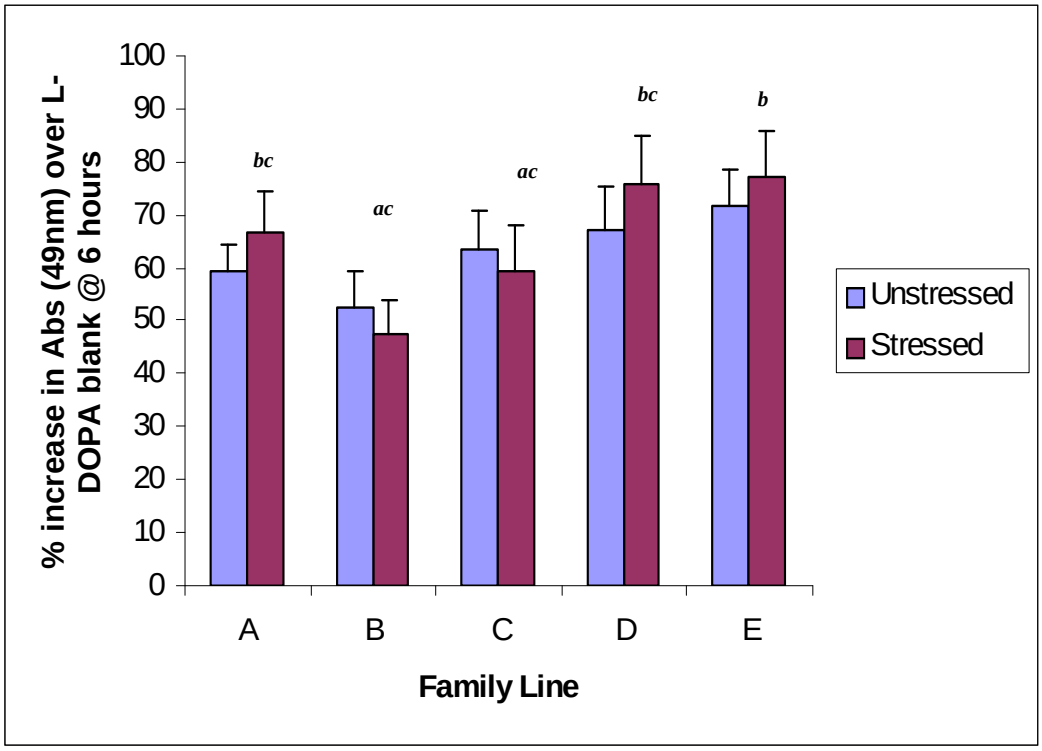


Figure 3.18: Mean (±SD) phenoloxidase activity (L-DOPA assay) (%) of five family lines of Pacific oysters (*C. gigas*) unstressed and stressed (n = 4). Letters ***a***, ***b*** and ***c*** denote significance between family line.

Tests of Between-Subjects Effects

Dependent Variable: ACTIVITY

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3253.034(a)	9	361.448	6.214	.000
Intercept	159077.303	1	159077.303	2734.933	.000
LINES	2791.925	4	697.981	12.000	.000
TIME	53.744	1	53.744	.924	.344
LINES * TIME	338.055	4	84.514	1.453	.242
Error	1686.784	29	58.165		
Total	164239.365	39			
Corrected Total	4939.819	38			

a R Squared = .659 (Adjusted R Squared = .553)

Table 3.8: Two-way analysis of variance (ANOVA) of the genetic lines experiment (L-DOPA assay)

Multiple Comparisons

Dependent Variable: ACTIVITY

	(I) LINES	(J) LINES	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Bonferroni	B	A	-12.92621(*)	3.813298	.020	-24.51119	-1.34123
		D	-21.42830(*)	3.813298	.000	-33.01328	-9.84333
		E	-24.61900(*)	3.947139	.000	-36.61060	-12.62741
	C	E	13.33131(*)	3.947139	.021	1.33972	25.32290

Table 3.9. Bonferroni *post hoc* multiple comparisons indicating significant difference ($P < 0.05$) in phenoloxidase activity (L-DOPA assay) between the five family lines of Pacific oysters (*C. gigas*)

3.4.2 HQ Assay

No HQ assay results could be included in this experiment to compare against the L-DOPA assay due to the removal of the 490nm filter from the 96-well microplate without prior consent. The filter was replaced with either a 530nm or 640nm filter during assaying, and thus the final results produced were not assayed at the 490nm wavelength and therefore could not be used.

Experiment 5: Salinity

3.5.1 L-DOPA Assay

Levels of phenoloxidase activity in treatments A (5ppt), B (15ppt), C1 (38ppt; instant ocean), C2 (38ppt; seawater) and D (50ppt) all follow a general linear trend over the four-day duration (***Figure 3.19***). It was noted that oysters in all three replicate tanks of treatment D spawned within the first two hours of experimentation on day 0, leaving a visible milky residue along the bottom of the tanks. Results from the two-way ANOVA (***Table 3.10***) indicate that there is no significant difference in levels of phenoloxidase activity between the independent factor SALINITY ($P > 0.125$) or between the interaction of TIME * SALINITY ($P > 0.696$), however there is a significant difference in phenoloxidase activity with the independent factor TIME ($P < 0.000$). Bonferroni *post hoc* analysis (***Table 3.11***) reveals that phenoloxidase activity on Day 4 is significantly higher to that on Day 1 ($P < 0.000$), Day 2 ($P < 0.001$) and Day 3 ($P < 0.001$).

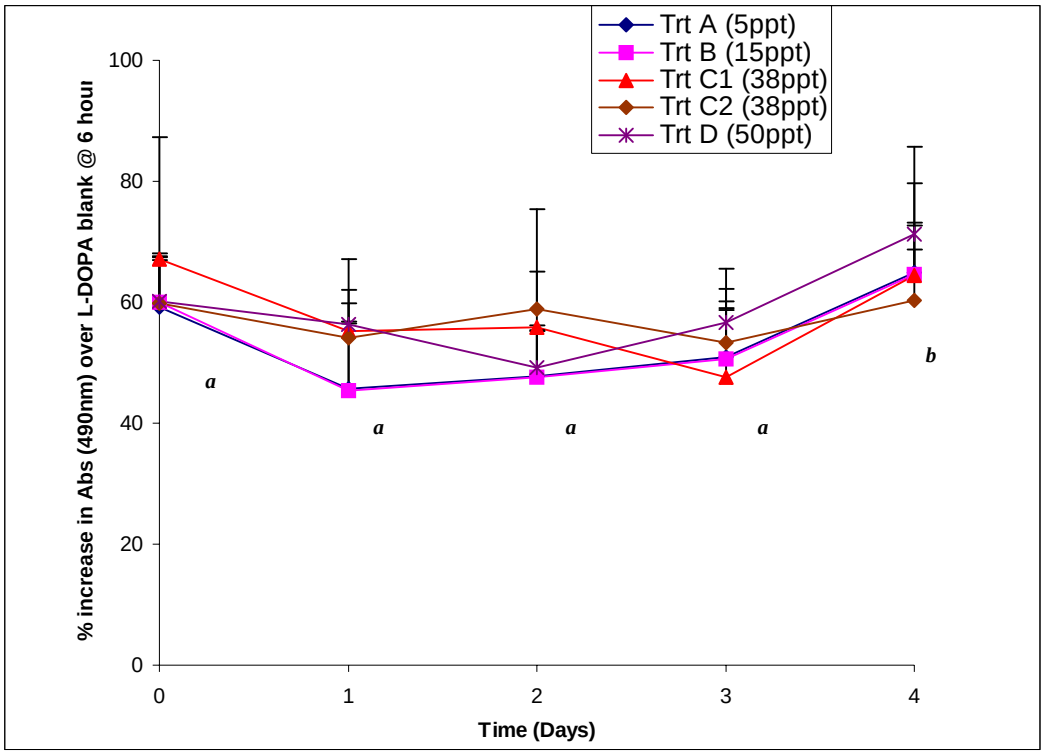


Figure 3.19: Mean (±SD) phenoloxidase activity (L-DOPA assay) (%) of Pacific oysters (*C. gigas*) exposed to the five salinity treatments; A (5ppt), B (15ppt), C1 (38ppt; Instant Ocean), C2 (38ppt; seawater) and D (50ppt) (n = 6). Letters ***a*** and ***b*** denote significance between days

Tests of Between-Subjects Effects

Dependent Variable: ACTIVITY

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7348.366(a)	24	306.182	2.035	.006
Intercept	455613.430	1	455613.430	3028.713	.000
TIME	4394.772	4	1098.693	7.304	.000
SALINITY	1109.633	4	277.408	1.844	.125
TIME * SALINITY	1897.575	16	118.598	.788	.696
Error	18202.195	121	150.431		
Total	481681.887	146			
Corrected Total	25550.562	145			

a R Squared = .288 (Adjusted R Squared = .146)

Table 3.10: Two-way analysis of variance (ANOVA) for the salinity experiment (L-DOPA assay)

Multiple Comparisons

Dependent Variable: ACTIVITY

	(I) TIME	(J) TIME	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Bonferroni	Day 4	Day 1	-13.9371(*)	3.22096	.000	-23.1472	-4.7270
		Day 2	-13.0875(*)	3.22096	.001	-22.2976	-3.8774
		Day 3	-13.2828(*)	3.19400	.001	-22.4158	-4.1498

Table 3.11: Bonferroni *post hoc* multiple comparison indicating significant difference ($P < 0.05$) in levels of phenoloxidase activity (L-DOPA assay) over the four day treatment period

3.5.2 HQ Assay

Initial levels of phenoloxidase activity recorded for treatment C1 (38ppt; instant ocean) on day 0 are consistently higher than that in the four other salinity treatments. Phenoloxidase levels in treatment C1 were revealed to be significantly higher when compared to treatment A ($P < 0.003$), treatment B ($P < 0.045$) and treatment C ($P < 0.011$) on Day 0 (***Figure 3.19***). Phenoloxidase levels in treatment C1 and D both have substantial increases in activity at Days 1 and 2 where they then plateau and remain relatively linear for the remaining experiment duration. Treatments A (5ppt), B (15ppt) and C2 (38ppt; seawater) maintain a consistent level of phenoloxidase activity over the four day experiment duration. It was noted that oysters in all three replicate tanks of treatment D spawned within the first two hours of experimentation on day 0, leaving a visible milky residue along the bottom of the tanks. Results from the two-way ANOVA (***Table 3.12***) reveal that there is significant difference in levels of phenoloxidase activity between both the independent factors TIME ($P < 0.000$) and SALINITY ($P < 0.000$) and between the interaction of TIME * SALINITY ($P < 0.000$). Pairwise comparison (***Table 3.13***) reveal that treatment C1 has significantly higher levels of phenoloxidase activity to treatments A ($P < 0.003$), B ($P < 0.045$) and C2 ($P < 0.011$) on day 0 and treatments C1 and D both have significantly higher levels of phenoloxidase activity to treatments A ($P < 0.000$), B ($P < 0.000$) and C2 ($P < 0.000$) on days 1, 2, 3 and 4 respectively.

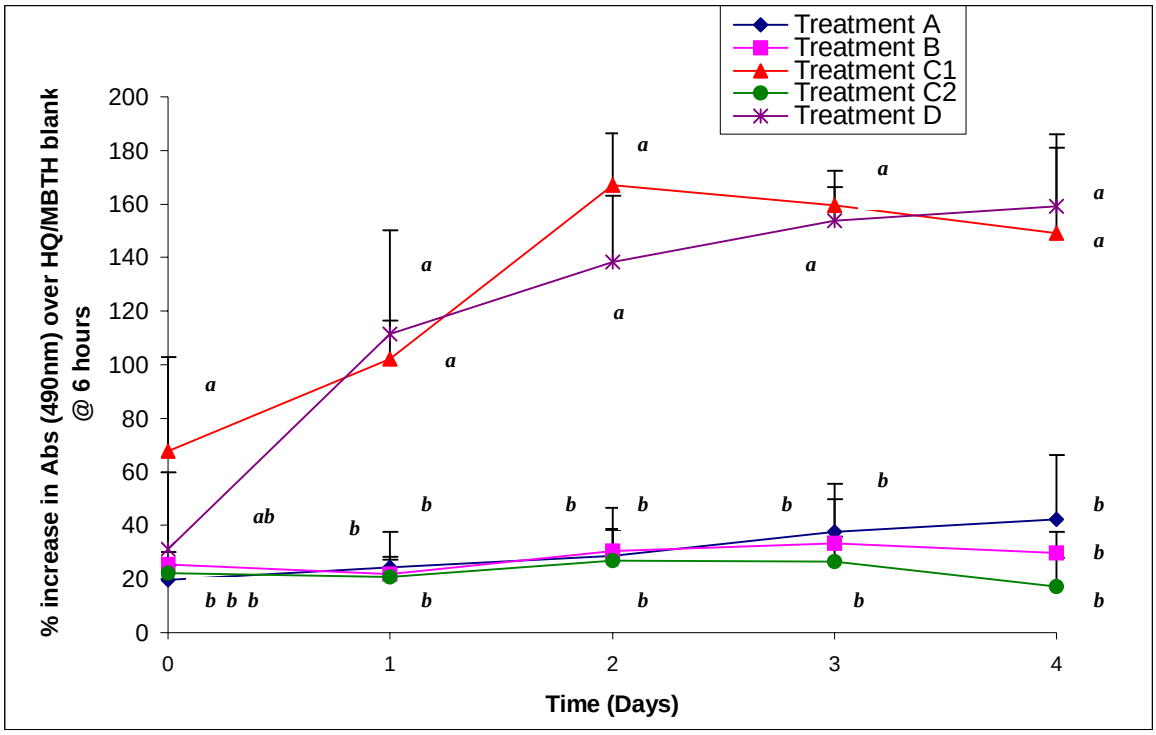


Figure 3.19: Mean (±SD) phenoloxidase activity (HQ assay) (%) of Pacific oysters (*C. gigas*) exposed to the five salinity treatments; A (5ppt), B (15ppt), C1 (38ppt; instant ocean), C2 (38ppt; seawater) and D (50ppt) (n = 6). Letters ***a*** and ***b*** denote significant difference between treatments on that particular day.

Tests of Between-Subjects Effects

Dependent Variable: SQRT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1508.141(a)	24	62.839	42.056	.000
Intercept	7744.991	1	7744.991	5183.484	.000
TIME	191.076	4	47.769	31.970	.000
SALINITY	1096.976	4	274.244	183.543	.000
TIME * SALINITY	180.708	16	11.294	7.559	.000
Error	177.806	119	1.494		
Total	9619.937	144			
Corrected Total	1685.947	143			

a R Squared = .895 (Adjusted R Squared = .873)

Table 3.12: Two-way analysis of variance (ANOVA) for the salinity experiment (HQ assay). Data was transformed using the square-root transformation (√x)

Pairwise Comparisons

Dependent Variable: SQRT

TIME	(I) SALINITY	(J) SALINITY	Mean Difference (I-J)	Std. Error	Sig.(a)	95% Confidence Interval for Difference (a)	
						Lower Bound	Upper Bound
Day 0	15ppt	38ppt-1	-2.145(*)	.740	.045	-4.262	-.028
	38ppt-1	15ppt	2.145(*)	.740	.045	.028	4.262
		5ppt	2.792(*)	.740	.003	.675	4.909
	38ppt-2	38ppt-1	-2.475(*)	.740	.011	-4.592	-.358
	5ppt	38ppt-1	-2.792(*)	.740	.003	-4.909	-.675
Day 1	15ppt	38ppt-1	-5.428(*)	.706	.000	-7.446	-3.409
		50ppt	-5.758(*)	.706	.000	-7.776	-3.739
	38ppt-1	15ppt	5.428(*)	.706	.000	3.409	7.446
		38ppt-2	5.608(*)	.706	.000	3.589	7.626
		5ppt	5.301(*)	.706	.000	3.283	7.320
	38ppt-2	38ppt-1	-5.608(*)	.706	.000	-7.626	-3.589
		50ppt	-5.937(*)	.706	.000	-7.956	-3.919
	50ppt	15ppt	5.758(*)	.706	.000	3.739	7.776
		38ppt-2	5.937(*)	.706	.000	3.919	7.956
		5ppt	5.631(*)	.706	.000	3.612	7.650
	5ppt	38ppt-1	-5.301(*)	.706	.000	-7.320	-3.283
		50ppt	-5.631(*)	.706	.000	-7.650	-3.612
Day 2	15ppt	38ppt-1	-7.529(*)	.706	.000	-9.548	-5.511
		50ppt	-6.336(*)	.706	.000	-8.355	-4.318
	38ppt-1	15ppt	7.529(*)	.706	.000	5.511	9.548
		38ppt-2	7.914(*)	.706	.000	5.895	9.933
		5ppt	7.634(*)	.706	.000	5.615	9.653
	38ppt-2	38ppt-1	-7.914(*)	.706	.000	-9.933	-5.895
		50ppt	-6.721(*)	.706	.000	-8.740	-4.703
	50ppt	15ppt	6.336(*)	.706	.000	4.318	8.355
		38ppt-2	6.721(*)	.706	.000	4.703	8.740
		5ppt	6.441(*)	.706	.000	4.423	8.460
	5ppt	38ppt-1	-7.634(*)	.706	.000	-9.653	-5.615
		50ppt	-6.441(*)	.706	.000	-8.460	-4.423
Day 3	15ppt	38ppt-1	-6.961(*)	.706	.000	-8.980	-4.943
		50ppt	-6.732(*)	.706	.000	-8.751	-4.714
	38ppt-1	15ppt	6.961(*)	.706	.000	4.943	8.980
		38ppt-2	7.532(*)	.740	.000	5.415	9.649
		5ppt	6.626(*)	.740	.000	4.509	8.743
	38ppt-2	38ppt-1	-7.532(*)	.740	.000	-9.649	-5.415
		50ppt	-7.303(*)	.740	.000	-9.420	-5.186
	50ppt	15ppt	6.732(*)	.706	.000	4.714	8.751
		38ppt-2	7.303(*)	.740	.000	5.186	9.420
		5ppt	6.397(*)	.740	.000	4.280	8.514
	5ppt	38ppt-1	-6.626(*)	.740	.000	-8.743	-4.509
		50ppt	-6.397(*)	.740	.000	-8.514	-4.280
Day 4	15ppt	38ppt-1	-6.720(*)	.740	.000	-8.837	-4.603
		50ppt	-7.164(*)	.740	.000	-9.281	-5.047
	38ppt-1	15ppt	6.720(*)	.740	.000	4.603	8.837
		38ppt-2	7.612(*)	.740	.000	5.495	9.730
		5ppt	5.810(*)	.740	.000	3.692	7.927

38ppt-2	38ppt-1	-7.612(*)	.740	.000	-9.730	-5.495
	50ppt	-8.056(*)	.740	.000	-10.173	-5.939
50ppt	15ppt	7.164(*)	.740	.000	5.047	9.281
	38ppt-2	8.056(*)	.740	.000	5.939	10.173
	5ppt	6.253(*)	.740	.000	4.136	8.370
5ppt	38ppt-1	-5.810(*)	.740	.000	-7.927	-3.692
	50ppt	-6.253(*)	.740	.000	-8.370	-4.136

Table 3.13: Pairwise comparison indicating significant differences ($P < 0.05$) in levels of phenoloxidase activity (HQ assay) between salinity treatments: A (5ppt), B (15ppt), C1 (38ppt; seawater), C2 (38ppt; seawater) and D (50ppt).

3.6 Experiment 6: Nutrition

3.6.1 HQ assay

The effect of feeding *Pavlova lutheri/Isochrysis* sp. (treatment A), *Nannochloris atomus* (treatment B) and no microalgae (treatment C) on levels of phenoloxidase activity over 28 days is shown in ***Figure 3.20***. Similar levels of phenoloxidase activity are measured for all treatments on day 0 (A: 18.08±4.3%, B: 19.5±8.2%, C: 25.5±11.8%) and day 1 (A: 14.8 ± 10.4%, B: 18.4 ± 8.9%, C: 13.2 ± 19.5%), followed by an increase in levels of phenoloxidase activity in all treatments by day 3. By day 7, all three nutrition treatments have similar levels of phenoloxidase activity that plateau and remain consistent for the duration of the experiment. Results from the two-way ANOVA (***Table 3.14***) reveal that both the independent factor TREATMENT ($P > 0.736$) and the interaction between TIME * TREATMENT ($P > 0.603$) are not significantly different and the independent factor TIME ($P < 0.002$) is significantly different in respect to levels of phenoloxidase activity. Bonferroni *post hoc* analysis (***Table 3.15***) reveals that levels of phenoloxidase activity on day 1 are significantly lower to that on Day 5 ($P < 0.029$) and Day 14 ($P < 0.031$).

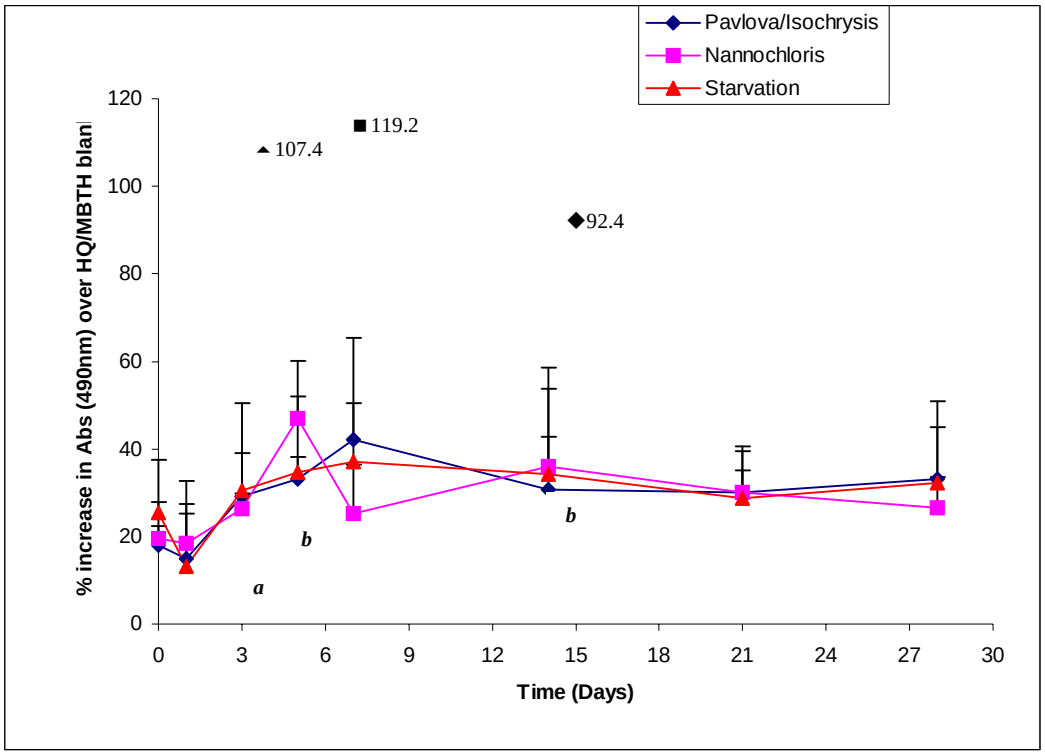


Figure 3.20: Mean (±SD) phenoloxidase activity (HQ assay) (%) of Pacific oysters (*C. gigas*) between the treatment factors: treatment A (*Pavlova lutheri/Isochrysis*), treatment B (*Nannochloris atomus*) and treatment C (Starvation) (n = 6). Symbols and values above time points represent outliers that have been omitted from the mean. Symbol ▲ (Starvation), ■ (*Nannochloris atomus*) and ◆(*Pavlova lutheri/Isochrysis*). Letters ***a*** and ***b*** denote significant difference between days.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6173.092(a)	23	268.395	1.529	.079
Intercept	105329.310	1	105329.310	600.141	.000
TIME	4325.838	7	617.977	3.521	.002
TRTMENT	107.965	2	53.982	.308	.736
TIME * TRTMENT	2114.854	14	151.061	.861	.603
Error	17024.250	97	175.508		
Total	135673.033	121			
Corrected Total	23197.342	120			

Table 3.14: Two-way analysis of variance (ANOVA) for the nutrition experiment (HQ assay).

	(I) TIME	(J) TIME	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Bonferroni	day 1	day 5	-17.57576(*)	5.188881	.029	-34.24547	-.90605
		day 14	-17.62936(*)	5.258875	.032	-34.52394	-.73479

Table 3.15: Pairwise comparisons indicating significant difference ($P < 0.05$) in levels of phenoloxidase activity (HQ assay) between the feeding treatments: A (*Pavlova lutheri*/Isochrysis), B (*Nannochloris atomus*) and C (Starvation).

3.6.2 Changes in Condition Index (CI)

Condition indices (CI) for treatment A (*Pavlova lutheri/Isochrysis*) remained constant for the 28 day period, whereas treatment B (*Nannochloris atomus*) and C (Starvation) recorded reduced condition index at the completion of the 28 day sampling duration (***Figure 3.21***). Results from the two-way ANOVA (***Table 3.15***) reveal that the independent factor TREATMENT ($P < 0.815$) is not significant and the independent factor TIME ($P < 0.000$) and interaction between TIME * TREATMENT ($P < 0.030$) is significantly different in respect to levels of phenoloxidase activity. Pairwise comparisons reveal that on day 0 ($P < 0.001$) treatment A (*Pavlova/Isochrysis*) has significantly lower levels of phenoloxidase activity than treatment C (Starvation).

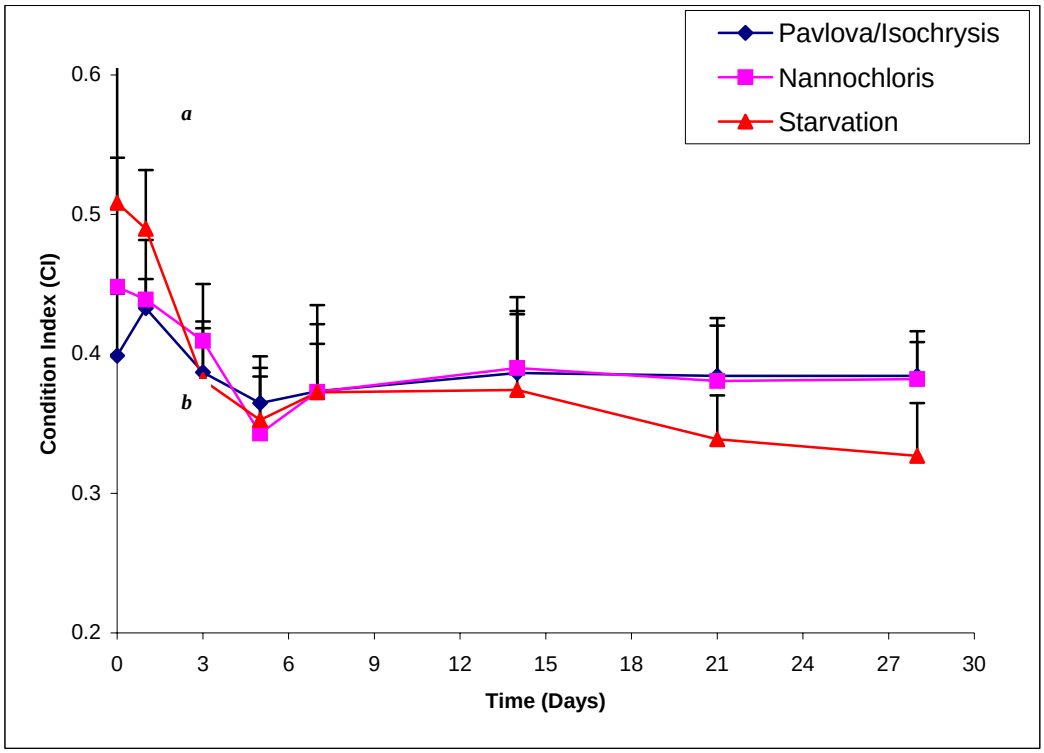


Figure 3.21: Mean (±SD) condition index (CI) for Pacific oysters from treatments A (*Pavlova lutheri/Isochrysis*), B (*Nannochloris atomus*) and C (Starvation) from various sampling days (n = 6). Letters ***a*** and ***b*** denote significant difference in condition index levels.

Dependent variable: CONDITION

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.265(a)	23	.012	4.310	.000
Intercept	22.186	1	22.186	8301.849	.000
TIME	.192	7	.027	10.255	.000
TREATMENT	.001	2	.001	.205	.815
TIME * TREATMENT	.072	14	.005	1.924	.030
Error	.321	120	.003		
Total	22.772	144			
Corrected Total	.586	143			

Table 3.15: Two-way analysis of variance (ANOVA) for the nutrition experiment (CI)

TIME	(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig. (a)	95% Confidence Interval for Difference (a)	
						Lower Bound	Upper Bound
Day 0	iso/pav	starve	-.110(*)	.030	.001	-.182	-.037

Table 3.16: Pairwise comparisons indicating significant difference (P < 0.05) in condition index (CI) between feeding treatments: A (*Pavlova lutheri*/Isocrysis), B (*Nannochloris atomus*) and C (Starvation)

Chapter 4: Discussion

This thesis primarily aimed to determine the likelihood of measuring hemolymph phenoloxidase activity as a potential indicator of stress in Pacific oysters (*Crassostrea gigas*). Stress is a common occurrence in commercial oyster aquaculture, which with current day culture techniques is unlikely to change. Therefore techniques are required for objectively measuring stress in Pacific oyster stock; a goal which is considered to be important to the oyster industry. With the quantification of stress inducing practices, comes the possibility of reducing stress and thereby the stress or disease related mortality associated with commercial aquaculture practices. Several groups around the world are currently researching the effects of physical and environmental stressors and their resultant physiological consequences on the neuroendocrine stress response or immunological function in cultured molluscs, including *C. gigas*. However, to date, relatively little work has been carried out to elucidate the link between the stress response and immune function. This study attempted to address this interaction by measuring the activity of an immune functioning enzyme as a potential stress response indicator in *C. gigas*.

This present study investigated the effects of several commonly encountered physical and environmental perturbations, similar to those that are associated with commercial Pacific oyster aquaculture in South Australia. Each experiment was specifically designed and implemented to replicate a common stress inducing perturbation, being either physical or environmental in nature, which was then quantitatively assessed by objectively measuring levels of hemolymph phenoloxidase activity. It must be remembered that this thesis was designed as an aquaculture-related project, so the primary focus was the aquaculture dimension, rather than a project focussing on the enzyme phenoloxidase, or the reliability of assays to measure it, in this species.

What do the assays used in this study actually measure?

Both the L-3, 4-dihydroxyphenylalanine (L-DOPA) and 4-Methoxyphenol (HQ) substrate based assays used for the measurement of phenoloxidase activity were sourced from previously published techniques. Indeed, this L-DOPA assay has been used by Smith & Soderhall (1991); Lanz *et al* (1993); Coles & Pipe (1994); Waite & Wilbur (1995); Asokan *et al* (1998); Mercado *et al* (2002) and Peters & Raftos (2003) to measure levels of phenoloxidase activity in various molluscs. An assay utilising the active substrate HQ and a chromogenic nucleophile 3-methyl-2-benzothiazolinone hydrazone (MBTH), was developed due to the poor sensitivity associated with the L-DOPA assay to measure phenoloxidase activity in cultivated fruits and vegetables. This HQ assay has been used in previous publications by Rodriguez-Lopez *et al* (1994); Espin *et al* (1995; 1997; 1998a,b) and Newton *et al* (2004) to measure monophenolase activity of phenoloxidase from a range of commercially cultivated fruits and vegetables (apples, pears, strawberries, artichoke, avocado, potato and mushrooms) and cultured Sydney rock oysters (*Saccostrea glomerata*). The L-DOPA and HQ assays used in the present study are identical (except for minor differences in equipment available) to these published methods.

Previous research by Martinez & Whitaker (1995) and Girelli *et al* (2004) have demonstrated the action of both enzymatic (phenoloxidase) and non-enzymatic oxidation of phenolic compounds that contribute to the process of melanogenesis. Non-enzymatic browning in fruits and vegetables can occur via the Maillard reaction, which is a series of autooxidation reactions that involve phenolic compounds and the formation of iron-phenol complexes. Therefore, the possibility exists that both L-DOPA and HQ assays are measuring the autooxidation of phenoloxidase-like substances, stock

substrate solutions and various other phenol or quinone products that are present within the hemolymph, rather than supposed catalytic action derived from enzymatic phenol oxidase activity.

Indeed, in the present study, it was evident that samples containing no hemolymph in both the L-DOPA and HQ assays underwent obvious melanogenic colour changes in the time course of the assay. Thus, hemolymph samples in both the L-DOPA and HQ assays had melanogenic activity corrected by subtraction of the background absorbance from the L-DOPA/FSW or HQ/MBTH blanks included in each assay. By subtracting these control blanks for phenoloxidase and monophenolase activities, and correcting for the minor absorbance of the hemolymph/reagent mixture at time = 0, it was assumed that the final value was an estimate of enzymatic and non-enzymatic phenoloxidase or monophenolase-like activities of hemolymph. However, several of the experiments described in this thesis suggest that both the L-DOPA and HQ assays displayed characteristics, which were of concern.

The first instance where phenoloxidase behaviour did not follow the general principles associated with enzymatic activity occurred during preliminary hemolymph dilution experiments for both L-DOPA and HQ assays. Enzymatic activity is generally expected to obey the principles involved with Michaelis-Menton Enzyme Kinetics. The typical Michaelis-Menton enzyme relationship is shown in ***Figure 4.1 (a)***.

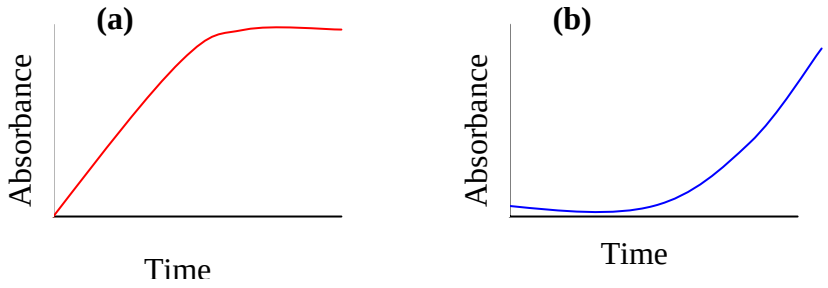


Figure 4.1:(**a**) A generalised Michaelis-Menton enzyme relationship, (**b**) an Allosteric enzyme relationship as shown by phenoloxidase assays.

Phenoloxidase activity displayed unusual behaviour in that levels of activity were seen to have an initial lag period that lasted up until the 3-hour time point for the L-DOPA assay and between 2 and 3-hour time points for the HQ assay. In terms of enzyme behaviour, this initial lag period of activity is considered as being exceptionally slow and does not obey generalised concepts that are associated with Michaelis-Menton Enzyme Kinetics. Fersht (1971) described such initial lag periods of activity, which is displayed by phenoloxidase, as being under allosteric control (***Figure 4.1 (b)***). Allosterism is described as being a conformational change in an enzyme that effectively changes its overall activity (Manning & Campbell 1961). Phenoloxidase therefore, is considered an allosteric enzyme, as it does not follow the general principles that are associated with Michaelis-Menton Enzyme Kinetics. However, the question remains, what factor(s) cause or influence the rate of allosteric change of phenoloxidase, and do these factors vary between individual oysters?

The second instance where doubt arose due to questionable experimental results occurred following the tropolone inhibition experiment. Kahn (1985) and Kahn & Andrawis (1985) have described tropolone as being the compound that most reliably and specifically inhibits phenoloxidase activity.

Indeed, results from both the L-DOPA and HQ assays support this; however, tropolone was also demonstrated to reduce the autooxidation of the substrate controls, suggesting that it is not a phenoloxidase specific inhibitor. This result was unexpected and, to date, is unexplained. Therefore, whatever these assays are measuring, it is not just enzymatic phenoloxidase activity.

Following the unusual enzyme behaviour observed from previously discussed experiments, a heating experiment was conducted to deliberately denature the enzymatic properties of phenoloxidase substances present in the hemolymph. Manning & Campbell (1961) state that enzyme inactivation and denaturation occurs virtually instantaneously at temperatures well below 100°C and in most cases at temperatures less than 70°C due to the break up of weak hydrogen bonds that effectively causes the enzymes to unfold or separate, leading to enzyme inactivation, denaturation and the cessation of catalytic action. Hemolymph samples were exposed to boiling water for 15 minutes and due to the relatively small volumes (< 1mL) of hemolymph that were boiled, it is highly likely that phenoloxidase and other enzymes present in the hemolymph should have been effectively denatured prior to assaying. However, no difference in levels of phenoloxidase activity was observed between hemolymph samples subjected to heat and no heat, which strongly suggests that the vast majority of phenoloxidase-like activity in *C. gigas* hemolymph is attributed to the non-enzymatic pathway (autooxidation).

Following the heating experiment, the native polyacrylamide gel electrophoresis (native PAGE) approach was utilised to try to quantify the number and sizes of phenoloxidase-active proteins present in *C. gigas* hemolymph. Native PAGE was based upon previously published methods by Nellaiappan & Vinayakam (1983) and Newton *et al* (2003) that demonstrated the presence of two distinct phenoloxidase protein bands in both a myriapod (*Thyropygus poseidon*) and the Sydney rock oyster (*S. glomerata*). In the present study native PAGE gel electrophoresis stained with Bromophenol blue effectively indicated the presence of multiple proteins present in the haemocyte supernatant and pellet hemolymph samples, which were distinguished as single distinct bands. However, several proteins present in both hemolymph samples were overlapping indicating unresolved protein bands making it difficult to determine total numbers of protein present in each of the hemolymph samples. These unresolved protein bands could have occurred due to either overloading the sample wells in the precast gels or from several proteins of similar molecular weight. A recommendation for future native PAGE would include the use of suitable molecular weight markers to allow for effective discrimination between individual protein bands based on molecular weights of corresponding proteins.

Both attempts to identify protein bands associated with phenoloxidase activity from hemolymph samples by staining with either L-DOPA or HQ stock substrate solution were unsuccessful. The L-DOPA stained gel was completely blackened with insoluble melanin following the 24-hour staining period. This blackening of the gel was attributed to a reaction between the L-DOPA substrate and the polyacrylamide used in the PAGE gel – an occurrence that was not mentioned in either of the studies by Nellaiappan & Vinayakam (1983) or Newton *et al* (2003). In contrast, the gel stained with HQ stock substrate solution produced no observable bands, suggesting that no detectable monophenolase activity was present in the hemolymph or haemocytes of *C. gigas*. However, previous work by Newton *et al* (2004) acknowledged this lack of observable monophenolase bands and attributed it to the very low haemocyte numbers present in *C. gigas* compared to *S. glomerata*. Native PAGE was expected to aid in the determination of numbers of enzymes capable of reacting with both L-DOPA and HQ substrates to produce observable protein bands, strengthening the argument that both assays were not measuring specific phenoloxidase activity. However, the lack of staining with either substrate cannot help elucidate the size(s) of proteins reacting in the spectrophotometric assays.

Despite all of these concerns, the widely published L-DOPA and HQ assay techniques were utilised in the remainder of the experiments to try to answer the original question posed.

Do the physical and environmental perturbations used in this study cause a state of stress in the oysters?

Recent research by Malham *et al* (2003) and Lacoste *et al* (2001a) demonstrated the effects of physical and environmental perturbations in *C. gigas*, by measuring fluctuating levels of catecholamines, primarily noradrenaline (NA) & dopamine (DA) in response to states of stress. Lacoste *et al* (2001b) demonstrated the effect of physical stress in the form of shaking oysters for 15 minutes by measuring a 7 fold increase in levels of noradrenaline (NA) and a 4 fold increase in dopamine (DA) at five and fifteen minutes following the completion of the disturbance. Furthermore, increasing levels of catecholamines contributed to immunosuppressive effects on the immune system, by reducing the number and migratory efficiency of circulating haemocytes, phagocytosis and production of reactive oxygen intermediates (ROI).

A very similar experiment was recently carried out at the SARDI laboratory by Qu (2004). Indeed, the oysters sampled by Qu (2004) were the same oysters sampled in the present study (Rumbling Experiment). The three-minute rumbling treatment produced a 3-fold increase in noradrenaline (NA) at five and ten minutes following cessation of the rumbling period (***Figure 4.2***). Even more recent research by (Zhang Unpublished data 2004) also demonstrated the effect of three different rumbling times; 1.5, 3 and 9 minutes on hemolymph lysosome activity (using a Neutral Red Retention assay). Zhang (Unpublished data 2004) successfully measured a 50% decrease in levels of lysosome with increasing rumbling duration, suggesting that this form and duration of physical stress activates the neuroendocrine stress response and affects immune function activity of extracellular lysosome in *C. gigas* (***Figure 4.4***). Given these results, it is safe to suggest that rumbling treatments do cause a stress response in Pacific oysters.

Previous work by Lacoste *et al* (2001b) has also demonstrated the effects of environmental stress in the form of decreasing levels of salinity (34ppt-24ppt) by measuring fluctuating levels of noradrenaline (NA) and dopamine (DA). Lacoste *et al* (2001) measured a 5-fold increase in levels of noradrenaline (NA) and a 2.5-fold increase in dopamine (DA) up to 24 hours following the transfer of Pacific oysters into diluted seawater. Recent work carried out by Qu (2004) measured fluctuating levels of noradrenaline (NA) in *C. gigas* when exposed to a 24-hour emersion stressor coupled with a temperature increase (from 15° to 27.5°C). Qu (2004) measured up to a 3-fold increase in level of noradrenaline (NA) over the 24-hour period of emersion (***Figure 4.3***). Again, levels of catecholamines measured by Lacoste *et al* (2001) and Qu (2004) indicate that environmental stressors promote a stress response in *C. gigas* that can be actively measured and quantified as a primary neuroendocrine response to stress.

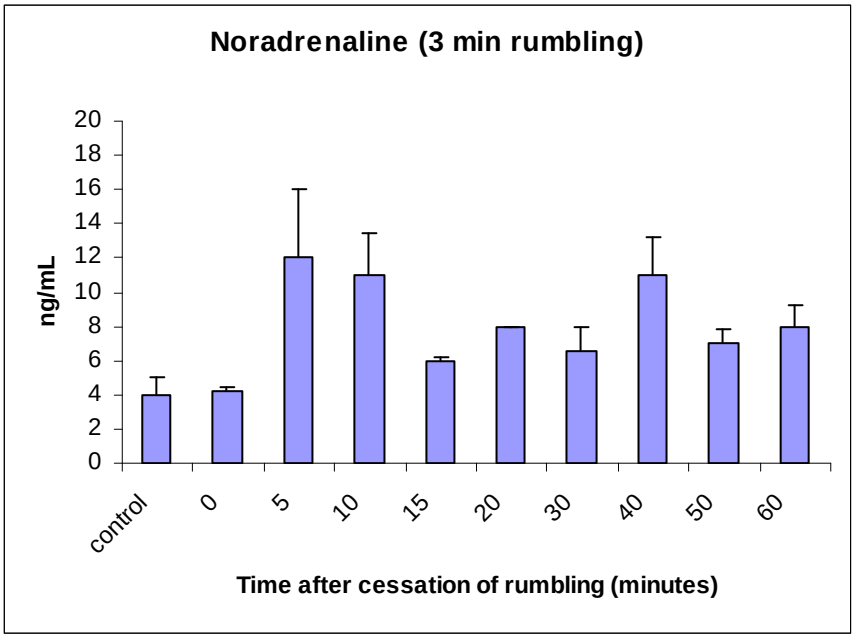


Figure 4.2: The effect of a 3 minute rumbling treatment on noradrenaline levels in the hemolymph of Pacific oysters (*C.gigas*). Experimental oysters were acclimated at 15ºC. Data expressed as mean ± standard error. Data reproduced from Qu (2004) with permission.

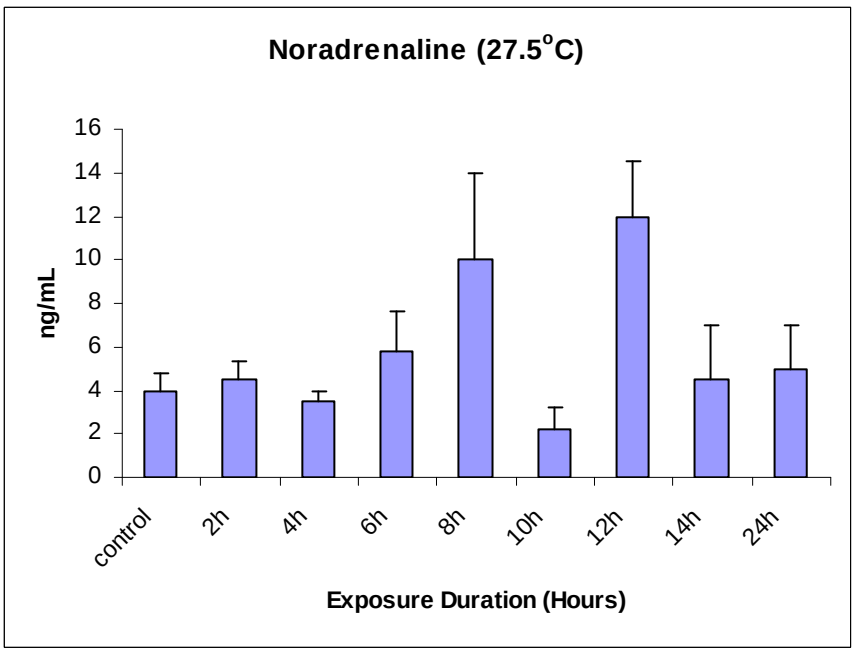


Figure 4.3: The effects of an emersion and temperature protocol (exposure to air and a 12.5°C increase in temperature) on noradrenaline levels in the hemolymph of Pacific oysters (*C. gigas*). The oysters were acclimatised at 15ºC prior to being used in the experiments. Data is expressed as mean ± standard error. Data reproduced from Qu (2004) with permission.

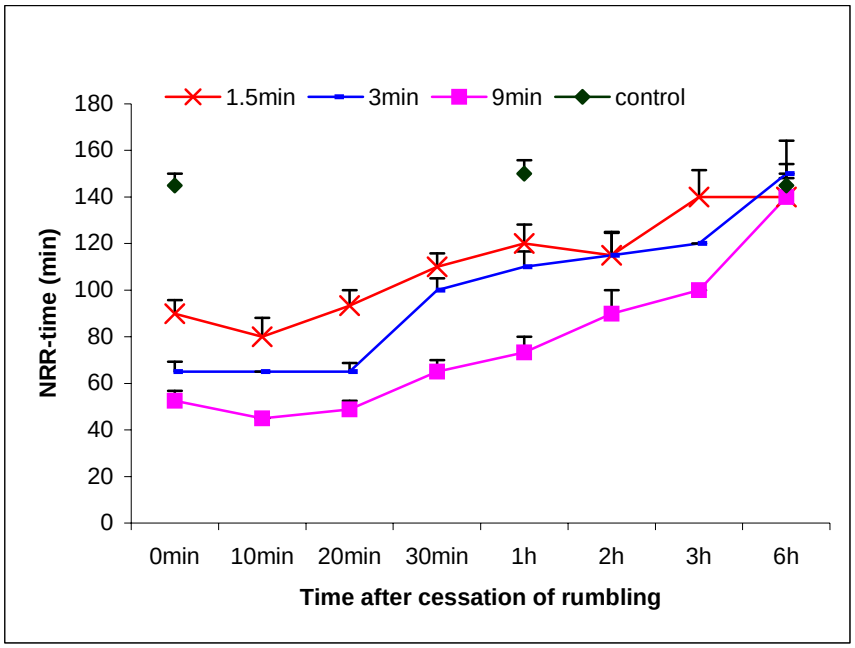


Figure 4.3. The effect of 1.5, 3 and 9 minutes of rumbling on Neutral Red Retention (an assay measuring lysosome activity) on Pacific oysters (*C. gigas*). Experimental oysters were acclimated at 15°C. Data expressed as mean ± standard error. Data reproduced from Zheng (2004) with permission.

In the vast majority of instances in this study, levels of hemolymph phenoloxidase activity remained relatively similar (as measured by both the L-DOPA and HQ assays) despite changes in catecholamines and/or lysosome activity (when the oysters were used for more than one project). Statistical analysis indicated that there were some significant interactions between the stressors and/or timepoints that affected levels of phenoloxidase activity. However, on a biological scale (and a physiological context as seen by the 3- to 7-fold increases in catecholamines), the magnitude (i.e. changes of +/- 15% of baseline) and duration (i.e. isolated timepoints – 15 mins to 6 hours) of these statistical differences suggest that phenoloxidase is unsuitable as a stress response indicator.

Stress and Immune Function

Stress has long thought to be an important factor that is capable of reducing an organism's ability to resist advances from pathogenic agents. Previous literature regarding vertebrate and invertebrate immune systems have demonstrated the link that exists between stress and increased mortality, supposedly due to reduced disease resistance (Colombo *et al* 1990; Lee *et al* 1996; Lacoste *et al* 2003).

Recent work by (Lacoste *et al* 2001a,b) where stress stimulated the release of noradrenaline also produced a transient state of immunosuppression in *C. gigas*. Thus, immune defence components such as haemocyte number, phagocytotic action and the production of reactive oxygen intermediates were all suppressed. Consequences of such transient states of stress-induced immunosuppression (Lacoste *et al* 2003) leave the organism less likely to be able to combat against pathogenic agents attempting to colonise the organism, resulting in disease.

Indeed, Peters & Raftos (2003) propose that *Martellia sydneyi*, the etiological agent of the fatal QX disease in Sydney rock oysters, must evade or suppress the host defence system to establish infection

and this happens when oysters are stressed by poor water quality. Peters & Raftos (2003) suggested that poor water quality in the Hawkesbury River causes a stress-induced immunosuppression of the prophenoloxidase activating system (proPO) in Sydney rock oysters and QX disease can result.

Several measures of immune function were shown to be affected by a 15 min rumbling stressor in *H. tuberculata* (Malham *et al* 2003). Thus, circulating haemocyte number, migratory activity, phagocytotic capacity and the production of reactive oxygen intermediates were all suppressed for 30-60 minutes post-stress. Interestingly, almost all of these parameters showed an increase between 100 and 480 minutes post-stress, clearly showing a more complex interaction between stress and immune function. Unfortunately, phenoloxidase activity was either not measured or not reported in this study.

Mitta *et al* (1999) descried haemocytes as being the main cellular effectors in invertebrate immunity. Movement of haemocytes out of the hemolymph and into the surrounding organs leaves the possibility that once activated, levels of phenoloxidase activity would reside within the organ(s) rather than entering the hemolymph, resulting in no increase in hemolymph levels of phenoloxidase activity despite activation of haemocytes. Future studies could include counts of haemocytes and measurement of phenoloxidase activity both in the circulation and various organs.

In almost all cases where the interaction between stressors and immune function has been examined there have been clear and profound changes in whatever immune function parameter(s) were measured. These changes are usually decreases from baseline, but occasionally increases are also measured, depending on the nature of the stressor, the timeframe and the immune parameter. The lack of any convincing stress-related change in phenoloxidase activity in the present study is contrary to the balance of similar studies in molluscs and other organisms. This conclusion then begs the question of whether phenoloxidase has a role in the immune function of invertebrates? Only after the uncertainties regarding the assay protocols have been answered will it be possible to address this question.

Levels of phenoloxidase or phenoloxidase-like activity in the present study

Almost all of the wide range of physical and environmental stressors, covering both acute and chronic scenarios, used in the present study failed to elicit substantial or convincing changes in phenoloxidase activity. The exception was the salinity experiment where there were two results that deserve attention. The first was that there was a significant and substantial increase in HQ determined monophenolase activity of phenoloxidase in two of the treatment groups over a period of 4 days. The second noteworthy finding was that this difference was not reflected in the levels of L-DOPA phenoloxidase activity measured in the same samples.

Dealing with the latter point first, this result strongly suggests that the two assays used in the present study were measuring different properties or activities in the oyster hemolymph. This experiment does not help to elucidate what these different properties were in a biochemical sense, but if future research were to be carried out on this topic, this would seem to be a point of difference that should be exploited.

Assaying using the HQ method revealed significantly higher levels of monophenolase activity in treatments C1 (38ppt instant ocean) and D (50ppt) on days 1, 2, 3 and 4, compared to treatments A (5ppt), B (15ppt) and C2 (38ppt seawater control). It could be concluded that levels of phenoloxidase activity increase at high salinities, however this is probably incorrect as daily measurements of salinity using a refractometer showed that both solutions were at 38ppt for the

entire experiment. Similarly other measures of water quality (temperature, dissolved oxygen, waste accumulation etc) did not differ between treatments.
One possibility remains. Did the synthetic salt used to make the 38ppt and 50ppt instant ocean treatments cause the high levels of phenoloxidase activity produced in each treatment? Artificial saltwater solutions may differ in ionic composition from natural seawater due to variation in concentration of compounds/elements that differentially precipitate out of solution during the evaporation process. Thus, the suggestion is that there may be a stimulatory or toxic effect of substances(s) present in the synthetic salt solution, producing high levels of phenoloxidase activity. As both 5ppt and 15ppt synthetic saltwater solutions did not produce the same high levels of phenoloxidase activity, it suggests that whatever element or compound is in the synthetic salt was not at a concentration sufficient to elicit the same stimulatory effect as observed in both 38ppt and 50ppt synthetic treatments.

Investigations by Oliver & Fisher (1999) have shown that contaminants (cadmium and copper) affect the functioning of immune defence mechanisms including haemocyte density, production of cytotoxic molecules and phagocytosis in *C. gigas*. Furthermore, Suresh & Mohandas (1990) measured elevated acid phosphatase levels in hemolymph during phagocytosis when the clams *Sunetta scripta* and *Villarita cyprinoids* were exposed to 0.2ppm copper for two days.

In the situation where it is suspected that a solution has an ionic imbalance that is suspected of having a bearing on the result, suspect solutions should be quantitatively analysed by a specialised water quality laboratory (such as the South Australian Water Quality Centre at Bolivar). Such analysis could identify which ion(s) were in excess and further experimentation carried out. An experiment could be designed with the addition of known concentrations of the substance into natural seawater. Experimental oysters would then be placed into these solutions and phenoloxidase activity measured using the HQ assay as before.

Recent literature by Newton *et al* (2004) has suggested that low salinity levels are an environmental variable that can suppress phenoloxidase activity in Sydney rock oysters (*S. glomerata*). However, the 5ppt and 15ppt treatments used in the present study did not show this trend.

Why does levels of phenoloxidase activity differ between family lines?

There was a significant difference in levels of phenoloxidase activity between the five family lines; A, B, C, D and E. This suggests that there is a genetic component to phenoloxidase activity in *C. gigas*. However, it is not known whether line B, which had a significantly lower level of activity than lines A, D and E, has either an enhanced or reduced immune system to combat disease (this information is Commercial in Confidence). Recent work by Newton *et al* (2004) has involved a specific oyster-breeding program to develop QX resistance strains of Sydney rock oysters (*S. glomerata*). Newton *et al* (2004) found that QXR_3 oysters selected for resistance to outbreaks of QX disease had significantly (by approximately two times) higher levels of phenoloxidase activity than unselected wild type oysters. QXR_3 oysters were found to possess a novel isoform of the enzyme phenoloxidase, which Newton *et al* (2004) proposes results in an enhanced level of phenoloxidase activity that represents a specific disease resistance factor. Therefore, the possibility exists that family lines of *C. gigas* with high levels of phenoloxidase activity may confer an enhanced immune defence that is advantageous to the organism, effectively promoting improved resistance to disease. Further work with *C. gigas* family lines is suggested once the intellectual property issues have been resolved.

Major findings

L-DOPA Assay

- The L-DOPA assay used does not appear to measure levels of hemolymph phenoloxidase activity in Pacific oysters (*C. gigas*)
- The presence of other enzymes and/or non-enzymatic phenoloxidase-like substances are capable of utilising the L-DOPA substrate in catalytic reactions, resulting in confounded measurements of phenoloxidase activity

HQ Assay

- The HQ assay used does not appear to measure levels of monophenolase activity of hemolymph phenoloxidase activity in Pacific oysters (*C. gigas*)
- The presence of other enzymes and/or non-enzymatic phenoloxidase-like substances are capable of utilising the HQ substrate in catalytic reactions, resulting in confounded measurements of phenoloxidase activity

Phenoloxidase

- Measuring levels of hemolymph phenoloxidase activity does not provide a reliable means of stress response in Pacific oysters (*C. gigas*)
- Further experimentation using treatments based on an artificial seawater may reveal more information about (a) what the different phenoloxidase assays are actually measuring, and (b) what factor(s) increase the levels of HQ assayed monophenolase activity in the hemolymph of Pacific oysters (*C. gigas*)

Prospects for Future Research

- Development of new and improved spectrophotometric assay methods that objectively measure levels of hemolymph phenoloxidase activity in Pacific oysters (*C. gigas*)
- Repeat all results found concerning phenoloxidase from this study in Pacific oysters (*C. gigas*) and compare with Sydney rock oysters (*Saccostrea glomerata*)
- Interaction of levels of phenoloxidase activity with immune defence functions including haemocyte migrations, phagocytosis, luminol-dependent chemiluminesence (CL), intracellular anion production, extracellular superoxide anion production and antimicrobial peptides when subjected to states of stress in Pacific oyster (*C. gigas*)
- Measuring other enzymes present in Pacific oysters (*C. gigas*) hemolymph including; acid phosphatase, alkaline phosphatase, β-glucuronidase, lysosome, lysozyme, esterase esterase lipase, lipase, leucine arylamidase, valine arylamidase as potential indicators of stress
- The elicitation of biological stressors via xenobiotic assaults on levels of hemolymph phenoloxidase activity in Pacific oysters (*C. gigas*)
- Production of a purified phenoloxidase standard for use in assay and native PAGE methods
- Quantification of possible isoenzymes of phenoloxidase present in Pacific oyster (*C. gigas*) hemolymph, role of these isoenzyme(s) in immune defence and the possible development of a selective breeding program to produce lines of Pacific oysters with enhanced disease resistance
- The development of diagnostic techniques that actively measure tissue and organ concentrations of phenoloxidase activity

Chapter 5 References

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