The Effects of Environmental Stressors on Immunological Activity in *Pinctada imbricata*

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Rhiannon Philippa Kuchel

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List of Abbreviations

ACTH: Adrenocorticotropin hormone

AFLP: Amplified fragment length polymorphism

ANOVA: Analysis of variance

AVD: Akoya viral disease

BSA: Bovine serum albumin

CA: Catecholamines

cDNA: Complementary deoxyribonucleic acid

CI: Condition index

cm: Centimetres

CRH: Corticotrophin-releasing hormone

DAB: 3,3′-diaminobenzidine tetrahydrochloride

df: Degrees of freedom

DIC: Differential interference contrast

DII: Department of Industry and Investment

DO: Dopamine

DOC: Dissolved oxygen content

DMSO: Dimethyl sulfoxide

DPI: Department of Primary Industries

EF-1: Elongation factor-1

FAO: Food and Agriculture Organisation

FITC: Fluorescein isothiocyanate

FOV: Field of view

FREPs: Fibrinogen-related proteins

FSW: Filtered sea water

g: Grams

GPx: Glutathione peroxidase

GST: Glutathione-S-transferase

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

h: Hours

HH: Hypoxia hypercapnia

HL: Haemocyte lysates

HRP: Streptavidin-horseradish peroxidase

HSP-70: Heat shock protein-70

KMRS: Kimberly Marine Research Station

L: Litres

LPS: Lipopolysaccharides

L-dopa: 3, 4, -dihydroxyphenylalanine

MA: Mechanical agitation

MBTH: 3-methyl-2-benzothiazoline hydrazone

MDS: Multi-dimensional scaling

m: Meters

M: Moles

mg: Milligrams

min: Minutes

mL: Millilitres

mm: Millimetres

mM: Millimoles

NA: Noradrenaline

NBT: Nitroblue tetrazolium chloride

NSW: New South Wales

ng: Nanograms

nM: Nanomoles

nm: Nano molar

OD: Optical density

OOD: Oyster oedema disease

OH: Hydroxyl radicals

OsHV: Ostreid herpes virus

PAMPS: Pathogen associated molecular patterns

PEI: Ethylene imine polymer solution

PI: Propidium iodide

PIPES: 1,4 piperazine diethanesulfonic acid

PCR: Polymerase chain reaction

PBS: Phosphate buffered saline

pNPP: p-nitrophenylphosphate

ppt: Parts per thousand

PO: Phenoloxidase

ProPO: Prophenoloxidase

PRRs: Pattern recognition receptors

PRx: Peroxiredoxin

QLD: Queensland

qPCR: Real time polymerase chain reaction

QX disease: Queensland unknown

RLO: Rickettsia-like organisms

ROS: Reactive oxygen species

ROOH: Hydroperoxides

RNA: Ribonucleic acid

RT: Room temperature

SEM: Scanning electron microscope

sem: Standard error of the mean

SS-III: Super Script-III

SIMS: Sydney Institute of Marine Science

s: Second

TBS: Tris buffered saline

TBT: Tributyltin

TEM: Transmission electron microscope

THC: Total haemocyte counts

TUNEL: Terminal dUTP nick-end labeling

US$: United States of America dollar

v/v: Volume to volume

VHSV: Haemorrhagic septicaemia virus

w/v: Weight to volume

WA: Western Australia

AUS: Australian dollars

µ: Micro

ºC: Degrees Celsius

4-HA: Hydroquinine monomethyl ether

Note on Thesis Preparation

Chapters 2, 3, 4 and 7 are presented as published manuscripts or submitted for publication (Chapters 5 and 6). As such there is some necessary repetition of information in the General Introduction (Chapter 1) and the General Discussion (Chapter 8), when compared to the Introduction and Discussion sections of Chapters 2 to 7.

Author Contributions

Chapter 1: General Introduction and research objectives.

I performed the review of literature and writing of this chapter. My supervisor David Raftos provided constructive feedback.

Chapter 2: Japanese pearl oyster (*Pinctada imbricata*) haemocytes: Morphology and function.

I performed the research and wrote this chapter. Debra Birch and Nicole Vella provided technical assistance with the microscopy. My supervisor David Raftos provided direction and constructive feedback.

Chapter 3: The Effects of Environmental Stressors on Immunological Function in *P. imbricata.*

I performed the research and wrote this chapter. My supervisors David Raftos and Sham Nair provided direction and constructive feedback.

Chapter 4: *In vitro* effects of noradrenaline on Akoya pearl oyster (*Pinctada imbricata*) haemocytes.

I performed the research and wrote this chapter. My supervisor David Raftos provided direction and constructive feedback.

Chapter 5: Phenoloxidase activity as an indicator of stress in the silver-lip pearl oyster, *Pinctada maxmia.*

Both myself and Alison McCarthy, from Kimberly Marine Research Station, were responsible for the collection of haemolymph samples. I wrote this chapter. My supervisor David Raftos provided direction and constructive feedback.

Chapter 6: Changes in the transcriptional expression of oxidative stress response genes in Akoya pearl oysters (*Pinctada imbricata*) exposed to air and mechanical agitation.

I performed the research and wrote this chapter. My supervisors David Raftos and Sham Nair provided direction and constructive feedback.

Chapter 7: Environmental stress and disease in pearl oysters, focusing on the Akoya pearl oyster (*Pinctada fucata*).

I was responsible for undertaking the review of literature and writing this chapter. Wayne O'Connor provided key information. Both Wayne O'Connor and my supervisor David Raftos provided direction and constructive feedback.

Chapter 8: General Discussion.

I summarized the major finding form this thesis and outlined implications for further research. Critical input and constructive feedback was provided by my supervisor David Raftos.

Supplementary Data Part A (not part of the thesis), Chapter 9: Cytoskeletal rearrangements in human erythrocytes induced by snake venoms: light microscopy of shapes and nuclear magnetic resonance studies of membrane function.

I was responsible for the confocal fluorescent microscopy component of this manuscript. In addition to this I also provided constructive feedback during the drafting and submission process.

Supplementary Data Part B (not part of the thesis), Chapter 10: The Environment, Immunity and Disease in Oysters and other Marine Invertebrates.

In this chapter I was involved in editing and constructing the manuscript. I also contributed some written work. Dan Butt and Saleem Adalaileh also contributed to writing this chapter. Critical input and constructive feedback was provided by my supervisor David Raftos.

Publications From This Thesis

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Kuchel, R. P., Raftos, D. A., Nair, S. (2010). The Effects of Environmental Stressors on Immunological Function in *P. imbricata. Journal of Fish and Shellfish Immunology*, **29**: 930-936.

Kuchel, R. P., Raftos, D. A. (2011). *In vitro* effects of noradrenaline on Akoya pearl oyster (*Pinctada imbricata*) haemocytes. *Fish and Shellfish Immunology*, **31**: 365-372.

Kuchel, R. P., O'Connor, W. A., Raftos, D. A. (2011). Environmental stress and disease in pearl oysters, focusing on the Akoya pearl oyster (*Pinctada fucata*). *Reviews in Aquaculture*, **3**: 138-154.

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Conference Oral Presentation and Poster

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List of Tables and Figures

Summary

Pearl oyster farming is a significant component of the Australian mariculture industry. However, disease often threatens its productivity. Previous work has identified a strong relationship between environmental stress and altered immunological activity in many molluscs, often resulting in the spread of opportunistic pathogens. My studies investigate this relationship, focusing on the effect of stress induced neuroendocrine secretions.

The First Chapter provides a general introduction to the history and existing knowledge of Akoya pearl oyster aquaculture. This Chapter examines subjects that are to be further investigated by this thesis, namely; the relationship between farmed marine invertebrates and the influence of husbandry and handling on their immune and endocrine systems.

The Second Chapter provides a detailed introduction of the morphology and cytochemistry of *P. imbricata* haemocytes; granulocytes, hyalinocytes, and serous cells. Granulocytes represent the most abundant cells type (62%), and are shown to have the highest capacity to phagocytose Congo red-stained yeast cells. Granules contained within granulocytes were also found to stain positively for a number of immuno-reactive enzymes or products including; phenoloxidase (PO), peroxidase, superoxide, and melanin. Hyalinocytes were the second most abundant cell type (36%), and also engulfed Congo red-stained yeast cells. Serous cells represented the smallest component of the haemocyte population (2%) and were not involved in clearance activities.

Chapter Three investigates the effects of common environmental stressors (low salinity, mechanical agitation (MA), and exposure to air) on the immunological activity in *P. imbricata* haemocytes. Both phagocytosis and PO activity decreased

significantly when oysters were exposed to all three stressors. The responses to other parameters tested (granulocyte to hyalinocyte ratio, total haemocyte counts, acid phosphatase activity, and total protein content) were found to vary, and the different immunological parameters tested were influenced uniquely according to the type of stressor.

Chapter Four addresses the relationship between stress and hormone-induced apoptosis in defensive haemocytes from *P. imbricata*. Haemocytes were exposed to 0.0, 2.5, 5.0 and 10.0 ng noradrenaline (NA) per µg of protein. Both DNA fragmentation and Annexin V-FITC staining was greatest in haemocytes exposed to 10 ng NA/ µg protein. The ability of haemocytes to adhere to glass slides was also negatively affected by NA exposure. Cytoskeletal re-arrangement was evident in NA-treated haemocytes stained with the F-actin selective stain, Phalloidin Alexa Fluor 488. Morphological and ultrastructural changes in NA-exposed haemocytes identified by transmission and scanning electron microscopy included; chromatin and cytoplasmic condensation, the formation of apoptotic bodies, vacuolization and blebbing.

Husbandry and handling are often the cause of, or exacerbate, stress in farmed marine species. Chapter Five explores this relationship by investigating the effects of antifouling practices (treatment with high pressure water jets, being struck with a chisel, a combination of the high pressure water jets and being struck with a chisel and exposure to air) on mature pearl oysters in the field (*P. maxima*). We also investigated the effects of air exposure on juvenile oysters. Phenoloxidase activity declined by ≥ 40% after 48h exposure in all mature treated oysters. After 96h, PO activity recovered to greater than control values. Comparatively, PO activity increased significantly in juvenile oysters exposed to air at both 24 and 96h.

The Sixth Chapter investigates the effects of MA and exposure to air on the expression of antioxidant genes in *P. imbricata*. Significant declines in the expression of glutathione-S-transferase (GST) and peroxiredoxin (PRx) were recorded after 60 min exposure to both MA and exposure to air. Similarly, glutathione peroxidase (GPx) expression declined significantly after 60 min exposure to MA. Hierarchical cluster analysis and multidimensional scaling indicated that the suppression of antioxidant expression was transient and had begun to return to control (unperturbed) levels within 6 hours. The MDS analysis also showed there to be no difference between the MA and air treatments, suggesting that air (the common component of both treatments) was the causative agent of stress. Given that the expression of HSP-70, a common marker of 'stress', was not significantly affected when oysters were exposed to MA and air, monitoring antioxidant genes in pearl oysters may represent a more informative suit of biomarkers in response to husbandry and handling stressors.

Chapter Seven reviews the effects of environmental stress and the spread of disease in *P. fucata* (also known as *P. imbricata* throughout Australia), referring to experiences with related species. It provides a comprehensive and detailed summary of factors implicated with disease genesis and on-going outbreaks.

Overall, the data presented in this thesis demonstrates a strong association between environmental stress and immunological suppression.

CHAPTER ONE

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1.1 General Biology of P. imbricata

1.1.1 Taxonomy and Distribution

Pearl oyster farming can be highly lucrative, as the animals are nucleated for the production of pearls and can also be harvested for their meat (Urban, 2000). Current figures estimate that one hundred grams of cultured pearls have a market value of up to US $2,600 (Anon, 2007; Mamangkey, 2009). Pearl oysters belong to the order Pterioida in the superfamily Pteriacea (Wada, 1978; Chellam et al., 1981; Pit, 2004). The Akoya or Japanese pearl oyster, *Pinctada imbricata* (*P. imbricata*) has a cosmopolitan distribution including the northern and eastern coastlines of North and South America, the eastern coast of Africa, Korea, Southern China, and Australia, as well as the Mediterranean, the Indian Ocean and the Red Sea (Fig. 1; Shirai, 1994; O'Connor et al., 2003; Kuchel et al., 2011). Within Australia *P. imbricata* is endemic to Shark Bay in the west, around the perimeter of the northern coastline extending down the eastern seaboard to the northern tip of northern Victoria (Hynd, 1955; O'Connor et al., 2003).

There has been much confusion regarding the taxonomic classification of *P. imbricata* and it often varies based on geographical location (Yu et al., 2006; Acosta-Salmón, 2004). The species is referred to as either *P. fucata* (Gould, 1850), *P. vulgaris* (Schumacher, 1817), *P. fucata martenseii* (Dunker, 1850), or *P. radiata* (Leach, 1814; O'Connor et al., 2003; Pit, 2004). A number of taxonomic studies have focused on this species complex in an attempt to settle the conflicting classifications (O'Connor et al., 2003). It is thought that the extended larval period has played a

significant role in the rather considerable distribution of this genus (Cunha et al., 2011).

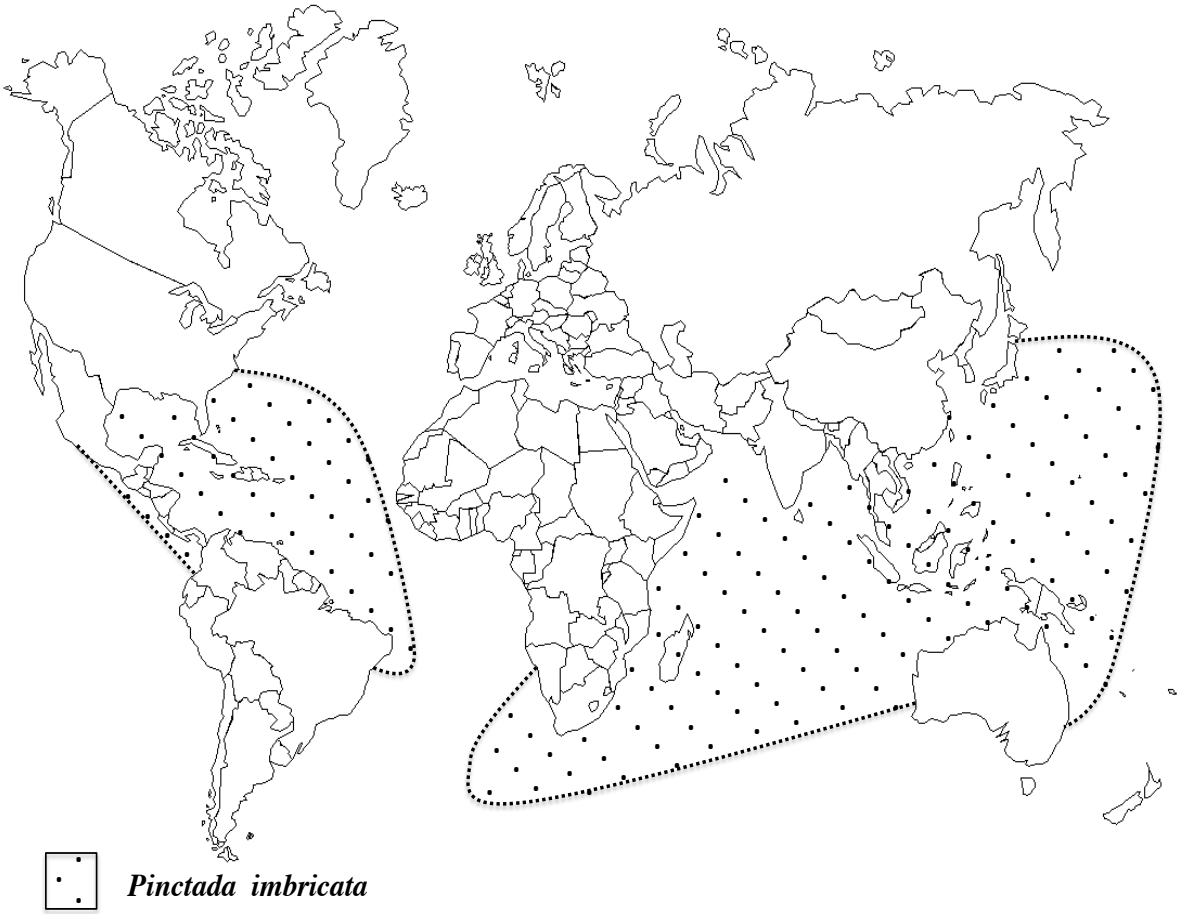

Figure 1. The global distribution of *P. imbricata*. *P. imbricata* is endemic to the northern and eastern coastlines of North and South America, the eastern coast of Africa, Korea, Southern China, and Australia, as well as the Mediterranean, the Indian Ocean and the Red Sea (adapted from Shirai, 1994; O'Connor et al., 2003; Kuchel et al., 2011).

In 1961, Ranson classified *P. imbricata* as the Atlantic pearl oyster, *P. radiata*, based on its distribution in the Atlantic and Pacific Ocean (Wada, 1978). Further investigative studies identified the Japanese pearl oyster, *P. martensii*, to be a separate species from *P. radiata* (Wada, 1978). Other studies by Kuroda et al. (1971),

reassessed Ransons's classification and reported the Japanese pearl oyster to be a subspecies of the tropical Pacific oyster, *P. fucata*, and renamed it *P. fucata martensii* (Wada, 1978). Colgan and Ponder (2002) compared genetic materials from Australian bred *P. imbricata* with samples from oysters (*P. fucata*) in Honshu, Japan (O'Connor et al., 2003). Results from this study confirmed the samples to be conspecific (Colgan and Ponder, 2002; O'Connor et al., 2003). Similarly, Yu et al. (2006) investigated the relatedness of *P. fucata* (China), *P. imbricata* (Australia), and *P. fucata martensii* (Japan). With the use of amplified fragment length polymorphism (AFLP) markers they concluded that all three taxa are conspecific with *P. fucata* (Yu et al., 2006). For the purpose of this thesis, I have accepted the taxonomic classification *P. imbricata* throughout Chapters 2, 3, 4, 6 as detailed by Shirai (1994) and *P. fucata* throughout Chapter 7.

1.1.2 Reproduction

Due to the lucrative nature of pearl cultivation, there has been substantial interest in the reproductive condition and associated events of *P. imbricata* (Alagarswami et al., 1989; O'Connor and Lawler, 2002). A thorough understanding of reproductive biology assists in many aspects of protocol development for pearl farming, including hatchery production, wild spat collection, and most importantly, timing of nuclei implantation and pearl extraction (Wada et al., 1991, 1995; O'Connor and Lawler, 2002; O'Connor et al., 2003). Studies of reproductive states of *P. imbricata* have found them to vary significantly depending upon geographical location. Subtropical oysters demonstrate two discrete spawning periods (typically November and March in NSW, Australia), whilst tropical species tend to spawn year

round (O'Connor et al., 2003). This reproductive pattern is typical of tropical regions, as there is little variance in both water temperature and food supply (Urban, 2000).

P. imbricata are protandrous hermaphrodites and reach sexual maturity from approximately 6 months to a year in age (Tranter, 1958c; Gervis and Sims, 1992; O'Connor et al., 2003; Pit, 2004). Sexual ratios tend to be male dominated in the first few years of development, and female in the latter (Hynd, 1955). This suggests that sexual dimorphism is influenced by environmental conditions, as oogenesis requires a significantly higher food ration than spermatogenesis (Tranter, 1958 a, b; Pit, 2004). For this reason, the period of "maleness" typically dominates regions of lower year-round food abundance, such as the conditions experienced in temperate waters (Tranter, 1958 a, b; Pit, 2004).

Most oysters reproduce by broadcast spawning. Based on both water temperature and tidal cycle, gametes (both sperm and eggs, 2n = 28) are released into the water column, whereupon external fertilization occurs (Loosanoff and Davis, 1963; Wada, 1978; Acosta-Salmón, 2004; Pit, 2004). After fertilisation early embryos (24h) develop a protective shell and are termed 'D-stage' veliger larvae (Fig. 2; Wada, 1991; Doroudi and Southgate, 2002; Pit, 2004). This developmental phase is independently mobile and can swim freely using ciliated velae (Gervis and Sims, 1992; Pit, 2004). This mobility is important because D-veligers exhaust their endogenous energy reserves within 24 hours, at which time they become phototaxic and remain close to the water's surface (Nayar et al., 1978; Acosta-Salmón, 2004; Pit, 2004). Larvae develop into an *umbo* veliger at 8-10 days (Acosta-Salmón, 2004; Pit, 2004), and shortly after a pigmented 'eye' spot forms (Chellam, 1987; Gervis and Sims, 1992; Acosta-Salmón, 2004; Pit, 2004). Settlement occurs in conjunction with the development of a functional foot and is associated with a number of metamorphic

events, including the developments of labial palps and gill filaments (Plantigrade; Chellam, 1987; Pit, 2004).

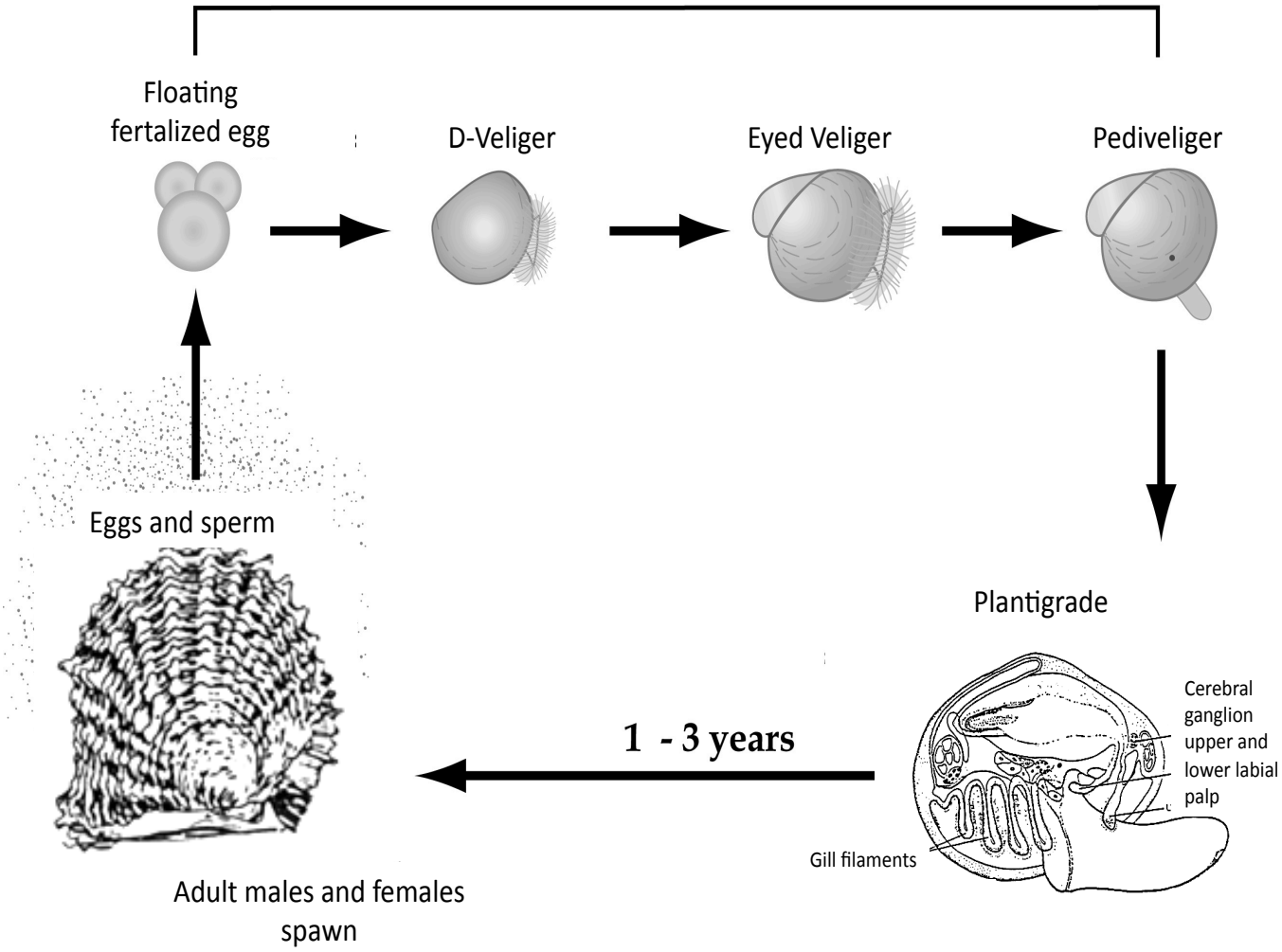

Figure 2. Generalised life cycle of *P. imbricata* (adapted from Gervis and Sims, 1992).

1.1.3 External Anatomy

The anatomy of *P. imbricata* described in this section relates to organs and structures that are relevant to the research conducted in this study. The body and shell

of *P. imbricata* are asymmetrical. The shell is shaped like a horseshoe and is comprised of two valves joined by an elastic ligament (Acosta-Salmón, 2004). The left valve is deeper than the right and the byssal notch is located on the anterior side (Wada, 1991; Acosta-Salmón, 2004). The shell is comprised of three distinct layers; conchiolin (the outermost), calcite crystals (prismatic layer) and aragonite crystals fused in a protein matrix (nacre, inner most layer; Acosta-Salmón, 2004). External shell colour varies, typically as a combination of brown, green, sliver, gold and pink (Fig. 3). Growth processes are located on the surface of the shell and form scale-like growth lines (Wada, 1991). These structures are tapered with finely rounded tips and arranged in radial rows (Wada, 1991).

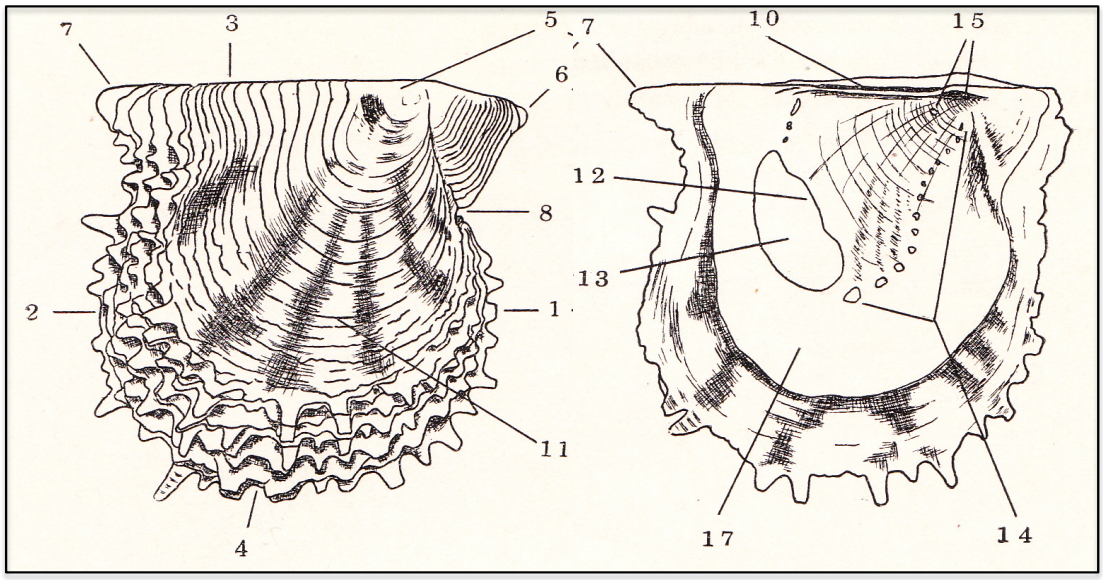

Figure 3. The shell of *P. imbricata*. 1, anterior margin; 2, posterior margin; 3, dorsal margin; 4, ventral margin; 5, umbo; 6, anterior ear; 7, posterior ear; 8 byssal notch; 10, ligament; 11, growth line; 12, impression of retractor; 13, impression of adductor; 14, impression of pallial muscles; 15, impression of levators; 16, pallial line; 17, pearl layer (adapted from drawings by Katsou Takayama).

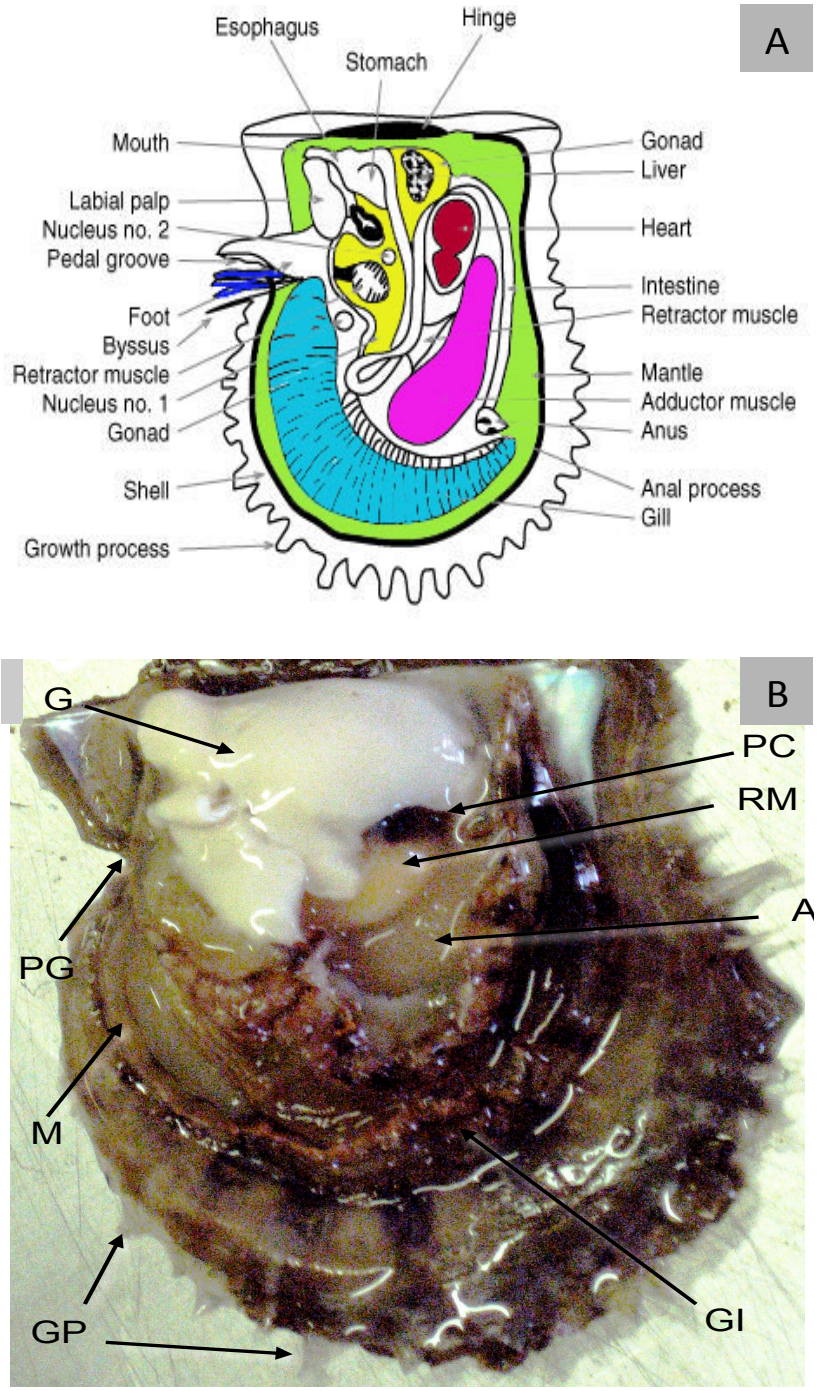

Figure 4. A, schematic representation of the internal anatomy of *P. imbricata*. Note the pericardial cavity (adapted from Gervis and Sims, 1992). **B**, the internal anatomy of *P. imbricata*: (GI) gills, (RM) retractor muscle, (G) gonad, (PC) pericardial cavity, (A) adductor muscle, (M) mantle, (GP) growth processes, (PG) pedal groove.

1.1.4 Internal Anatomy

Detachment of the adductor muscle from the dorsal aspect of the shell exposes the oyster's internal anatomy; the fatty deposit, the mantle, gills, byssus, gonad, foot, and adductor muscle (Fig. 4). The pericardium is located beneath the fatty deposit and next to the gonad (Fig. 5).

1.2 Pearl Production

1.2.1 The History of Pearl Production

Fishing for pearls and pearl by-products is an ancient practice (Chellam et al., 1981). Records from India date back to approximately 4000 B.C. when oysters were harvested for Mother-of-Pearl (MOP; Wada, 1991; Pit, 2004). In these times, MOP was acquired for practical uses, namely for the manufacture of buttons, implements, and to a lesser extent, decorations (Acosta-Salmón, 2004; Pit, 2004). Natural pearls were first discovered as a result of foraging and were subsequently used in cultural and religious ceremonies (Strack, 2006). In the early 19th century (before the intervention of man) natural pearls were exceptionally rare and obtaining one was costly. This was due to the potential threats to divers which included shark attacks and drowning (Kunz and Stevenson, 1908; Mamangkey, 2009). It was not until the mid 19th century that wild oyster stocks began to decline, providing an impetus for artificial pearl cultivation (Saville-Kent, 1890; Pit, 2004).

The history of modern pearl production began with experiments conducted by William Saville-Kent, on *P. maxima*, in 1890. However, the history of mass pearl production is commonly associated with Kokichi Mikimoto, who successfully produced half pearls (mabe; Fig. 5; Wada, 1991; Pit, 2004). Shortly after Mikimoto's

discovery, Tokichi Nishikawa and Tatsuhei Mise discovered that by introducing a nucleus and donor mantle tissue (saibo) into the gonad of an oyster, a pearl sac would form around the nucleus resulting in mineralization and nacre deposition (Pit, 2004). A joint patent was awarded to Nishikawa and Mise. However, a year later, Mikimoto applied for a similar patent, the only difference being his technique included 'round' pearl production (Fig. 6; Cahn, 1949; Wada, 1991; Pit, 2007). This protocol has since dominated the industry and Mikimoto has long been considered its 'founding father' (Wada, 1991; Pit, 2004).

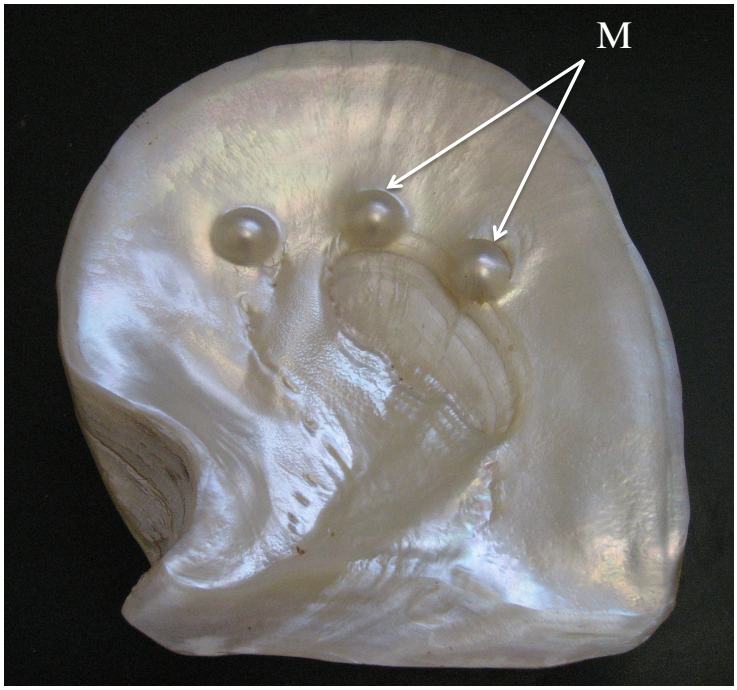

Figure 5. Half pearls, or mabe (M), are hemispherical pearls grown against the inside nacre of the shell. Mabe are significantly cheaper than round cultured pearls and are often set in jewellery such as rings, pendants and brooches (photograph by Rhiannon Kuchel).

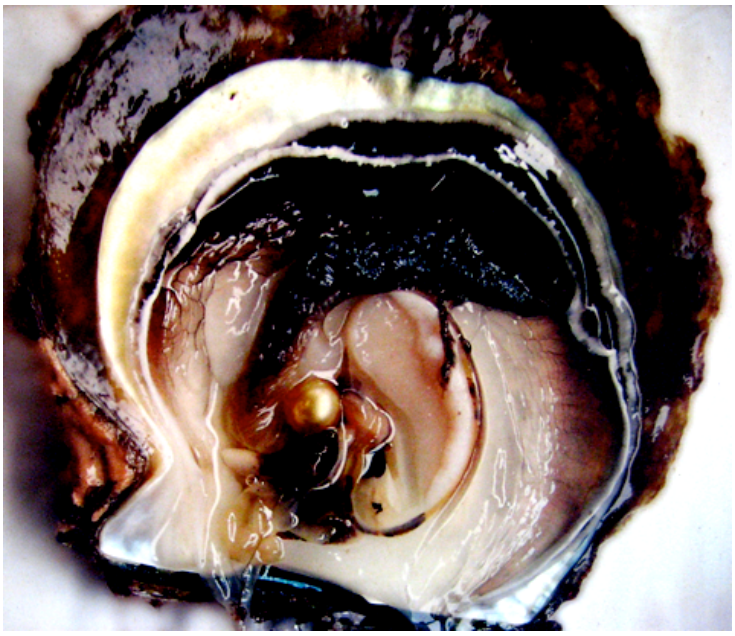

Figure 6. Man-made pearls mimic the natural process of pearl production. An irritant, i.e. nucleus, is inserted into the gonad with a section of saibo tissue. After 7 days a pearl sac forms around the nucleus and at approximately 40 days nacre is deposited. Oysters are typically seeded with a round nucleus (photograph by Rhiannon Kuchel).

1.2.2 Pearl Production

Today, Japanese cultured Akoya pearls total approximately 2.2% of both the fresh and saltwater pearl market, but represent at least 20% of the overall value (US $128 million; Anon, 2007; Mamangkey, 2009). The process of artificial pearl production is often referred to as 'seeding' or 'grafting' an oyster (Pit, 2004). Prior to seeding, oysters undergo a month long conditioning phase, inducing both nutritional and physiological stress (Taylor and Strack, 2008; Mamangkey, 2009). In *P. maxima*, the oysters are exposed to air just before nucleation, forcing them to gape (Mamangkey, 2009). Recently, research has investigated the possibility of chemically

'relaxing' oysters with propylene phenoxetol prior to extracting saibo, therefore eliminating the necessity to expose the oysters to air (Norton et al., 1996; Mamangkey, 2009). These studies have shown that 'relaxed' tissue performs better than that of non-relaxed oysters (Mamangkey, 2009).

Modern-day pearls are produced by inserting a nucleus milled from a bivalve shell, typically that of a Mississippi freshwater clam. The nucleus itself is a complex of a protein matrix and an aragonite and calcite crystal structure (Kobayashi and Samata, 2006). The nucleus is coupled with a small 3mm square section of donor mantle tissue (saibo - nacre secreting) and is inserted into the gonad of the recipient (host) oyster (Fig. 7 & 8; Gervis and Sims, 1992). Saibo tissue is selected for and based on both nacre colour and lustre, as these characteristics are thought to contribute to the resulting pearl (Taylor, 2002; Mamangkey, 2009; McGinty et al., 2010). The orientation of the saibo tissue in relation to the nucleus is of significant importance. If the cells that secrete nacre are facing the opposite direction to the nucleus, nacre deposition will not occur (Kafuku and Ikenoue, 1983; Pit, 2004). A nacreous layer of aragonite crystals is deposited around 40 days after nucleus insertion at approximately 0-7 layers per day. Industry standards for Akoya pearls requires nacre thickness of ~ 0.2 mm -0.5 mm (Fig. 9; Pit, 2004).

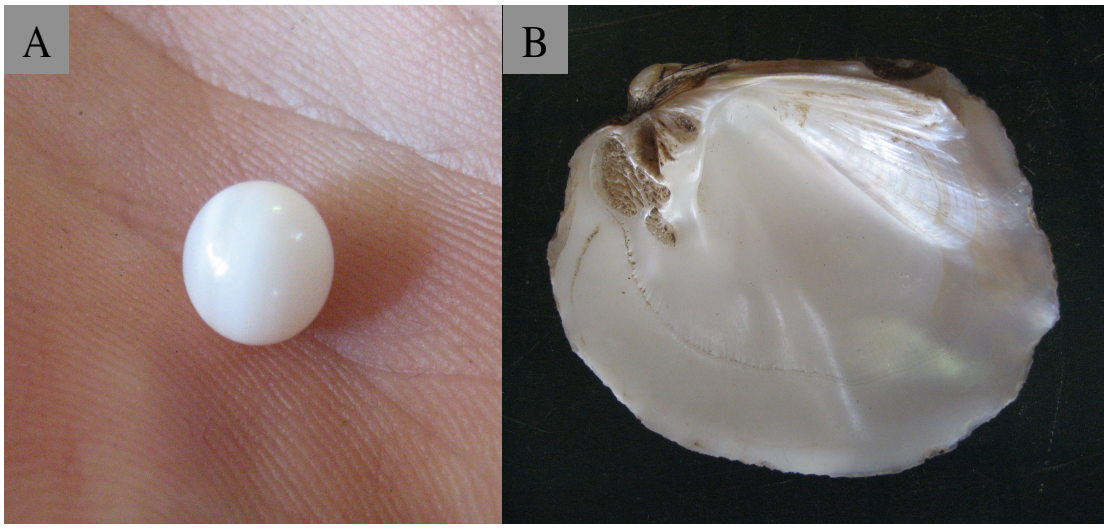

Figure 7. **A**, a nucleus milled from a freshwater clam shell. **B**, a freshwater clam shell (photograph by Rhiannon Kuchel).

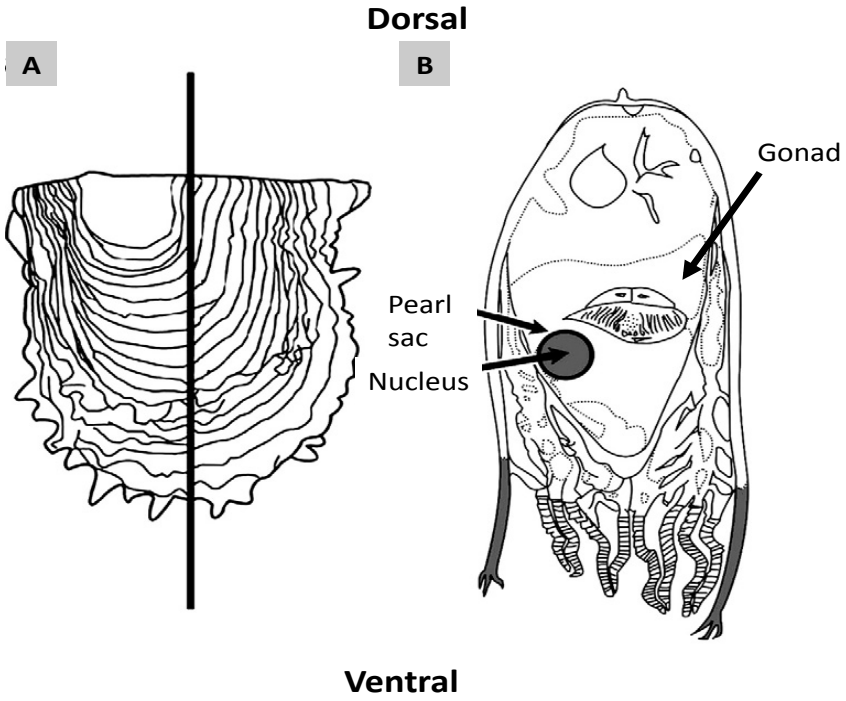

Figure 8. Nucleation of *P. imbricata*. The nucleus is inserted into the gonad through a wound located near the foot (adapted from Inoue et al., 2010).

The overall quality of a pearl is assessed by five different criteria: shape, size, lustre, surface texture, and colour (Gervis and Sims, 1992; Acosta-Salmón, 2004). Nacre deposition is significantly influenced by environmental conditions, the overall health of the recipient oyster and the quality of donated saibo tissue (Gervis and Sims, 1992; Acosta-Salmón, 2004). At best, only 30 - 35% of nucleated oysters survive the process and produce a pearl (Matlins, 2002). Resulting pearls are commonly graded by the AAA-A system (Mamangkey, 2009).

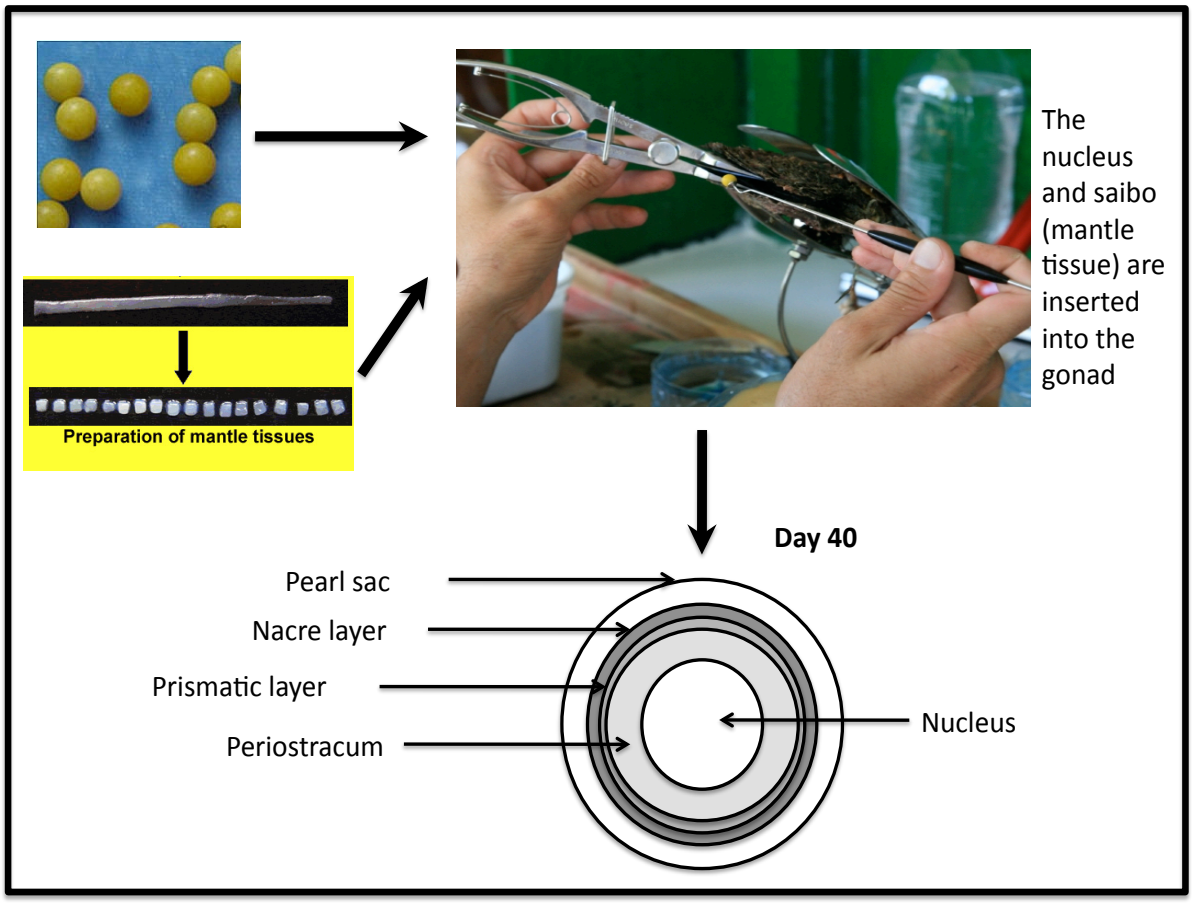

Figure 9. Diagrammatic representation of nacre deposition and pearl formation. A pearl sac forms around the nucleus within the first seven days of grafting. Subsequently, a further three other layers develop around the nucleus; the periostracum (~15 days), the prismatic (~30 days), and nacre (~40 days; adapted from Pit, 2004).

1.3 The Akoya Pearl Industry

1.3.1 History of the Akoya Pearl

Commercial Akoya pearl culture was first established in Japan in the 1920's (Kafuku and Ikenoue, 1983; Pit, 2004). However, by 1996 productivity declined significantly due to the proliferation of Akoya viral disease (AVD), from which 150 million oysters perished (Beard and Wade, 2002; Pit, 2004). By early 2000, the Japanese Akoya pearl industry represented only 21% of the total world market, and by 2002, pearl production fell to ~ 30 tonnes, a mere 13% of overall production in 1996 (Dettmar, 2002; Pit, 2004). The outbreak of disease and its continuing proliferation has been still linked with increasing urbanisation and industrialisation (O'Connor et al., 2003).

1.3.2 Akoya Pearl Production in Australia

The decline in Japanese cultured Akoya pearls has created an opportunity for other pearling nations to enter the market, including Australia. A survey conducted by Colgan and Ponder (2002) revealed that *P. imbricata* is endemic to the coastline of Australia, from Shark Bay in the west to the tip of Victoria in the east (O'Connor et al., 2003). In 2003, the New South Wales Department of Industry and Investment (NSW DII) and Radiata Pty Ltd began to assess the viability of Akoya pearl culture in Port Stephens, NSW (O'Connor et al., 2003). Results from initial studies were encouraging and a commercial pearl oyster venture is now in operation.

The production of high quality Akoya pearls, combined with the development of a robust industry in Australia is very promising. There are a number of advantages in farming *P. imbricata* over the already popular *P. maxima* and *P. margaritifera* (Pit,

2004). For example, the time taken for *P. imbricata* to produce a pearl from hatchery spat to harvest is significantly shorter than for other pearl species. This is because *P. imbricata* can be nucleated within a year (~ 50 mm dorso-ventral length), compared to with two years (100 – 200 mm) for *P. maxima* and *P. margaritifera* (Wada, 1986; Shirai, 1994; Pit, 2004). The time taken to produce a pearl by *P. imbricata* is approximately 2.5 – 3 years, whereas *P. maxima* and *P. margaritifera* require a minimum of 3.5-4 years (Pit, 2004). In addition, *P. imbricata* can be implanted with multiple nuclei, whilst the other farmed species are restricted to a single nucleus (Shirai, 1994).

1.4 Invertebrate Immunology

1.4.1 Invertebrate Immune Systems

The development of a robust and lucrative pearl industry relies on the production and maintenance of healthy oysters. Pearl quality is ultimately a reflection of immunological status, which can be jeopardised by stress and disease. Multicellular organisms have evolved complex immune systems to protect against infection (Lee and Söderhall, 2002). During metazoan evolution, two forms of immunity developed; adaptive (acquired) and innate (natural) immunity (Lee and Söderhall, 2002). Innate immunity is phylogenetically more ancient than adaptive immunity and is present in both vertebrates and invertebrates (Clatworthy, 1998).

Traditionally adaptive immunity is associated with vertebrates. However, a number of recent studies have investigated the possibility that variable forms of specific and adaptive immunity also exist within invertebrates (Butt et al., 2007; Kurtz and Franz, 2003b; Pham et al., 2007). For example, Kurtz and Franz (2003b)

discovered that priming injections in the copepod, *Macrocyclops albidus*, decreased re-infection by the same parasite, but did not affect resistance to other unrelated parasites. Similarly, Pham et al. (2007) have shown that the mosquito, *Drosophila melanogaster*, can adapt to immune challenges when injected with sublethal doses of *Streptococcus pneumonia*. Secondary exposure to the original bacteria was not found to result in infection (Pham et al., 2007).

It is thought that adaptive immunity, at least in vertebrates, developed over 400 million years ago and is considered to be more "sophisticated" than innate immunity (Fearon and Locksley, 1996). Acquired immunity possesses more complex molecular mechanisms than that of innate immunity, including immunological memory, specific recognition of foreign antigens, and hypervariable pathogen-specific receptor proteins (Janeway and Medzhitov, 2002; Aladaileh et al., 2007a). Specific immunological memory is represented by pathogen-specific receptor proteins which are generated by somatic gene rearrangements and clonal amplification (Aladaileh et al., 2007a).

The invertebrate immune response has received significant attention in the last two decades (Canesi et al., 2002). The first and basic lines of natural immunity are chemicophyisical barriers (mucus, external skeletons, and cuticles), which help to protect against initial microbial invasion (Deaton et al., 1999; Canesi et al., 2002). If one of these primary barriers is breached the secondary component of innate immunity is engaged. This secondary response is represented by both cellular and humoral defence mechanisms mediated by circulating haemocytes (Medzhitov and Janeway, 2002; Deleporte et al., 2006).

The cellular component of innate immunity is based on haemocyte function and involves enhanced cell migration, phagocytosis, encapsulation, the release of

antimicrobial proteins, nodule formation and the production of reactive oxygen species (ROS), such as superoxide and peroxide (Armstrong et al., 1993; Anderson et al., 1994; Epand and Vogel, 1999; Stuart and Ezekowitz, 200; Butt et al., 2007).

A number of studies have investigated the structure and function of bivalve haemocytes, and although there has been some confusion over their classification. There are two distinct types, hyalinocytes and granulocytes (Cheng, 1981; Auffret, 1988; McCormick-Ray and Howard, 1991; Cajaraville and Pal, 1995; Ballarin and Cima, 2005; Chang et al., 2005). Both granulocytes and hyalinocytes are involved in immunological defense responses. Granulocytes differ from hyalinocytes in that they contain cytoplasmic granules, which have been found to produce a number of intracellular antimicrobial compounds, including superoxide anions and melanin (Aladaileh et al., 2007a), as well as defensive enzymes, such as phenoloxidase (PO), acid phosphatase, and peroxidase.

Unlike vertebrates, invertebrates do not produce antigen-specific lymphocytes or immunoglobulins (Anderson, 1996). The molluscan humoral immune response is based on the activity of proteolytic cascades involving pattern recognition receptors (PRR's; Cerenius and Söderhall, 2004). Pattern recognition receptors include lectins, prophenoloxidase (proPO) activating molecules, fibrinogen-related proteins (FREPs) and encapsulation-promoting peptides that are either situated on haemocyte surfaces or are dissolved within haemolymph serum (Peters, 2003; Fisher, 1986; Anderson, 1996). Lectins bind to carbohydrate moieties on the surface of pathogens and are primarily responsible for "self" and "non-self" recognition (Anderson, 1996). This recognition system initiates a cascade of immunological responses, including opsonization and increased phagocytic clearance activities (Fisher, 1986).

1.4.2 Phagocytosis

Phagocytosis is an important component of host defence and facilitates the removal of invading pathogens and cellular debris (Fig. 10; Stuart and Ezekowitz, 2005). It is thought that phagocytosis evolved from the feeding mechanisms of unicellular organisms (Ratcliffe et al., 1985).

Phagocytosis is initiated when pathogens are recognised as "non-self" by the complex process of molecular "pattern recognition" (Söderhall and Cerenius, 1998). Research investigating molluscan defense dates back to the late 1890's (Metchnickoff 1893; De Bruyne, 1983; De Bruyne, 1896), whilst studies investigating the effects of non-organic particles such as talc expoure and "india ink" occurred in the late 1960's (Pauley and Sparks 1967; Cheng et al., 1970). Because most bacterial walls contain a rigid component of peptidoglycan (a carbohydrate), they are easily distinguished from "self" (Söderhall and Cerenius, 1998). Gram-negative bacteria also have an external layer of lipopolysaccharides (LPS) which encases peptidoglycan (Peters, 2004; Stuart and Ezekowitz, 2005). Eukaryotic pathogens are identified by the presence of plasmalemma-associated carbohydrates, such as ß-glucans (Fryer and Bayne, 1995). Peptidoglycans, LPS and ß-glucans represent unique pathogen-associated molecular patterns (PAMPs) that are detected by specific PRRs (lectins, proPO, FREPs and encapsulation-promoting peptides; Tiscar and Mosca, 2004; Stuart and Ezekowitz, 2005). The activation of haemocytes by the presence of PAMPs and other non-organic particles may be regulated by proteins homologous to vertebrate cytokines (Marson et al., 2001; Kambris et al., 2006).

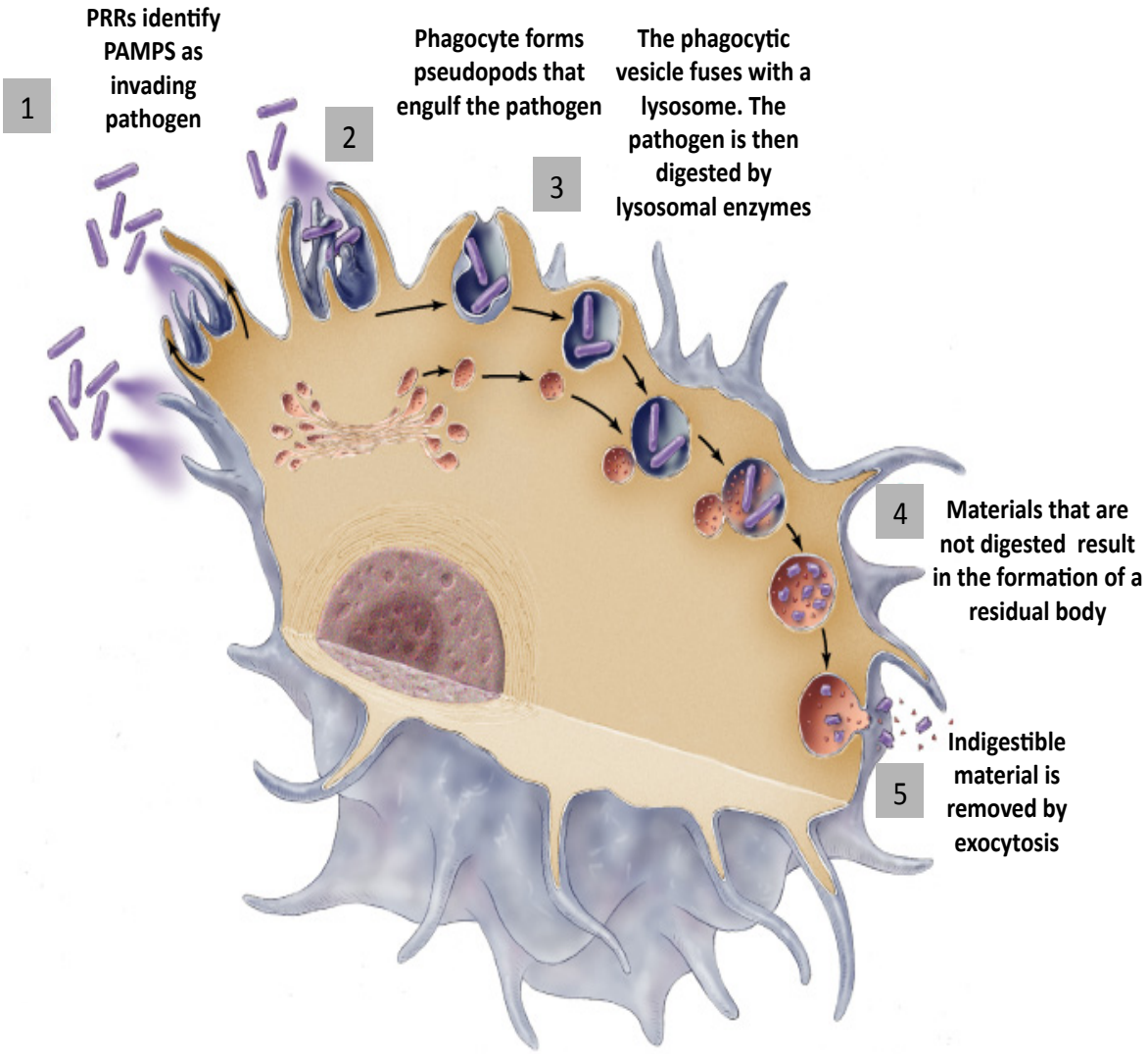

Figure 10. Schematic representation of phagocytosis. This process is characterised by five steps: 1, recognition; chemotaxis and pseudopodia development; 2, ingestion and phagosome formation; 3, fusion of lysosomes to the phagosome to form a phagolysosome and digestion of ingested pathogen by enzymes; 4, formation of a residual body containing indigestible materials; and 5, excretion of waste.

At the onset of phagocytosis, the recognition of PAMPS by PRRs initiates a "zippering" or cell-adhesion effects resulting in the phosphorylation of membrane associated proteins including profilin and gelsolin (Rodríguez-Domínguez et al.,

2006; Bugge et al., 2007). Actin microfilaments and the plasma membrane envelop the invading pathogen by developing pseudopodia (Söderhall and Cerenius, 1998). Once the invading pathogen is completely engulfed, the primary phagosome undergoes a series of fission and limited-fusion events with lysosomes to form a phagolysosome (Hauton et al., 2001). Microbial degeneration and digestion occurs as lysosomal agents, such as lysozome, PO, acid phosphatase and proteases are released into the phagosome (Munoz et al., 2006).

1.5 Environmental Stressors and Disease

Only 5% - 10% of pearls harvested represent the highest quality, yet they generate around 95% of the industry income (Haws, 2002; Mamangkey, 2009). For this reason, any advances in the industry could represent major financial benefits (Mamangkey, 2009). Most research thus far has focused on improving the production of pearls and their overall quality. While obviously this is of considerable interest to the industry, the effect that stress has on oyster mortality and the proliferation of disease should also be considered to be of the upmost importance.

Oysters are ectotherms. They are poikilothermic and osmoconforming. For this reason their haemocytes and internal organs are exposed to many of the same environmental conditions as their external environment (Fisher, 1986). Continual fluctuations away from "optimal conditions" can lead to acute physiological stress, often resulting in altered immunosurveillance and an increased chance of infection by opportunistic pathogens (Fig. 11; Cheng, 1988; Aladaileh et al., 2008).

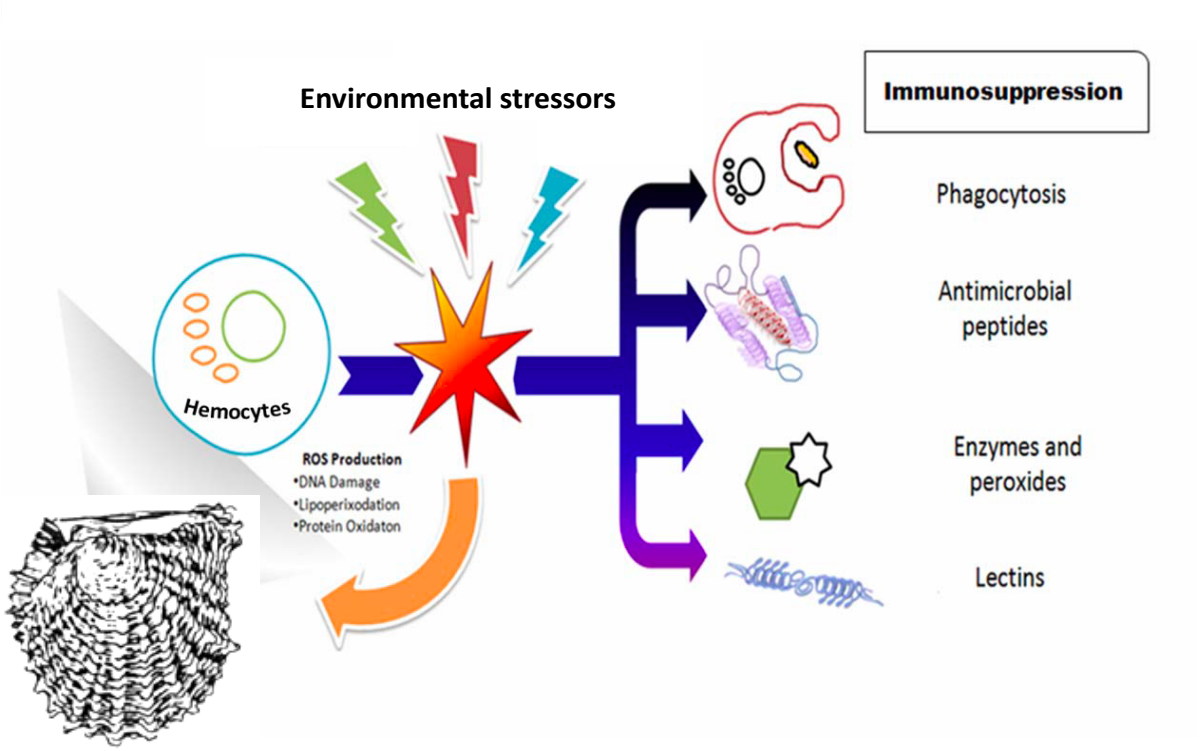

Figure 11. A schematic diagram representing the effects of environmental stressors on the immune system in *P. imbricata* (Figure adapted from Girón-Pérez 2010).

Typical stressors encountered in aquaculture include hypo- and hyper-thermia, declines in dissolved oxygen content (hypoxia), mechanical agitation induced by exposure to jet cleaning machines, fluctuations in salinity, and exposure to air. These stressors have been shown to compromise immune function (Matozzo et al., 2007). All organisms regulate their responses (both physiological and behavioural) to these biotic and abiotic stressors to achieve homeostasis, which is regulated in part by the endocrine system (Lacoste et al., 2001a-d; Aladaileh et al., 2008). Therefore, stress can induce a change in hormone regulation, which can alter homeostasis and associated biological functions (Chrousos and Gold 1992; Aladaileh 2007b).

In aquaculture, it is important to identify and recognise the early stages of bivalve stress (Sindermann, 1984). These typically include: mantle regression, regression of the digestive tubule epithelium, a pale digestive gland, oedema, haemocyte infiltration of tissues, a lag in gametogenesis, and an increased frequency of ceroids (brown bodies; Sindermann, 1984).

In this context, a link between immunological impairment and environmental stress has been identified in a number of invertebrates (Hegaret et al., 2003; de Almeida et al., 2007). For example, stress induced by mechanical agitation (associated with husbandry and handling) in *C. gigas* resulted in the release of endocrine stress hormones, including noradrenaline and dopamine (Lacoste et al., 2002b). Results from their study also indicated that there was a transient decrease in a range of immunological parameters.

Given these findings, a number of studies have examined 'stress indices' as potential indicators of disease susceptibility. Jeffries (1972) found that stressed clams (*Mercenaria mercenaria*) are more likely to be invaded by the pathogen, *Polydora* (Sindermann, 1984). His studies focused specifically on amino acid ratios, in particular taurine/glycine ratios of the gill and mantle tissues (Jeffries, 1972). Ratios above 3:1 indicated stress, whilst anything exceeding 5 was an indication of acute stress and an associated increase in disease susceptibility (Jeffries, 1972; Sindermann, 1984). Similarly, Feng et al. (1970) used taurine/glycine ratios to examine stress responses and infection in *Crassostrea virginica*. Other indices that have been used for the identification and analysis of stress include "scope for growth" (measuring for the potential for somatic growth and gamete production) and oxygen/nitrogen ratios (Corner and Cowry, 1968; Bayne and Scullard, 1977; Sindermann, 1984).

Fluctuations in environmental conditions may also be implicated with increased disease epizootics by increasing the virulence of infectious agents (Butt 2007). In 1982, Haskin and Ford found density gradients of the pathogen *Haplosporidium nelsoni* (MSX disease) to be directly related to salinity concentrations. At salinities ranging from 9 - 18 p.p.t., the parasite was found to enter *C. virginica*, but its development was severely retarded (Haskin and Ford, 1982). It should be noted that the developmental cessation of *H. nelsoni* might also be the result of a decrease in salinity, in combination with favorable conditions for *C. virginica*. Similar studies by Butt et al. (2006a) showed that salinity significantly affects infection rates of *Saccostrea glomerata* with *Marteilia sydneyi* (QX disease). Their studies also highlighted that decreases in the defensive enzyme, PO, was also associated with a decline in salinity, which resulted in increased infection with *M. sydneyi* (Butt et al., 2006b).

1.6 The Neuroendocrine System and Stress

1.6.1 The Neuroendocrine System

Homeostasis is maintained by both behavioural and physiological responses, and it is regulated by the endocrine system (Oehlmann and Schulte-Oehlmann 2003). Invertebrate endocrine systems lack the complexity of those in vertebrates. However, they do possess discrete endocrine organs that consist of neurosecretory cells (Oehlmann and Schulte-Oehlmann 2003).

The stress response occurs as a coordinated series of metabolic events (Goedken et al., 2005). Corticotropin releasing hormone (CRH) and adrenocorticotropin hormone (ACTH) are the main mediators of physiological stress

responses in both vertebrates and invertebrates (Ottaviani et al., 1998). Both CRH and ACTH control the secretion of catecholamines (biogenic amines; CA), such as dopamine (DO) and noradrenaline (NA), which act primarily as neuroregulators (i.e. neurotransmitters and neuromodulators; Lacoste et al., 2001b-d). Hormonal messengers, such as DO and NA, have been detected in a range of invertebrates, such as scallops (*Placopecten magellanicus*), cnidarians (*Renilla koellikeri*), and bivalve molluscs including *S. glomereata* and *C. gigas* (Lacoste et al., 2001b; Aladaileh et al., 2008). Catecholamines have been found to control respiration, feeding activity, metamorphosis, and reproduction (Sakharov and Salanki 1982; Teyke et al., 1993; Beiras and Widdows 1995; Wang et al., 2006, Aladaleih 2007b).

Lacoste et al. (2001b) conducted a series of experiments to determine the effects of NA on *C. gigas* haemocytes (Lacoste et al., 2001a-d). Results from their studies indicated that NA has a dose-dependent inhibitory effect on phagocytosis and modulates the production of ROS. Recent studies by Aladaileh et al. (2008) showed NA injections in *S. glomerata* cause a decrease in the immunologically active enzyme phenoloxidase (PO), as well as declines in total haemocyte counts and overall phagocytic activity (Aladaileh 2007b). Both of these studies support the hypothesis that neuroendocrine responses can compromise immunological function.

1.6.2 Environmental Stressors and the Endocrine System

A link between compromised immune efficiency and stress has been established in a number of invertebrates (Malagoli et al., 2007; Lacoste et al., 2001a-d; Aladaileh et al., 2008). This decline in immunosurveillance may be implicated in disease outbreaks and their ongoing persistence.

A study performed on *C. gigas* demonstrated that mechanical agitation (MA) and temperature or salinity variations are associated with the secretion of NA (Lacoste et al., 2001c). NA secretion resulted in the decline of many immunological parameters, such as total haemocyte counts and total protein content (Lacoste et al., 2002b). Furthermore, temperature induced stress was found to increase infection and mortality rates in juvenile oysters infected by the pathogen *Vibrio splendidus* (Lacoste et al., 2001a). In a similar study, the abalone *Haliotis turberculata* were subject to 15 min of MA. Assays identified the presence of both DO and NA within their circulatory system (Cheng et al., 2006; Malham et al., 2003). Immune parameters such as total haemocyte counts, migratory activity, phagocytic and respiratory bursts were also found to be compromised (Cheng et al., 2006).

A number of studies have also identified a correlation between hormonal stress responses and apoptotic events (Lacoste et al., 2002a; Aladaileh et al., 2008). Sokolova et al. (2004) found that heavy metals alter haemocyte morphology and induce apoptosis in the Eastern oyster, *C. gigas*. Similarly, Tri-n-butyltin (TBT) and herbicide 2,4-dichlorophenoxyacetic acid in the mussel, *Mytilus galloprovincialis*, were found to affect haemocyte proliferation and increase the incidence of apoptosis. These studies suggest that apoptosis of defensive haemocyte populations may be a contributing factor in stress-associated immunosuppresion.

1.7 Research Plan

1.7.1 Aims and Objectives

The aim of my thesis is to define the effects of environmental stress on immunological activity in pearl oysters. My studies will:

1. Provide a morphological and functional classification of *P. imbricata* haemocytes.
2. Test whether environmental stress (temperature, salinity and MA) alters key immunological responses such as; PO and acid phosphatase activity, phagocytosis, total haemocyte counts, granulocyte abundance, and total protein content.
3. Study the cellular basis and the relationship between the stress hormone, NA, and impaired immune function, focusing on hormone-induced apoptosis among defensive haemocytes.
4. Demonstrate the regulation of PO in stressed *P. maxima*, and its applications for early detection with on farm applications.
5. Investigate the effects of environmental stressors (MA and exposure to air) on the expression of antioxidant genes.
6. Review the Akoya pearl industry, focusing on invertebrate immunology and the effects of environmental stressors and their interactions with the spread and persistence of disease.

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CHAPTER TWO

HAEMOCYTE MORPHOLOGY AND FUNCTION IN THE AKOYA PEARL OYSTER, *PINCTADA IMBRICATA*

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Preface

As detailed in Chapter One of this thesis, there is a definable link between exposure to environmental stress and increased disease susceptibility due to immunnosuppression. The innate immune response is mediated by a combination of humoral and cellular effectors. The presence of pathogen associated molecular patterns (PAMPs) initiates non-self recognition which can result in the phagocytosis and intracellular killing of invading organisms. This provided impetus for my initial research, as the characterisation of haemocytes is an imperative first step in defining immunological responses to disease and infection because invertebrate blood cells are primary mediators of host defence. This work was important because the results form a foundation for further research investigating environmental stressors and associated immunosuppression in *P. imbricata*. A range of immunological assays and both light and electron microscopy were employed to identify haemocyte structure and function. The results from this Chapter show there are three distinct blood cell populations, granulocytes, hyalinocytes and serous cells. Granulocytes were found to be the most immuno-reactive cell population, engulfing the greatest amount of Congo red-stained yeast and staining positively for a range of cytotoxic enzymes. Hyalinocytes were also involved in clearance activities, but to a lesser extent. These results demonstrate the importance of haemocytes in pearl oyster defence.

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Haemocyte morphology and function in the Akoya Pearl Oyster, *Pinctada imbricata*

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ABSTRACT

The morphology and cytochemistry of *Pinctada imbricata* haemocytes were studied *in vitro*. Three distinct blood cell types were identified; hyalinocytes, granulocytes, and serous cells. Haemocytes were classified based on the presence/absence of granules, and nucleus to cytoplasm ratio. Granulocytes were the most common cell type (62 ± 2.81%), followed by hyalinocytes (36 ± 2.35%), and serous cells (2 ± 0.90%). Granulocytes, and hyalinocytes were found to be immunologically active, with the ability to phagocytose Congo red stained yeast. Of the cells involved in phagocytosis, granulocytes were the most active with 88.8 ± 3.9% of these haemocytes engulfing yeast. Cytochemical stains (phenoloxidase, peroxidase, superoxide, melanin, neutral red) showed that enzymes associated with phagocytic activity were localised in granules within granulocytes. Based on their affinities for Giemsa/May–Grünwald stain, haemocytes were also defined as either acidic, basic or neutral. Hyalinocytes and serous cells were found to be eosinophilic, whilst granulocytes were either basophilic (large granulocytes), eosinophilic (small granulocytes) or a combination of the two (combination granulocytes). Light, differential interference contrast and epifluorescence microscopy identified three sub-populations of granulocytes based on size and granularity; small (4.00–5.00 μm in diameter, with small granules (0.05–0.5 μm in diameter), large (5.00–9.00 μm in diameter, with large granules (0.50–2.50 μm in diameter) and combination (5.00–9.00 μm in diameter, with both large and small granules). These observations demonstrate that *P. imbricata* have a variety of morphologically and functionally specialized haemocytes, many of which maybe associated with immunological functions.

1. Introduction

The increasing prevalence of infectious diseases in aquaculture has become a significant problem for many previously lucrative industries (Huchette and Clavier, 2004; Travers et al., 2008). Between 1993 and 1996 the Japanese Akoya pearl industry experienced a significant slump in production of both shell and pearls, representing a decline in productivity of almost 50% (O'Connor et al., 2003). This disruption in the market was attributed to coastal degradation and the proliferation of disease (O'Connor et al., 2003). A thorough understanding of immunological responses by aquatic species may help to lessen the impact of disease, especially within monocultures. The characterisation of haemocytes within invertebrate aquatic species is a useful first step in defining immunological processes because these cells are thought to be primary mediators of host defence.

In bivalve molluscs, haemocyte-mediated immune responses include nodule formation, encapsulation, phagocytosis, melanisation and the production of reactive oxygen species (ROS; Bayne, 1990; Pipe, 1992; Hégaret et al., 2003; Aladaileh et al., 2007; Butt and Raftos, 2008), whilst humoral responses involve the synthesis of effector proteins and antimicrobial enzymes (Bayne, 1990; Boulanger et al., 2006; Aladaileh et al., 2007). Aside from their immunological functions, mollusc haemocytes are also thought to be involved in shell mineralization, excretion, metabolite transport, digestion and wound repair (Cheng, 1981; Mount et al., 2004).

A number of studies have investigated the structure and function of bivalve mollusc haemocytes (Cheng, 1984; Auffret, 1988; McCormick-Ray and Howard, 1991; Cajaraville and Pal, 1995; Ballarin and Cima, 2005; Chang et al., 2005; Aladaileh et al., 2007). They have been based on a variety of techniques including density gradient centrifugation (Cheng et al., 1980; Friebel and Renwrantz, 1995), flow cytometry (Xue et al., 2001; Hégaret et al., 2003), and transmission, light and scanning electron microscopy (Morona and Mingyi, 1989; Zhang et al., 2006; Travers et al., 2008). Early characterisations of bivalve haemocytes were often contradictory because a variety of techniques were used for their classification; as a result numerous terminologies were adopted. It was not until Cheng (1981, 1984) and Hine (1999) synthesised the literature that the existence of three major circulating haemocyte classes, un-differentiated cells, hyalinocytes and granulocytes, were hypothesised.

Both hyalinocytes and granulocytes are phagocytic and play a central role in host defence (Auffret, 1988). In a recent study by Aladaileh et al. (2007), granulocytes from the Sydney rock oyster (*Saccostrea glomerata*) were found to be more phagocytically active

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than hyalinocytes. Similar granulocytes have also been identified in many invertebrate species other than molluscs, and have been classified as either eosinophilic, basophilic or neutrophilic (McCormick-Ray and Howard, 1991; López et al., 1997). Cytochemical assays have demonstrated that granules within granulocytes contain a number of intracellular antimicrobial compounds, including superoxide anion and melanin (Aladaileh et al., 2007), as well as defensive enzymes, such as phenoloxidase (PO), acid phosphatase, and peroxidase.

In contrast to granulocytes, bivalve haemocytes containing few or no cytoplasmic granules are defined as hyalinocytes. In 1999, Hine described hyalinocytes as having a hyaline cytoplasm of a "silky" appearance (Hine, 1999). Superficially, hyalinocytes can be divided into two classes; small hyalinocytes with large nuclei and scanty cytoplasm lacking organelles, and large hyalinocytes with reniform or irregular nuclei, large cytoplasms, and often a variety of organelles.

Despite these data on haemocyte morphology and function in other bivalve species, there is no comparable information for the Akoya pearl oyster (*Pinctada imbricata*). *P. imbricata* is currently being assessed by The Department of Industry and Investment New South Wales (NSW DII) as the basis of a local pearl oyster industry (O'Connor, 2003). The Akoya pearl oyster is native to the east coast of Australia, from NSW to the northern tip of Queensland. It is typically fished for pearls in both Japan and China (O'Connor et al., 2003).

This study characterises *P. imbricata* haemocytes on the basis of their morphology and function, particularly potential immunological activities including the production of ROS, the presence of PO, and phagocytosis.

2. Materials and methods

2.1. Haemolymph collection

P. imbricata were supplied by The Department of Industry and Investment New South Wales (NSW DII), who obtained them from Broken Bay Pearls Pty Ltd. (Tuncurry, NSW). Oysters (15 per tank) were housed at the Sydney Institute of Marine Science (SIMS) in 45 L flow-through sea water system maintained at 22 °C. Oysters were removed from aquaria 5 min prior to haemolymph extraction to drain excess water from their mantle cavities. Oysters were opened by severing the adductor muscle. Haemolymph was withdrawn from the area surrounding pericardial cavity and adductor muscle using 27-gauge needles fitted to 1 mL syringes.

2.2. Live cell analysis

Thirty microlitre aliquots of whole haemolymph were placed on acid-alcohol washed slides and allowed to adhere for 20 min. The haemocytes were then covered with glass coverslips and viewed at high magnification (60× objective, oil immersion) with an Olympus BH-2 microscope equipped with both epi-fluorescence and differential interference contrast (DIC) optics. The haemocytes were assessed on their ability to adhere to a glass slide, and the motility of adherent haemocytes was observed. Differential haemocyte counts were made to calculate the relative percentage of different haemocyte sub-populations; twenty random fields of view from five separate haemolymph samples were analysed.

2.3. Cytological analyses

Cytological analyses were performed by adding 30 μL of whole haemolymph onto acid-alcohol cleaned slides. Cells were allowed to adhere for 25 min in a humid chamber before being stained by the following protocols. After staining, haemocytes were observed with an Olympus BH-2 microscope equipped with both epi-fluorescence and differential interference contrast (DIC) optics. All of the reagents and buffers for cytological analysis were from Sigma–Aldrich (Castle Hill, NSW), unless indicated otherwise. For each treatment, haemolymph from seven oysters were analysed separately.

2.4. Giemsa/May–Grünwald stain

Haemocytes were stained with Romanowsky's Giemsa/May–Grünwald stain, to characterise basic cellular morphology (Aladaileh et al., 2007). Adherent haemocytes were fixed for 20 min with formaldehyde (4% w/v in filtered sea water, FSW). Slides were then immersed in May–Grünwald stain for 6 min, before being counterstained with Giemsa for a further 30 min and washed in phosphate buffered saline (PBS; 136 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, adjusted to pH 7.5). Slides were air-dried, mounted with Ultramount No. 7 (Fronine Laboratory Supplies, Taren Point, NSW). Under these conditions basic granules stained blue whilst acidic granules stained pink (Chang et al., 2005).

2.5. Neutral red staining for lysosomes

Neutral red was used to further differentiate between acidic and basic vesicles in *P. imbricata* haemocytes. A stock solution was prepared by dissolving 20 mg of neutral red in 1 mL dimethyl sulfoxide (DMSO). The stock solution was then filtered through Whatman No. 2 filter paper and diluted 1:5 in PBS (Lowe and Pipe, 1994). 20 μL of the diluted neutral red was then overlaid onto unfixed adherent cells for 5 min. Neutral red stained vacuoles from red (acidic) to yellow (basic; Lowe and Pipe, 1994).

2.6. Peroxidase staining

Adherent cells were fixed with formaldehyde (4% w/v in FSW) for 10 min before being washed in PBS and transferred to a Coplin jar containing 5 mg/mL^{-1} 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 60 mM H_2O_2 (7.2 pH). Haemocytes were incubated in this solution at 37 °C for 2 h before being rinsed with PBS (adapted from Graham and Karnovsky (1966)). Peroxidase staining appeared as a yellow/brown precipitate in cytoplasmic granules using bright field optics (Cima et al., 2001; Aladaileh et al., 2007).

2.7. Intracellular phenoloxidase activity

Thirty microlitres of 20 μg mL^{-1} lipopolysaccharide (LPS), 5 mM hydroquinine monomethyl ether (4HA; Fluka, Buchs, Switzerland) and 5 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH) in FSW were overlaid onto live adherent haemocytes. The cells were stained for 25 min at room temperature before being mounted and inspected by light microscopy (Aladaileh et al., 2007). Quinones, the product of PO activity on polyphenol substrates, were detected under bright field illumination as a pink/red colouration within haemocytes (Cima et al., 2001; Aladaileh et al., 2007).

2.8. Staining for intracellular lipids

Adherent haemocytes were fixed in 4% paraformaldehyde for 30 min, before being bathed in 70% ethanol for 2 min. Sudan black B stain was prepared by adding 25 mg of Sudan black B to 50 mL of 70% ethanol. The fixed cells were then stained with Sudan black B for 15 min, rinsed with 70% ethanol and finally washed in distilled water. The slides were mounted and examined under DIC for purple/black deposits associated with Sudan black deposition.

2.9. Detection of superoxide anions

Thirty microlitres of nitroblue tetrazolium (NTB, 1 mg mL^{-1} in PBS) was spotted onto acid washed slides and incubated for 1 h at 37 °C in a humid chamber. 20 μL of whole haemolymph was then added to the NBT spots and allowed to stand for 30 min at room temperature (Song and Hsieh, 1994). After staining, intracellular superoxide anions ($O_2^{\cdot-}$) were detected as blue granules within haemocytes (Song and Hsieh, 1994; Aladaileh et al., 2007).

2.10. Intracellular melanin

Adherent haemocytes were rinsed with PBS and placed in Coplin jars containing a Fontana–Masson silver solution at 60 °C for one hour. This solution (Sheehan and Hrapchak, 1980) was prepared by adding concentrated ammonium hydroxide to 20 mL 10% silver nitrate drop by drop until the solution turned from dark brown to clear. After staining, cells were fixed with 5% w/v sodium thiosulphate for 2 min and then mounted. Under bright field illumination, melanin appeared within the cells as a brown precipitate (Sheehan and Hrapchak, 1980).

2.11. Phagocytic activity

Congo red stained *Saccharomyces cerevisiae* (Baker's yeast type II), were used as target cells for phagocytosis. Two hundred and fifty milligrams of *S. cerevisiae* were suspended in 5 mL of FSW containing 0.8% Congo red and autoclaved at 90 °C for 20 min. The suspensions were then washed twice by centrifugation at 3000*g*, 4 °C (for 5 min per wash). The final pellet was re-suspended in 10 mL FSW and stored at −20 °C. Immediately before use, yeast were diluted to 5×10^{-5} mL^{-1} in PBS.

One hundred microlitres of diluted yeast were spotted onto poly-L-lysine-coated glass slides and allowed to settle for 30 min. The supernatant was then removed before 30 μL of whole haemolymph was placed over the yeast and left to adhere for 25 min in a humid chamber. The slides were then rinsed in FSW, mounted, and examined at high magnification (1000×, oil immersion) using epifluorescence and DIC optics. 20 random fields of view each for five haemolymph samples from different oysters were analysed. Phagocytic activity was calculated by counting the number of granulocytes and hyalinocytes that had phagocytosed one or more yeast.

2.12. Confocal microscopy

Fifty microlitres of whole haemolymph were allowed to adhere to acid-alcohol washed slides for 25 min before being fixed with 10% w/v paraformaldehyde in FSW for 30 min. Cell membranes were permeabilised with 0.1% w/v Triton–X (in PBS) for 5 min before the cells were stained with 5 μL of phalloidin Alexa® in 200 μL PBS for 30 min to detect F-actin. Nuclei were counter-stained with propidium iodide for 2 min. Haemocytes were then examined using an Olympus Fluoview 300 confocal microscope.

2.13. Scanning electron microscopy (SEM)

Five hundred microlitre aliquots of whole haemolymph from 10 oysters were fixed with 500 μL of 8% w/v paraformaldehyde and 5% w/v glutaraldehyde in 0.1 M 1,4 piperazinediethanesulfonic acid (0.1 m PIPES buffer; pH 7.2 with 0.3 M sucrose) for 2 h. Cells were then washed in PIPES buffer and post-fixed with 1% w/v osmium tetroxide in 0.1 M PIPES buffer for 40 min, before being adhered to acid-alcohol washed coverslips for 30 min using 0.1% w/v ethylene imine polymer solution (PEI). Adherent cells were dehydrated in a graded ethanol series (50–100% in steps of 10%) and processed with a critical-point dryer (Emitech K850, Kent, Ashford). Coverslips were then mounted on aluminum stubs and coated with gold using a sputter coater (Emitech K550, Kent, Ashford). Samples were examined using a JEOL 6480 LA (NSW, Frenchs Forrest) scanning electron microscope.

2.14. Transmission electron microscopy (TEM)

Five hundred microlitre aliquots of whole haemolymph from 10 different oysters were fixed for 10 min at 4 °C in 4% w/v paraformaldehyde, 2.5% glutaraldehyde and 0.3 M sucrose in 0.1 M PIPES buffer (Sigma–Aldrich, pH 7.2). Fixed cells were then centrifuged at 400*g* for 10 min at room temperature and the supernatant was aspirated. The pellets were re-suspended in fresh fixative overnight at 4 °C. The haemocytes were then embedded in 2% w/v agar, washed with PIPES (2 × 1 h), and post-fixed in 1% w/v osmium tetroxide (OsO_4, in 0.1 M PIPES buffer pH 7.2) for 40 min. The pellets were rinsed (2 × 10 min washes in 0.1 M PIPES ph 7.2) and submerged in 2% w/v filtered aqueous uranyl acetate for 20 min (*en bloc* staining). After *en bloc* staining, the samples were washed with PIPES buffer (10 min) and dehydrated through a graded ethanol series (50–100%). Samples were embedded in L.R. White resin (80% polyhydroxy substituted bisphenol, a dimethacrylate resin 19.6%, C12 methacrylate ester, 0.9% benzoyl peroxide) and oven-dried in a gelatin capsule at 70 °C for 48 h. Ultrathin sections were cut using a Reichert Ultracut-S ultramicrotome and mounted onto cleaned 300 copper-mesh grids. Ultrathin sections were stained with filtered saturated uranyl acetate for 40 min and filtered Reynolds lead citrate for 5 min. The sections were examined with a Philips CM 10 transmission electron microscope.

3. Results

3.1. Light microscopy

Live cell and cytological analyses identified three haemocyte types, granulocytes, hyalinocytes and serous cells in the haemolymph of *P. imbricata*. These cell types were classified based on their size, shape, nucleus:cytoplasm ratio (N:C), and presence or absence of cytoplasmic granules, as follows.

Hyalinocytes represented 36.0% ± 2.3% (±represents standard error of the mean) of the total haemocyte population (Fig. 1J). Small hyalinocytes (3.0 ± 1.0 μm diameter) were round in shape and had a large central nucleus (a high N:C ratio: Fig. 1A). When stained with Giemsa/May–Grünwald they were eosinophilic (Fig. 2A), and their cytoplasm was limited to a thin peripheral layer. Medium to large hyalinocytes were characterised by the presence of few, if any, cytoplasmic granules and varied in size from 4 to 10 μm in suspension, and up to 35–50 μm when adhered to slides (Fig. 1B). Staining with Giemsa/May–Grünwald showed that medium to large hyalinocytes were eosinophilic (Fig. 2B). The nuclei of hyalinocytes were variable in shape, being either reniform or ovoid, with or without nucleoli. Once adhered to slides, hyalinocytes commonly develop pseudopodia and spread on the slide with little locomotory movement. Two distinct morphological forms of adherent hyalinocytes were observed; those that remained spherical, projecting only a few small pseudopodia, and those that spread significantly, forming triangular to star-shaped cell bodies.

Granulocytes were identified as large polymorphic cells possessing numerous cytoplasmic granules. They represented 62.0 ± 2.8% of the total haemocyte population (Fig. 1J), and were between 4 and 10 μm in diameter in suspension. Adherent granulocytes measured up to 30 μm in diameter. Granulocytes had small nuclei in comparison to the cytoplasm (low N:C ratio). Their nuclei were ovoid, spherical, or eccentric. Once adhered to slides, granu-

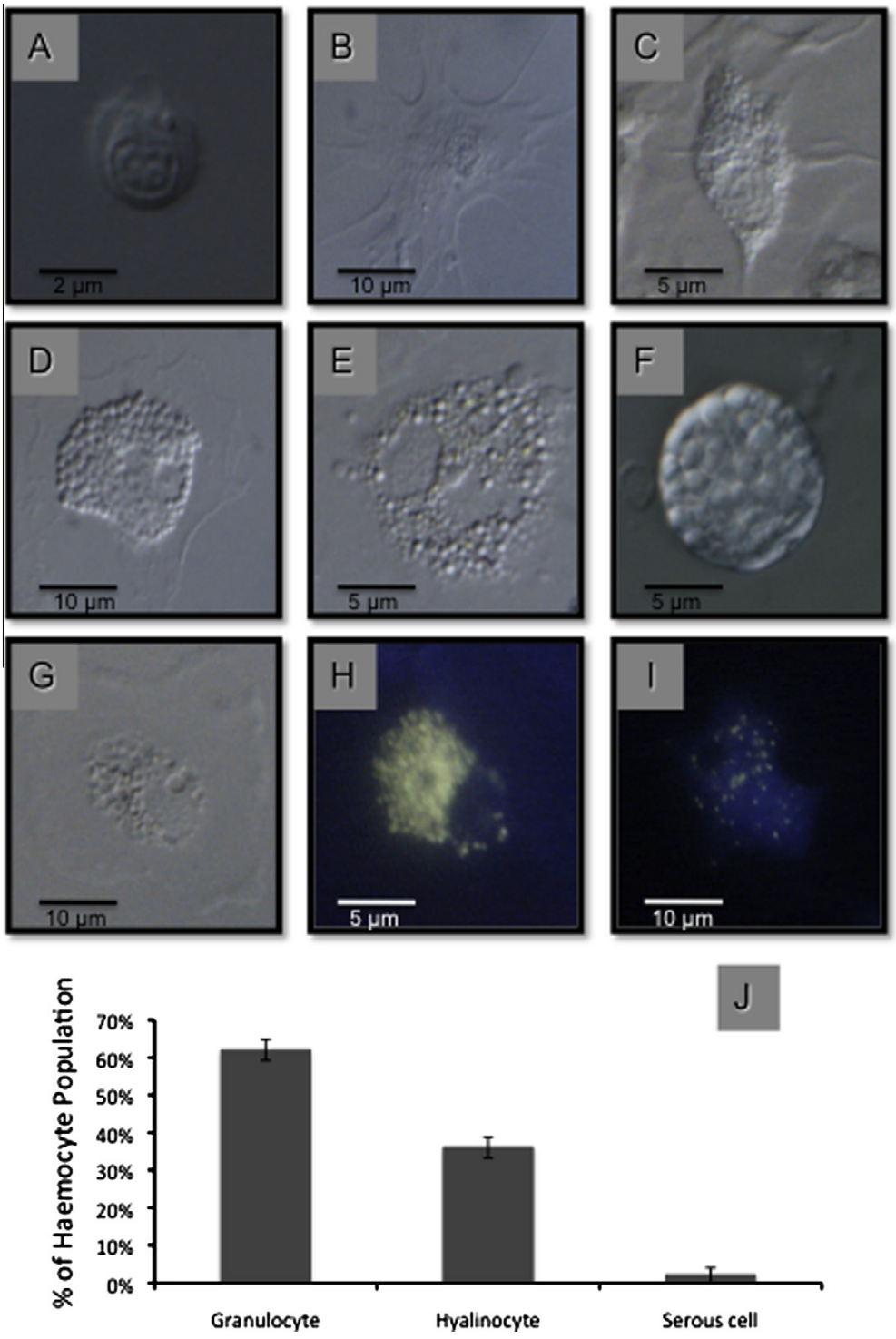

Fig. 1. Differential interference contrast light micrographs of adherent haemocytes from *P. imbricata* (A–G) and epifluorescent micrographs illuminated at 305 nm (H–I). (A) Small hyalinocyte, (B) large hyalinocyte, (C) small granulocyte, (D) large granulocyte, (E) combination granulocyte, (F) serous cell, (G) degranulated granulocyte, (H) fluorescent small granulocyte, (I) fluorescent large granulocyte. (J) Mean relative percentage of the different haemocyte types in haemolymph ($n = 7 \pm$ standard error of the mean).

locytes often projected numerous long filamentous pseudopodia (Fig. 1C–E), were highly motile and they were often observed degranulating (Fig. 1G).

Three sub-classes of granulocytes were evident based on their Giemsa/May–Grünwald staining characteristics, granule size and autofluorescence. The sub-classes were; small eosinophilic granulocytes (Figs. 1C and 2D), with cytoplasms that were densely packed with small (0.05–0.5 µm diameter) highly fluorescent granules: large basophilic granulocytes (Figs. 1D and 2C), which were densely packed with large (0.5–2.5 µm diameter) moderately fluo-

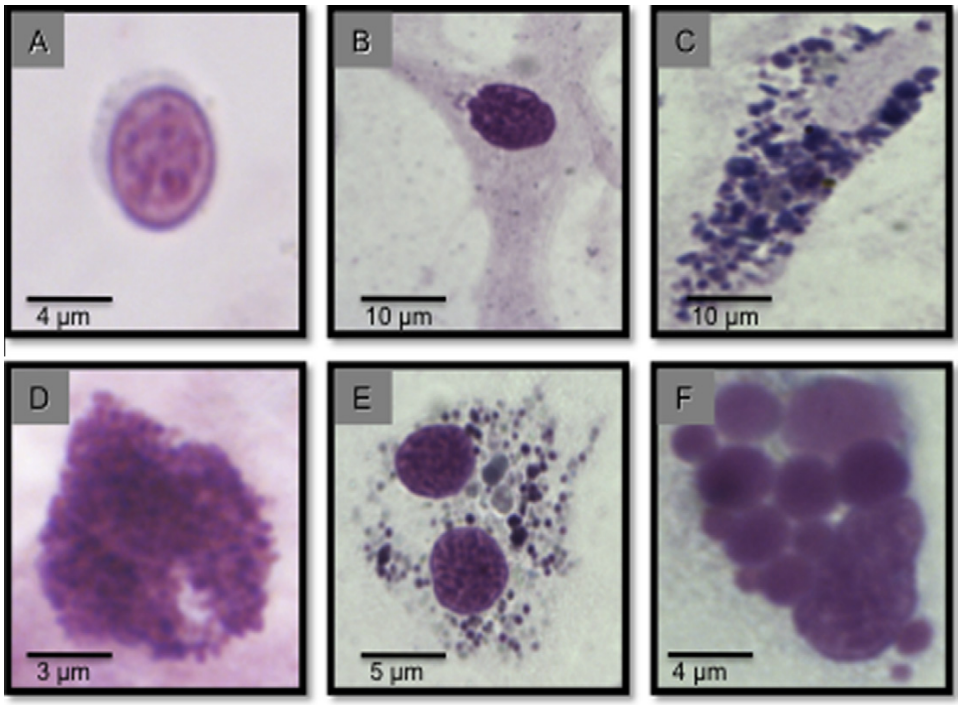

Fig. 2. Light micrographs of fixed haemocytes from *P. imbricata* stained with Giemsa/May–Grünwald. (A) Small eosinophilic hyalinocyte, (B) eosinophilic hyalinocyte, (C) large basophilic granulocyte, (D) small eosinophilic granulocyte, (E) combination granulocyte, and (F) serous cell.

rescent granules; and "combination" granulocytes, which had a mixture of both eosinophilic and basophilic granules (Figs. 1E and 2E) that were either large (0.5–2.5 µm diameter) or small (0.05–0.5 µm diameter) with variable autofluorescence emissions when excited at (305 nm). The colour of autofluorescence in granules of the different sub-classes of granulocytes was variable. Small granulocytes had granules that fluoresced white-yellow (Fig. 1H), large granulocyte granules fluoresced green-yellow (Fig. 1I), whilst the granules in combination or 'transitional' cells fluoresced both white-yellow and green-yellow.

The third cell type identified was the serous cell (Fig. 1F). These cells comprised 2.0 ± 0.9% of the total haemocyte population (Fig. 1J), and measured between 5 and 12 µm (in diameter) in suspension. Serous cells were densely packed with large, non-fluorescent eosinophilic granules (up to 3.5 µm diameter; Fig. 2F). In suspension, these cells were typically spherical or ovoid in shape. The nuclei of serous cells were rarely visible.

3.2. Cytochemistry

Neutral red staining was observed in granulocytes as either a pink, red, red-orange, or orange-yellow colouration within vesicular compartments. Granules in small granulocytes exclusively stained bright pink (Fig. 3F), whilst those in large granulocytes were often red-orange (Fig. 3H). Combination granulocytes had vesicles that stained in all of the above-mentioned colours, except bright pink (Fig. 3G).

Peroxidase activity appeared as dark brown/pink deposits localised within the perinuclear region of haemocytes (Fig. 3D). Staining was most pronounced in small granulocytes. Both PO and melanin activity was seen primarily in small granulocytes (Fig. 3A and E respectively), while they appeared as localised deposits in other granular and hyaline cells. Lipid deposits were identified by their blue-purple colouration in the cytoplasm of both granulocytes and hyalinocytes (Fig. 3B). Lipid deposits had either a perinuclear or homogenous distribution throughout the cytoplasm. Superoxides were seen predominantly in granulocytes, and appeared in either cytoplasmic granules, or around the perimeter of vacuoles (Fig. 3C).

3.3. Phagocytosis

Hyalinocytes and granulocytes ingested Congo red stained yeast (Fig. 4A–H). Granulocytes were most actively phagocytic. Fig. 4I shows that 88.0 ± 3.9% of the haemocytes that engulfed yeast cells were granulocytes, in comparison with hyalinocytes (11.2 ± 3.9%). Both hyalinocytes and granulocytes were able to phagocytose large numbers (up to 18) of yeast cells (Fig. 4B–E). They engulfed yeast by projecting small filopodia around the target. The filapodial arms containing the engulfed yeast cell then retracted into the cell body (Fig. 4H).

After about 40 min of co-incubation with haemocytes some ingested yeast exhibited a colour change (observed under epi-fluorescence microscopy) from orange-red to purple-blue around the periphery of the phagosome (Fig. 4F). A number of hyalinocytes that had ingested yeast were also found to have autofluorescent vacuoles when exposed to UV light (Fig. 4G).

3.4. SEM

Three morphologically distinct cell types were identified by SEM. These were; small spherical cells (most likely small hyalinocytes), with shallow surface indentations and fine protruding lobes (Fig. 5A); cells with large continuous pseudopodial extensions giving the cell a cabbage-like appearance (most likely hyalinocytes; Fig. 5B); and cells with numerous fine filopodia projecting from a rounded cell body (most likely granulocytes; Fig. 5C and D). Their filopodial extensions were often lumpy in appearance with spherical protrusions at their termini.

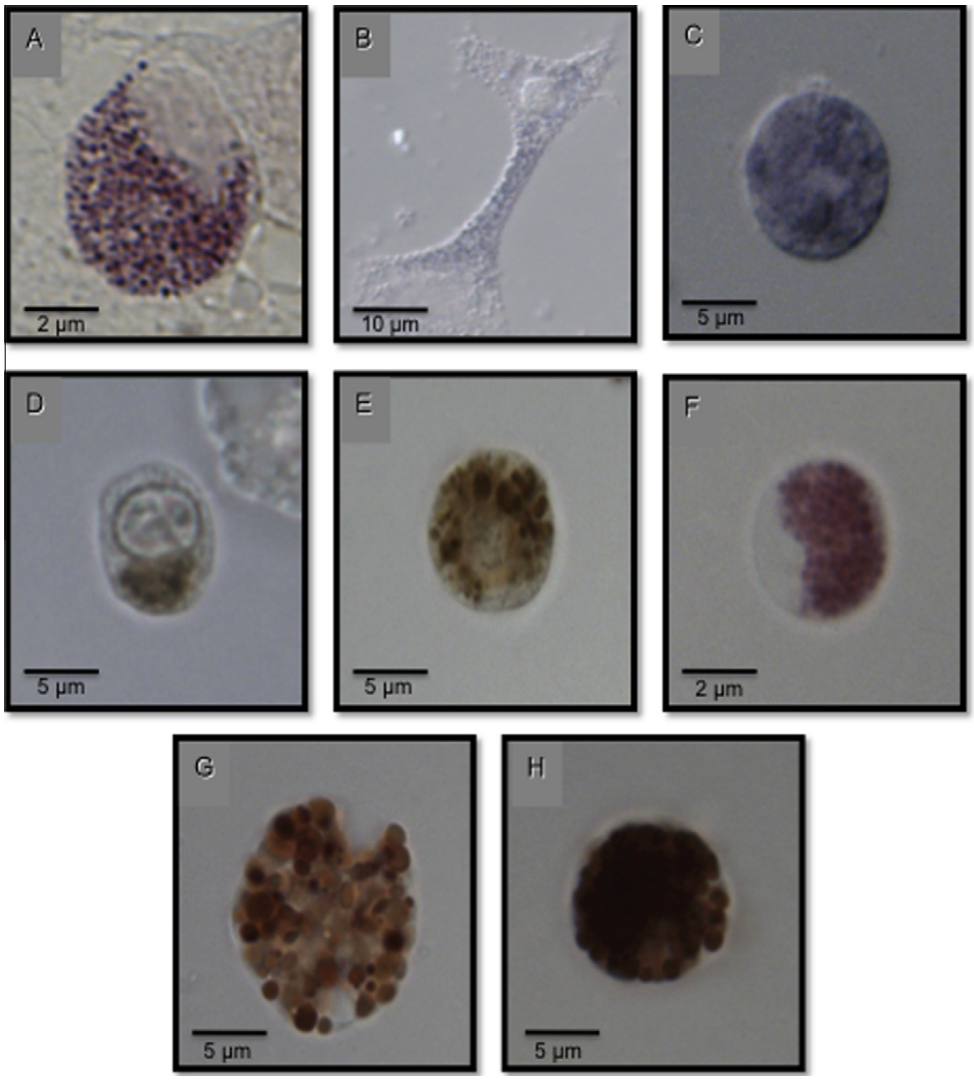

Fig. 3. Differential interference contrast (A–D) and conventional light micrographs (E–H) of *P. imbricata* haemocytes showing cytoplasmic localisation of: (A) phenoloxidase, (B) lipid, (C) superoxide, (D) peroxidase, (E) melanin, and (F–H) neutral red in: (F) small granulocyte, (G) combination granulocyte, (H) large granulocyte.

3.5. Fluorescence confocal microscopy

Fluorescence confocal microscopy showed that polymerisation of F-actin was closely associated with pseudopodium formation and was characteristic of particular cell types. F-actin fluorescent staining was most pronounced at the tips of pseudopodia and the periphery of haemocytes. Pseudopodia were seen as bright green F-actin-positive extensions from the cell body. Small hyalinocytes had many fine F-actin-stained filopodia radiating from the central body of the cell (Fig. 5E). F-actin-positive pseudopodia of medium to large hyalinocytes appeared as thick extensions, which often bifurcated into fine lamellipodia (Fig. 5F). Small granulocytes tended to have many F-actin-positive filopodia extending from the body of the cell (Fig. 5G). In contrast, large granulocytes had many small F-actin-positive stained nodes or focal adhesions around the periphery of the cell (Fig. 5H).

3.6. TEM

3.6.1. Hyalinocytes

TEM identified three distinct cell types that were comparable to those observed by light microscopy (hyalinocytes, granulocytes and serous cells). In TEM, small hyalinocytes were round or oval, and were the smallest class of haemocytes (Fig. 6A). As in light microscopy, they were characterised as having single large nuclei and scanty cytoplasm. Within the nuclei, the chromatin was often dispersed and organised in small clumps located away from the nuclear membrane. These cells exhibited a well-developed rough endoplasmic reticulum with free ribosomes and were often packed with mitochondria.

Larger more developed hyalinocytes varied significantly in morphology based on size and organelle complexity (Fig. 6B–D). Medium-sized hyalinocytes (5–6 µm in diameter; Fig. 6B) had a dense cytoplasm containing numerous mitochondria (Fig. 6E). Their nucleus to cytoplasm ratio was generally greater than that of small hyalinocytes and they had round to ovoid shaped nuclei containing highly condensed chromatin. These cells exhibited conspicuous rough endoplasmic reticulum and some vacuoles, and they had the ability to develop filopodia.

Large hyalinocytes (6–8 µm in diameter; Fig. 6C) often had polymorphic or lobed nuclei. These cells exhibited a well-developed Golgi apparatus with secretory vesicles, abundant mitochondria and partially condensed chromatin.

The largest form of hyalinocyte (Fig. 6D) had small eccentric nuclei with compact, condensed chromatin assemblages. These cells also contained some electron-dense granules.

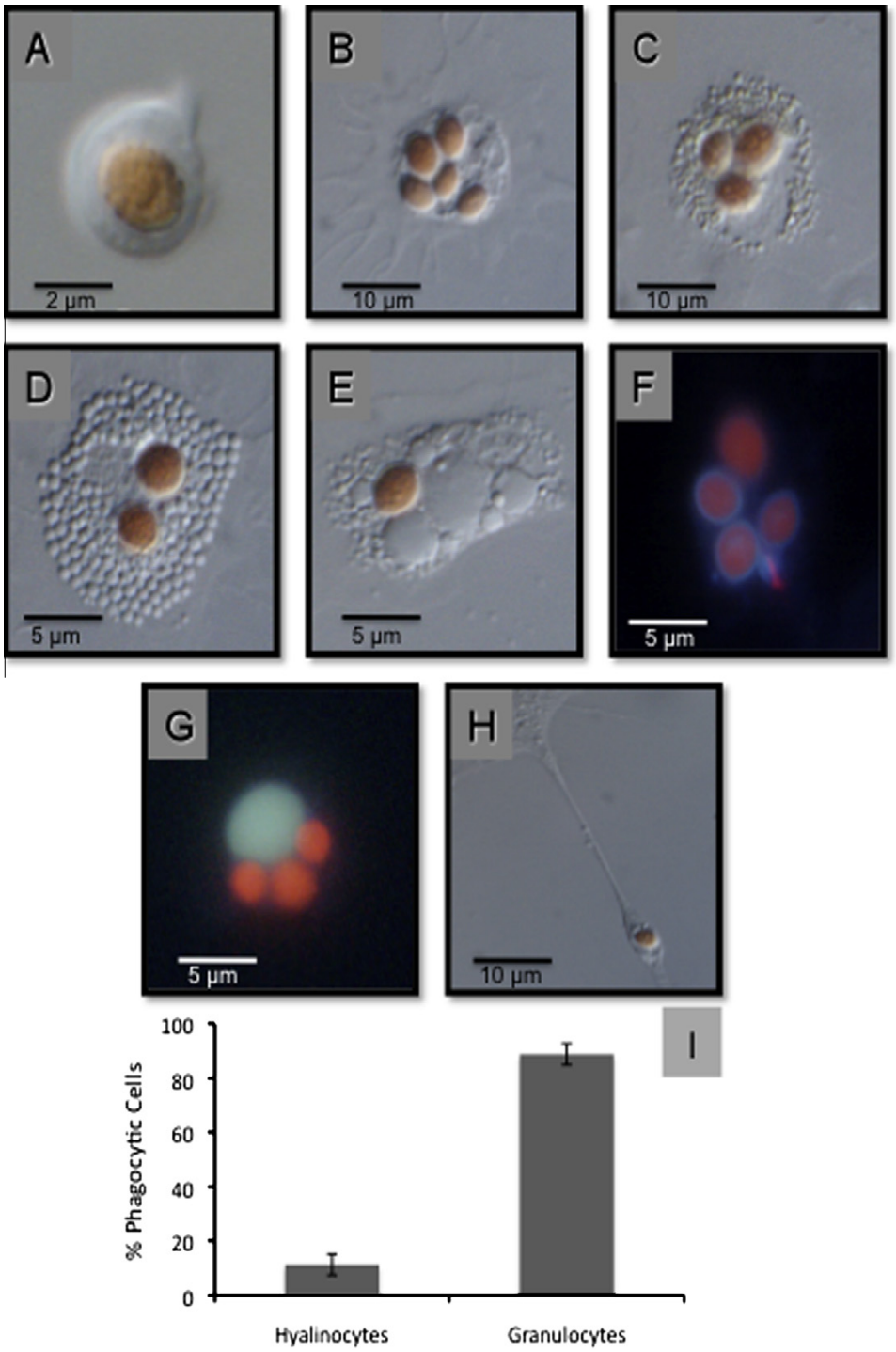

Fig. 4. Differential interference contrast (A–E and H) and epifluorescent micrographs (F–G) of *P. imbricata* haemocytes ingesting yeast. (A) Small hyalinocyte with one engulfed yeast cell, (B) large hyalinocyte with five engulfed yeast cells, (C) small granulocyte with three engulfed yeast cells, (D) large granulocyte with two engulfed yeast cells, (E) combination granulocyte with one engulfed yeast cell, (F) engulfed yeast cells, (G) fluorescent vacuole under UV light, (H) hyalinocyte extending a pseudopodium around a yeast cell, and (I) relative frequency (%) of phagocytic haemocytes in haemolymph.

3.6.2. Granulocytes

Three distinct types of granulocytes were observed, based on their granularity and size. Some granulocytes (Fig. 7A) had a large central ovoid nucleus with small-dispersed chromatin assemblages. These cells could be distinguished by the presence of membrane-bound granules enclosing fine granular material (Fig. 9A, B and F), as well as numerous mitochondria and small vacuoles.

The second type of granulocytes were small-to-medium in size (5–6 µm in diameter) and were characterised by the presence of numerous (up to 150 granules per cell) electron-dense granules in the cytoplasm (Fig. 7B; Fig. 9G–H). Mitochondria in these granulocytes were sparse, and their nuclei were often offset and ovoid, with moderately condensed chromatin. Vacuoles were abundant throughout these cells.

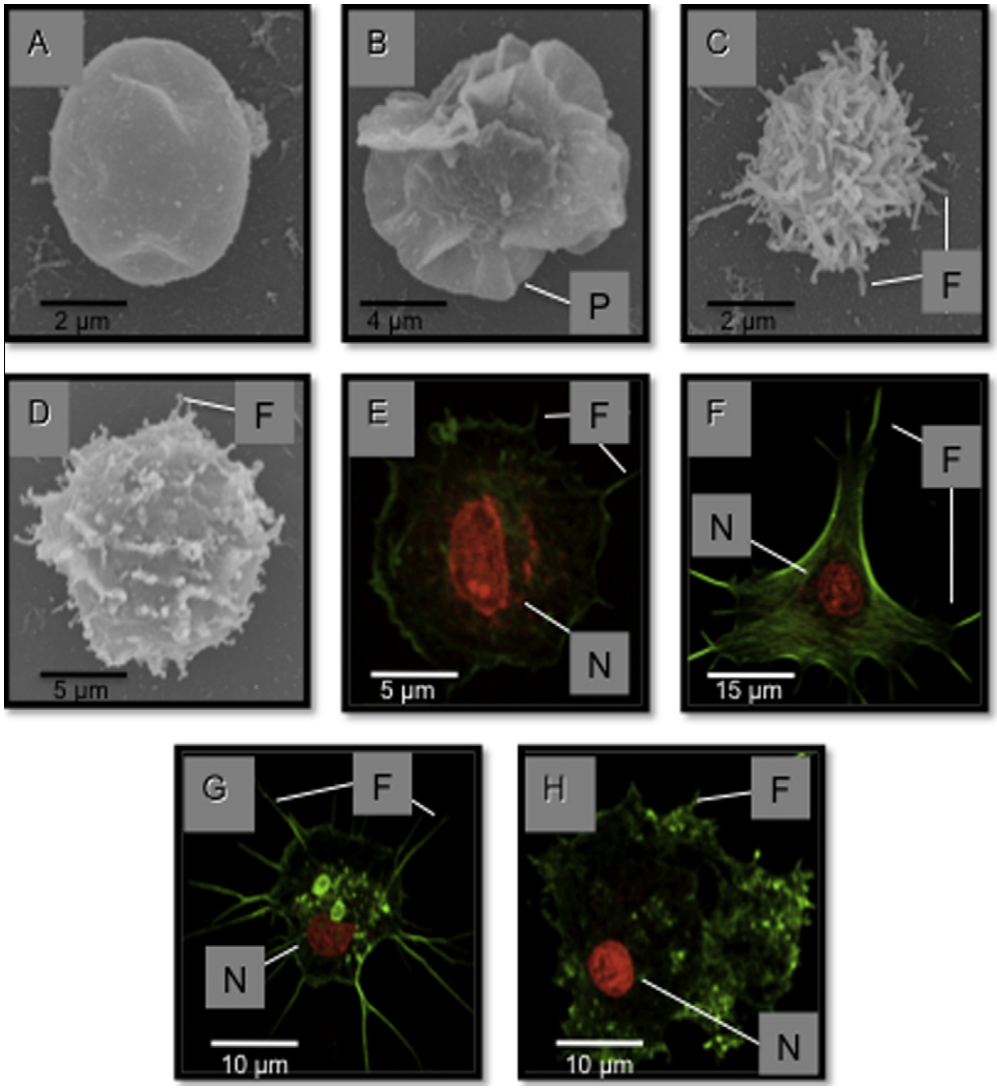

Fig. 5. Scanning electron micrographs (A–D) and fluorescent confocal micrographs (E–H) of *P. imbricata* haemocytes. (A) Small hyalinocyte, (B) large hyalinocyte, (C) small granulocyte, (D) large granulocyte, (E) small hyalinocyte, (F) large hyalinocyte, (G) small granulocyte, and (H) large granulocyte. N, nucleus; F, filopodia; P, pseudopodia.

The third granulocyte sub-type had many electron-lucent membrane-bound granules (Figs. 7C, D and 9D, F). These granules surrounded the nucleus, which was ovoid in shape. The cytoplasm contained few mitochondria and vacuoles, whilst rough endoplasmic reticulum was often very conspicuous.

All of the granulocyte sub-types possessed well-defined Golgi apparatuses (Fig. 7E–F), that were often associated with secretory vesicles and digestive lamellae.

3.6.3. Serous cells

Serous cells ranged in size (9–13 µm in diameter), granule density, and granule abundance (Fig. 8A and B). Some serous cells had a single large electron-lucent granule (Fig. 8A). Other serous cells had more numerous granules, which appeared less electron dense to serous cells that had a single granule (Fig. 8B).

4. Discussion

The haemocytes of bivalve molluscs have been studied extensively. However, their classification has been the subject of confusion and debate. Cheng (1984) reviewed the existing classifications to reach a consensus. He defined three main haemocyte types: undifferentiated cells, hyalinocytes, and granulocytes (Cheng, 1984). In the current study, haemolymph from *P. imbricata* was found to be comprised of these two major cell types, and a third, less common form, the serous cell.

Despite their suspected origin from un-differentiated cells, the developmental pathways of granulocytes and mature hyalinocytes remain unclear. A model proposed by Mix (1976) suggests that hyalinocytes are a precursor to granulocytes. In this context, Wen et al. (1994) suggested that the extensive presence of Golgi apparatuses are associated with cellular differentiation, which results from the progressive formation of membrane-bound granules. In the current study, Golgi apparatuses were seen in both of the major cell types, but not serous cells. Hence, the simple presence or absence of Golgi apparatuses may not reflect the developmental state of *P. imbricata* haemocytes.

Three distinct sub-classes of granulocytes could be identified in the haemolymph of *P. imbricata* based on granule size and electron density. These three sub-types were; small granulocytes, large granulocytes, and combination granulocytes. Studies examining haemocyte function in *Mytilus galloprovincialis* identified similar diversity among granulocytes (Cajaraville and Pal, 1995). Cajaraville and Pal (1995) proposed that variation in granule size and

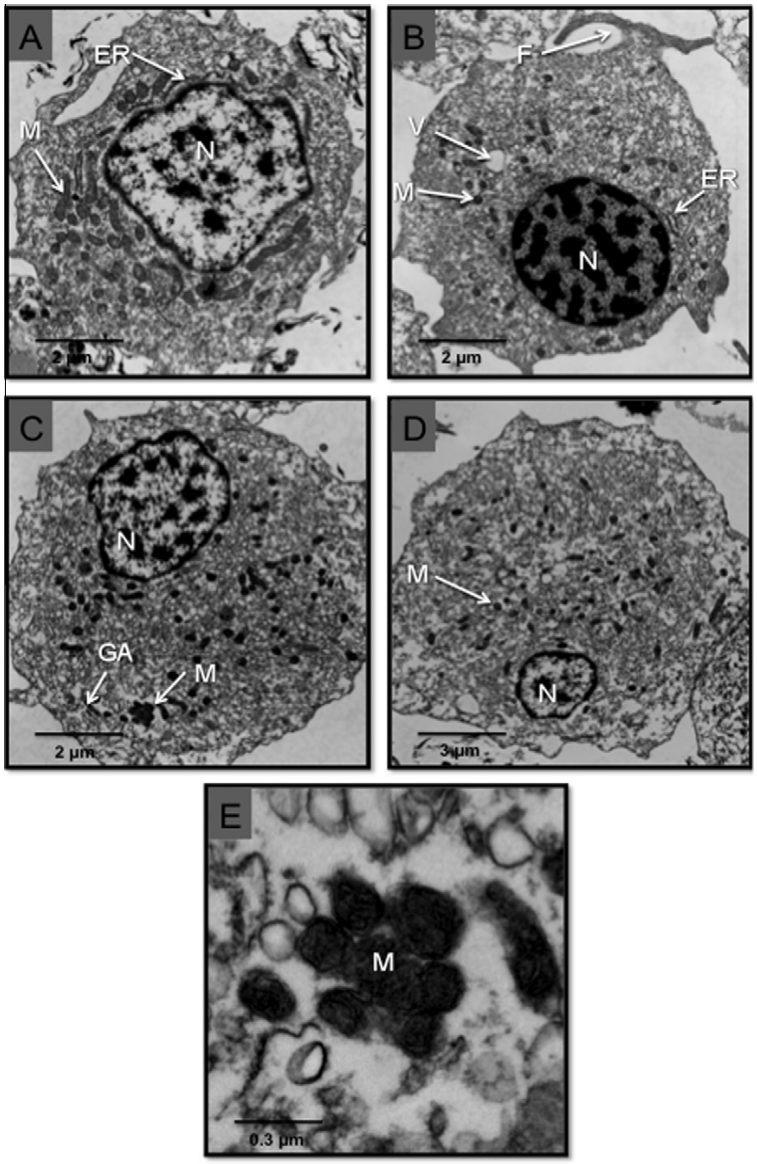

Fig. 6. Transmission electron micrographs of hyalinocytes in *P. imbricata* haemolymph. (A) Small hyalinocyte, (B) medium hyalinocyte, (C) large hyalinocyte, (D) large mature hyalinocyte, and (E) bundle of mitochondria. N, nucleus; M, mitochondria; ER, endoplasmic reticulum; F, filopodia; V, vacuole; GA, Golgi apparatus.

abundance may be a function of cellular differentiation and maturation. However, there is no direct evidence to support this contention.

Differential affinities of granules to Giemsa/May–Grünwald stain have also been suggested as markers of haemocyte differentiation (Cheng, 1981). In the current study, granulocytes were distinguished as either eosinophilic, basophilic, or had both acidic and basic granules. According to Cheng's scheme, younger smaller granulocytes contain basophilic granules, while mature large granulocytes are acidic (Cima et al., 2000). However, in the peppery furrow shell, *Scrobicularia plana*, Wootton and Pipe (2003) attributed differential staining to cell function, as well as progressive developmental state, and some bivalves, such as *Tricdacna crocea* and *Perna perna*, do not contain basophilic granulocytes (Nakayama et al., 1997; Barracco et al., 1999). Similarly, in *P. imbricata*, it appears that the granule types within different granulocyte sub-populations differ from other bivalves. Small granulocytes tended to contain acidic granules, while larger granulocytes had either acidic and basic granules, or were exclusively basic.

TEM confirmed that granulocyte sub-populations contain different types of granules. Dark electron-dense granules occurred predominantly within small granulocytes, while the granules of large granulocytes were often electron-lucent. Based on ultrastructure, these electron-lucent granules resemble those identified in other oyster species as basophilic granules (Chang et al., 2005).

In addition to granularity, the cell types identified in the current study also differed in their cellular projections and capacity for amoeboid movement. Observations using light microscopy demonstrated motile behaviour to be related to the occurrence of cytoplasmic granules. Granulocyte locomotion was amoeboid and directional, whilst hyalinocytes often adhered to a substrate and spread multidirectionally. Therefore, hyalinocytes appeared to be less mobile than granulocytes.

Despite different levels of adhesiveness and mobility, both of the major cell types in *P. imbricata* had the ability to engulf foreign material. Granulocytes were found to be the most phagocytically active. These results compliment those found in a number of other marine bivalves including *S. glomerata* and *Mercenaria mercenaria*

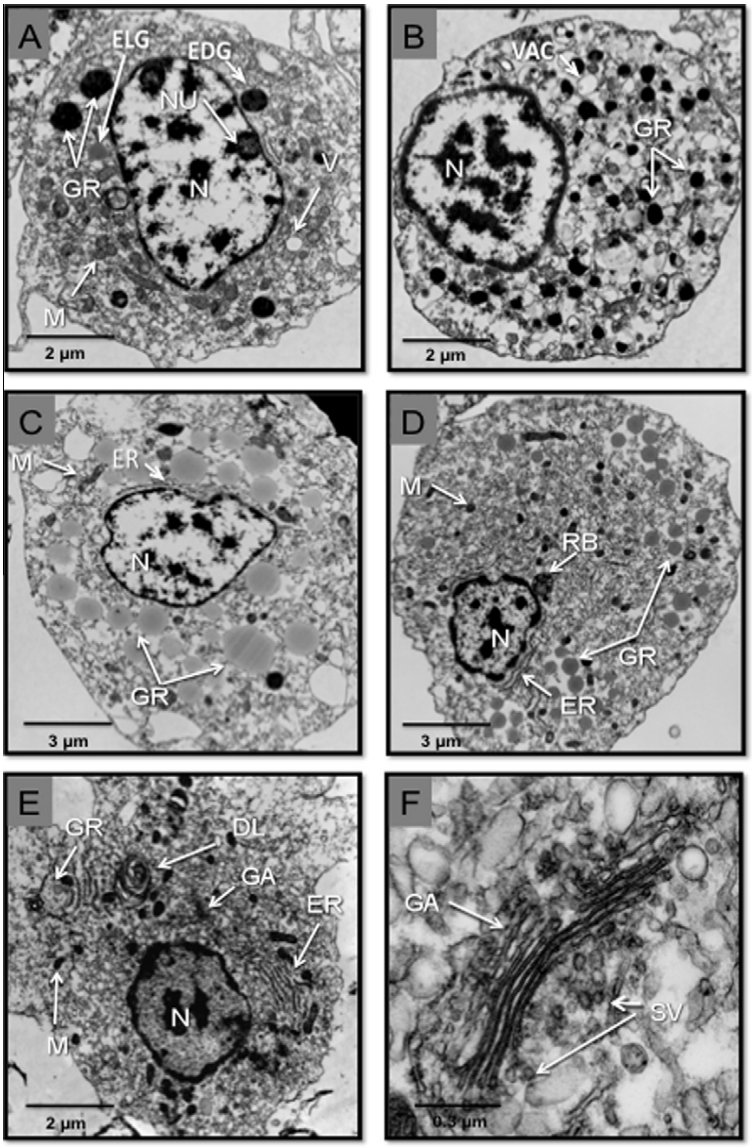

Fig. 7. Transmission electron micrographs of *P. imbricata* granulocytes. (A) Granulocyte with both electron-dense (EDG) and electron-lucent granules (ELG), (B) granulocyte with small EDG's and numerous vacuoles (VAC), (C) granulocyte with ELG's, (D) granulocyte with medium electron density granules, (E) granulocyte with digestive lamellae (DL), and (F) Golgi apparatus (GA) in a granulocyte. N, nucleus; M, mitochondria; ER, endoplasmic reticulum; GR, granule; SV, secretory vesicles; RB, residual body.

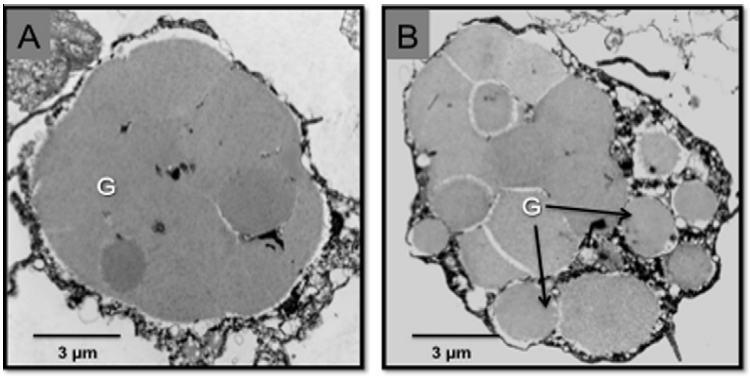

Fig. 8. Transmission electron micrographs of serous cells from *P. imbricata*. (A) Serous cell with a single large segmented granule and (B) serous cell with multiple granules.

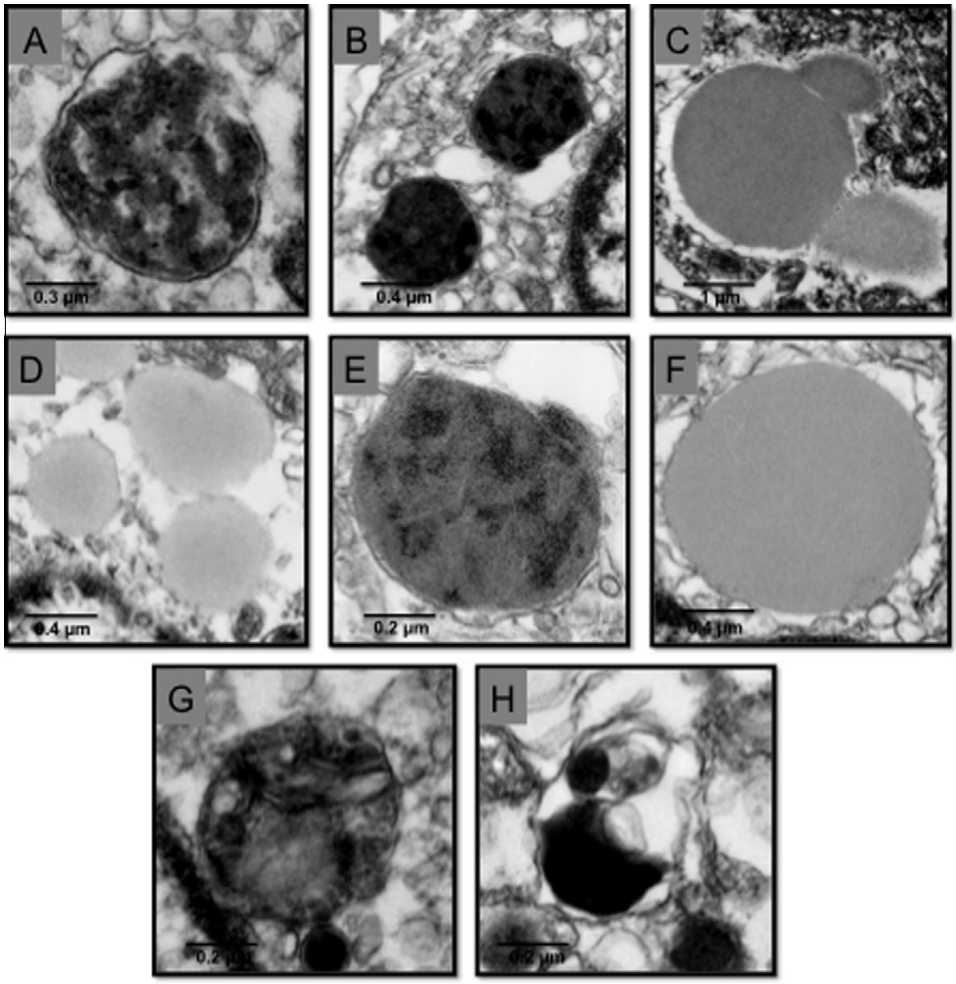

Fig. 9. Transmission electron micrographs of different types of granules (A–H) found in *P. imbricata* haemocytes.

(Moore and Eble, 1977; Aladaileh et al., 2007). However, other studies have demonstrated that certain cell types preferentially phagocytose different target cells. For example, granulocytes in *Crassostrea gigas* engulf more yeast and bacteria than hyalinocytes, while hyalinocytes phagocytose more latex beads (Terahara et al., 2006).

In the current study, the polymerisation of actin in both granulocytes and hyalinocytes was often localised around the perimeter of the cell, where pseudopodia developed. Large granulocytes often appeared to have focal adhesions on the cells surface whilst small granulocytes were observed projecting multiple actin-rich filopodia from a spherical cell body. These filopodia often appeared to have terminal swellings. A similar morphology is reported by Cheng et al. (1979) and Morona and Mingyi (1989), who concluded that the swellings were associated with the exocytosis of lysosomes (Mohandas et al., 1985).

Lysosomes promote the formation of an acidic environment, which alters metabolic processes of ingested microbes (McDade and Tripp, 1967; Cheng, 1993). In our study, lysosomal activity was indicated by a colour change in the pH-sensitive Azo dye, Congo red. In many cases, distinct colour changes from red to blue were detected around the periphery of yeast engulfed by haemocytes for >45 min. This suggests that lysosomal contents had been deposited into the phagosomes of *P. imbricata*. Similarly, some granular contents within both granulocytes and hyalinocytes were found to be acidic in nature, as identified by the accumulation of neutral red.

Ultrastructural studies with TEM also identified granules with morphologies similar to both primary and secondary lysosomes, as well as residual bodies, in granulocytes. These structures were identified as being membrane-delimited with complex granular contents. In other ultrastructural studies, Cheng and Foley (1975) correlated the appearance of digestive lamellae with intracellular digestion through primary and secondary phagosome formation. Concentric structures with similar structure to digestive lamella were observed in granulocytes of *P. imbricata*.

There was also evidence that the granules of haemocytes are well equipped with intracellular antimicrobial killing systems. Superoxide formation and peroxidase activity were evident in small granulocytes and hyalinocytes, with the former detected around the perimeter of large vacuoles. Superoxide generation has also been reported in haemocytes from a number of other bivalve species, including *Mytilus edulis*, *Cerastoderma edule*, and *Ensis siliqua* (Wootton et al., 2003).

PO was also evident in cytoplasmic granules of *P. imbricata*. In other species, PO is known to adhere to the surface of foreign particles forming aggregates (Ling and Yu, 2005). The enzymatic end product of the PO pathway, melanin, retards the growth of microbes (Cerenius and Söderhall, 2004). PO activity has been implicated with intracellular phagolysosomal processes in *S. glomerata* (Aladaileh et al., 2007), *M. edulis* (Renwrantz et al., 1996), *M. galloprovincialis* (Carballal et al., 1997), and *Scapharca inaequivalvis* (Holden et al., 1994). Both PO and melanin were identified in *P. imbricata* haemocytes and were often associated with small granu-

locytes, even though some larger granulocytes and hyalinocytes displayed localised deposits.

There is a limited literature describing serous cells in bivalves (Haigler, 1964). These cells are thought to function in either waste sequestration or as storage compartments. In *Tapes philippinarum* it was hypothesised that serous cells are probably produced in Keber's gland, a pericardial organ that is part of the excretory system (Cheng, 1981; Cima et al., 2000). The globules within serous cells appear to have a complex chemical composition. In 1964, Haigler described their contents to be similar to that of lipofuscin, whilst Cheng and Burton (1966) found them to contain mucoprotein, glycoprotein, glycolipid and acid mucopolysaccharides. In the current study, serous cells were characterised by the presence of large yellow-brown globules of pigment, which often concealed the nucleus (Nakayama et al., 1997). They were also found to be acidic, as reflected by Giemsa/May–Grünwald and neutral red staining. It is unclear whether serous cells perform any direct immunological function in *P. imbricata*, as they did not stain for defensive enzymes or products, such as PO, peroxidase, melanin or superoxide, and did not undertake phagocytosis.

In summary, based on morphological and cytochemical analyses, *P. imbricata* haemolymph was found to be composed of hyalinocytes (large and small), granulocytes and large globular serous cells when oysters were housed in controlled conditions. Haemocyte density, cytochemistry and morphology may vary depending upon environmental condition and associated seasonal fluctuations. This population of haemocytes is generally consistent with that of other bivalve species. The involvement of haemocytes in immunological defence was demonstrated by the expression of defensive enzymes and enzymatic end products. This reactivity implicates granulocytes and to a lesser extent hyalinocytes, with immune responses.

Acknowledgments

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CHAPTER THREE

IMMUNOSUPPRESIVE EFFECTS OF ENVIRONMENTAL STRESSORS ON IMMUNOLOGICAL FUNCTION IN *PINCTADA IMBRICATA*

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Preface

After identifying the immunological function of *P. imbricata* haemocytes, the next aim of this thesis was to investigate the effect of common environmental stressors on defensive haemocyte populations. The Akoya pearl oyster is typically farmed throughout Australia in estuarine conditions at the southernmost extent of the species' range. For this reason, Chapter Three focuses on the effects of hypo-salinity, exposure to air, and mechanical agitation (a typical stressor associated with husbandry and handling) on *P. imbricata* haemocytes. We found that all three stressors significantly inhibited both phenoloxidase activity and phagocytosis. Other parameters such as total haemolymph protein content, total haemocyte counts, acid phosphatase activity and granulocyte abundance were found to be affected by stress. However, these responses varied depending upon the stressor applied.

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Immunosuppressive effects of environmental stressors on immunological function in *Pinctada imbricata*

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ABSTRACT

This study assessed the effects of mechanical agitation, hypo-saline conditions, and exposure to the air on the Akoya pearl oyster, *Pinctada imbricata*, focusing specifically on the immunological activity of haemocytes. Both phagocytosis and phenoloxidase activity decreased significantly when oysters were exposed to all three stressors. Transient decreases were also evident in total haemocyte counts after mechanical stress and exposure to air, while significant increases in total haemocyte counts were evident after exposure to low salinity. Acid phosphatase activity increased significantly when oysters were exposed to air. The frequency of granulocytes in the haemolymph increased significantly when oysters were stressed by hypo-saline conditions, whilst the relative frequency of granulocytes did not differ significantly after mechanical agitation or exposure to air. The total protein content of haemolymph increased significantly when oysters were stressed by mechanical agitation and low salinity. These results suggest that fluctuations in environmental conditions affect circulating haemocytes and their cytochemistry, and that the different immunological parameters tested were influenced uniquely according to the type of stressor.

1. Introduction

The successful cultivation of any marine organism is dependent upon a stable relationship with their environment. Fluctuations in environmental conditions can significantly affect homeostasis and lead to physiological stress [38]. Typical stressors associated with aquaculture include hypo- and hyper-thermia, declines in dissolved oxygen content (hypoxia), mechanical agitation, fluctuations in salinity and exposure to air resulting in desiccation and anoxia [4,7,8,16,17]. Many of these stressors have been shown to compromise immune function [27]. A number of studies have demonstrated that extended periods of stress can lead to immunosuppression, which can result in opportunistic infections [12,15,33].

Until recently, the Akoya pearl oyster formed the basis of a highly lucrative pearling industry in Japan. Between 1993 and 1996, productivity declined significantly from 118 000 kg to 63 000 kg [28]. It was postulated that a combination of factors facilitated the proliferation of disease, including environmental fluctuations and overcrowding [35]. Due to these declines in the Akoya market, Australian companies, in conjunction with Japanese investors, are developing an Akoya pearling industry in Port Stephens, NSW, Australia [28]. In order to develop a successful industry, it is imperative to discern optimal farming conditions, as well as those that may compromise productivity. For this reason, our study will focus on the physiological implications of environmental stress on *Pinctada imbricata*, specifically investigating key immunological parameters.

In marine bivalves, immunological defence is based on innate responses that are mediated by both cellular and humoral factors [32]. Cellular defence includes haemocyte-mediated responses such as coagulation, encapsulation, phagocytosis, phagolysosomal activity and the production of reactive oxygen species (ROS; superoxide anion, hydrogen peroxide and hydroxyl radical [2,6,30].

Granulocytes and hyalinocytes represent the two main types of immunocompetent haemocytes in oysters [34]. Granulocytes are highly defensive haemocytes containing hydrolytic enzymes that assist in the intracellular killing of pathogens and represent the cellular aspect of internal defence [2]. Contrasting to this, hyalinocytes contain few to no cytoplasmic granules and are said to have a "silky" appearance [20,39]. For this reason, granulocytes are

Abbreviations: two-way ANOVA, two-way analyses of variance; DIC, differential interference microscopy; FSW, filtered seawater; MBTH, 3-methyl-2-benzothiazoline hydrozone; NSW DPI, New South Wales Department of Primary Industries; PO, phenoloxidase; PBS, phosphate buffered saline; ROS, reactive oxygen species; SEM, standard error of the mean; TEM, transmission electron microscope; THC, total haemocyte counts; 4-HA, hydroquinine monomethyl ether.

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considered to be more immunologically active than hyalinocytes [2].

Oysters are filter feeders and represent a class of marine invertebrates which are osmo- and thermo-conformers [40]. Although the ocean is relatively stable, coastline and estuarine waters do vary in both temperature and salinity. This dependence on their external environment makes oysters highly vulnerable to these environmental fluctuations, which can result in physiological stress [4,7,8,17]. Recent studies have shown that hypo-saline conditions (25 ± 1 ppt) decrease haemocyte abundance in the mussel *Mytilus galloprovincialis* [26], and inhibit the activity of intracellular phenoloxidase in Sydney rock oysters, *Saccostrea glomerata* (*S. glomerata*; [7]. High water temperatures (23–27 °C) have also been shown to inhibit haemocyte locomotion and adhesion in the eastern oyster *Crassostrea virginica* (*C. virginica*; [18] as well as resulting in a decrease in haemocyte abundance and phagocytic activity in the clam *Ruditapes philippinarum* [3,17,40]).

Fluctuations in ambient water temperature and salinity have not only been shown to compromise immunological activity but they have also been implicated with increased parasite/pathogen loads by creating a more suitable environment for their survival and reproduction [27]. In 1982, Haskin et al. investigated the density gradient of the pathogen *Haplosporidium nelsoni* (MSX disease) in Delaware Bay (USA). They found disease proliferation to be directly correlated with salinity [19]. At low salinities (9–18 ppt) the parasite was found to enter *C. virginica*, but its development was severely retarded [19]. Similar studies by Butt et al. [7] showed that salinity significantly affects the presence and infection rates of *S. glomerata* with *Marteilia sydneyi* (QX disease). Increased infection was correlated with a decrease in the defensive enzyme phenoloxidase, associated with a decline in salinity [7].

The current study examines the effects of low salinity, mechanical agitation and exposure to air on the haemocytes of the Akoya pearl oyster, *P. imbricata*. These stressors were selected as typical perturbations experienced by pearl oysters farmed for aquaculture.

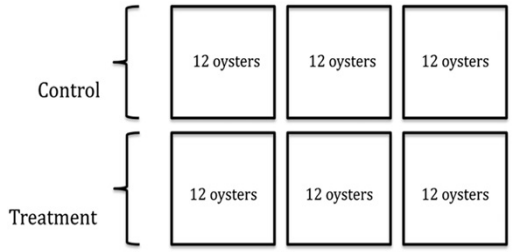
2. Materials and methods

2.1. Animals

Adult *P. imbricata* (50–80 mm in dorso-ventral height) were supplied by Port Stephens Fisheries Center, New South Wales (32°44′S, 152°08′E). Oysters were housed at the Sydney Institute of Marine Science (SIMS) and acclimatised for at least 14 days in temperature-controlled (22 °C, 45 L) flow through aquaria. The oysters were fed every two days with M-1 diet (Aquasonic Pty Ltd, Wauchope, NSW).

2.2. Experimental design

Three different types of environmental stressors were tested; low salinity (25 ppt), mechanical agitation (10 min, 300 rpm) and exposure to air (2 h periods every 24 h). To test the effects of altered salinity, oysters were transferred to closed aquaria containing seawater from Sydney harbour that had been diluted to 25 ppt with Milli-Q water for the duration of the experiment. Salinity was measured using a TPS WP-81 water quality meter (Enviroequip, Brisbane, Australia). Hypo-saline conditions chosen for exposure were based upon fluctuations recorded in the Port Stephens region (Wayne O'Connor pers. com.). Mechanical stress was simulated by placing oysters on a MS1 Minishaker (Crown, Scientific, NSW, Australia) and agitating them for 10 min at 300 rpm every 24 h. Once the oysters had undergone mechanical agitation they were returned to the tanks. In order to test air exposure treatment oysters were removed from the aquaria and placed on the lab bench every 24 h for 2 h. After the oysters were stressed, they were returned to the tanks. A total of 6 tanks housed 12 oysters each, three control tanks (36 oysters in total) and 3 treatment tanks (36 oysters in total; see diagram below). For each time point (0, 24, 72 & 120 h) three oysters were randomly selected from each of the three tanks corresponding to the treatment, totalling 9 oysters per time point for both control and for treatment. Partial water changes (50% or 25 L) were performed on closed tanks every 24 h. This experimental design was replicated for each stressor tested.



2.3. Sampling

Groups of 9 oysters ($N = 9$) from treatment and control tanks were removed from the aquaria 0, 24, 72 and 120 h after the beginning of each experiment (totaling 72 oysters). They were placed on absorbent towel for 10 min prior to haemolymph extraction to drain excess water from their mantle cavities. Haemolymph was extracted from each oyster by rupturing the pericardial cavity located near the adductor muscle using a 27-gauge needle fitted to a 1-mL syringe. Whole haemolymph was immediately transferred to 1.5 mL microcentrifuge tubes and held on ice before being stored at −80 °C. Haemolymph samples used to examine phagocytosis were overlaid onto acid–alcohol washed slides with adherent yeast. Cells collected for total and differential haemocyte counts were fixed in 4% paraformaldehyde.

2.4. Differential haemocyte counts

In order to differentiate between *P. imbricata* blood cells, haemocytes were stained with the Romanowsky stain, Giemsa/May-Grünwald [2]. Thirty microlitres of whole haemolymph was spotted onto acid–alcohol cleaned slides. Cells were allowed to adhere for 25 min in a humid chamber. After adhesion, samples were fixed for 20 min with paraformaldehyde (4% w/v in FSW; Sigma–Aldrich, Castle Hill, NSW). The slides were then immersed in May-Grünwald stain for 6 min. The cells were counterstained with Giemsa for a further 30 min and subsequently washed in Phosphate Buffered Saline (PBS; 136 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, adjusted to pH 7.5). Slides were air-dried and mounted with Ultramount No. 7 (Fronine Laboratory Supplies, Taren Point, NSW) and examined using Olympus BH-2 microscope. One hundred haemocytes were counted per oyster and classified as either granulocytes or hyalinocytes by the scheme of Cheng [10,11]. Although both hyalinocyte and granulocyte abundance were assessed, we have only presented data relating to granulocyte abundance as they play a more significant role in host defence [20].

2.5. Phagocytosis assay

Congo-red-stained yeast (*Saccharomyces cerevisiae*; Sigma–Aldrich), were used as target cells to test the phagocytic capacity of haemocytes. Two hundred and 50 mg of yeast in 5 ml FSW were mixed with 5 ml 0.8% Congo red prepared in FSW. The yeast were then autoclaved at 90 °C for 20 min. The suspensions were

centrifuged and washed twice at in FSW 3000×*g*, 4 °C, for 5 min. The final pellet was re-suspended in 10 mL FSW and stored at −20 °C.

Fifty microlitres of yeast (diluted to 5.5×10^6 mL^{-1} in FSW) were dotted onto poly-L-lysine-coated coverslips at room temperature (25 °C) in a moist chamber and allowed to settle for 10 min. The supernatant was extracted and 20 μL of whole haemolymph was pippetted onto the yeast and allowed to stand for a further 20 min. The slides were then viewed at high magnification (60× objective, oil immersion) with an Olympus BH-2 light microscope. The number of yeast ingested per haemocyte for one hundred haemocytes was recorded. The phagocytic index (PI) was calculated as the average number of yeast engulfed per haemocyte.

$$\text{PI} = \text{total number of yeast engulfed}/100$$

2.6. Total haemocyte counts

Fifty microlitres of whole haemolymph was fixed with 50 μL paraformaldehyde (4% w/v in filtered seawater; FSW). Ten microlitre aliquots of fixed haemocytes were pipetted onto an Improved Neubauer haemocytometer (Sigma–Aldrich, Castle Hill, NSW) for examination with a 60× objective (oil immersion). Cells were then counted to estimate the number of cells per mL.

2.7. Total protein content of haemolymph

Total protein content was determined using a Bio-Rad Bradford protein assay kit. Haemocyte lysates were prepared by freeze–thawing whole haemolymph at −80 °C. After thawing the samples, they were then centrifuged at 12000 rpm for 5 min. The supernatant was then extracted and the total protein concentrations of 50 μL aliquots were measured in triplicate per sample. Protein content was interpolated from a standard curve generated with bovine serum albumin (BSA; Bio-Rad).

2.8. Acid phosphatase assays

Acid phosphatase activity was measured using p-nitrophenylphosphate (pNPP; Sigma–Aldrich) as a chromogenic substrate (adapted from [7]. Eighty microlitres of haemolymph lysates were mixed with 120 μL of pNPP substrate (2.5 mg/mL in Milli-Q) in triplicate in the wells of a 96-well microtitre plate (3 replicate wells per sample). The plates were incubated for 1 h at 25 °C. Fifty microlites of NaOH (0.05 M) was added to each well before absorbance was measured at 405 nm. Samples were blanked against wells containing filtered seawater samples. The final concentrations of acid phosphatase were calculated from a standard curve generated using a pNPP standard [7].

2.9. PO assays

Whole haemolymph was freeze-thawed once (−80 °C/room temperature) to rupture haemocytes. The haemolymph was then centrifuged at 600×*g* at 4 °C for 10 min to remove cellular debris. Phenoloxidase (PO) assays were performed in 96-well flat-bottomed microtiter plates according to Peters and Raftos [29] (Sarstadt, Technology Park, SA). One hundred microlitres of lysate were added per well in triplicates for each sample followed by the addition of hydroquinine monomethyl ether (5 mM in FSW; Sigma–Aldrich, Castle Hill, Australia) containing 1 mM 3-methyl-2-benzothiazoline hydrazone (Sigma–Aldrich). The absorbance was measured at 490 nm immediately after the addition of the substrates using a Bio-Rad model 550-microplate spectrophotometer (Bio-Rad, Regents Park, NSW, Australia). A second reading was taken after 1 h of incubation at room temperature. Phenoloxidase activity was calculated by subtracting the initial reading (0 h) from the measurements made at 60 min. Specific enzyme activities are expressed as the change in optical density at 490 nm per microgram of protein.

$$\text{PO} = (\text{optical density at 60 min} - \text{optical density at 0 h})/\text{Protein per } \mu g$$

2.10. Numerical/statistical analyses

Fold difference is the comparison between control and treatment values. The values calculated represent a ratio relative to the control, where a value of 100% indicates that there is no difference between treatments and control. Fold difference was calculated for all treatments as follows:

$$\text{Fold difference \%} = ((\text{stress} - \text{control})/\text{control standard deviation}) \times 100$$

Statistical analyses were performed on normalized data. Two-way analysis of variance (ANOVA) followed by Post-Hoc multiple comparisons using the statistical program SPSS ver. 14.0 (SPSS Inc. Il, USA, 2006) were used to analyze the difference between the control and treatment. Individual students *t*-tests were then carried out to discern significant differences between individual time points. Results were considered significant when $P < 0.05$.

3. Results

3.1. Differential haemocyte counts

3.1.1. Differential haemocyte counts; between treatments (ANOVA)

Granulocyte numbers changed over time when exposed to low salinity ($F = 2.83$, $df = 1$, $p = 0.010$). Granulocyte abundance did not differ significantly from control values in either mechanically stressed or air-exposed oysters.

3.1.2. Differential haemocyte counts; between time points (T-tests)

After 24 h, the relative frequency of granulocytes in low salinity stressed oysters was 18% higher than those in control oysters (Fig. 1A; *T*-value = −4.43, $df = 12$, $p = 0.001$), whilst the overall frequency in mechanically agitated oysters decreased but not significantly. Granulocyte numbers in air-exposed oysters did not vary from control values between 24 and 72 h. At 72 h granulocyte numbers remained elevated in low saline exposed oysters and increased from values at 24 h in mechanically stressed animals. At 120 h granulocyte counts for low saline stressed oysters returned to near pre-treatment counts, whilst air-exposed granulocyte counts increased, but not significantly. Granulocyte abundance decreased by 24.1% in mechanically stressed oysters at 120 h.

3.2. Phagocytosis

3.2.1. Phagocytosis; between treatments (ANOVA)

Phagocytosis was significantly inhibited by all three stressors when compared to control values (air exposure: $F = 21.91$, $df = 1$, $p = 0.000$; mechanical stress: $F = 8.01$, $df = 1$, $p = 0.006$; low salinity: $F = 25.75$ $df = 1$, $p = 0.000$).

3.2.2. Phagocytosis; between time points (T-tests)

Phagocytosis declined most significantly for all three stressors at 24 h (Fig. 1B; air exposure: *T*-value = 4.79, $df = 10$, $p = 0.001$; mechanical stress: *T*-value = 3.45, $df = 16$, $p = 0.003$; low salinity: *T*-value = 4.27 $df = 12$, $p = 0.001$). At 72 h phagocytic activity

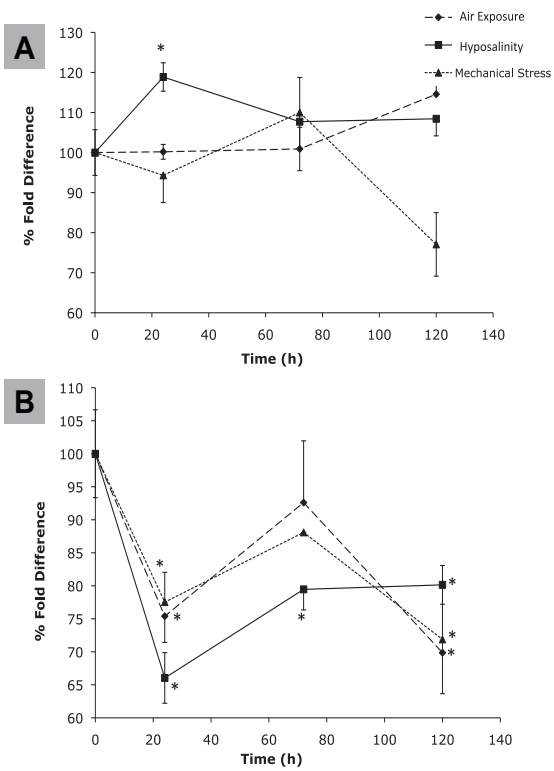


Fig. 1. Fold difference is the ratio between treatment and control values. The graphed values represent how the treatment varies against the control. (A) fold difference in granulocyte abundance (B) fold difference in phagocytosis. * denotes a significant difference between treatment and control values for that time point (p-value ≤ 0.05). Error bars represent standard error of the mean.

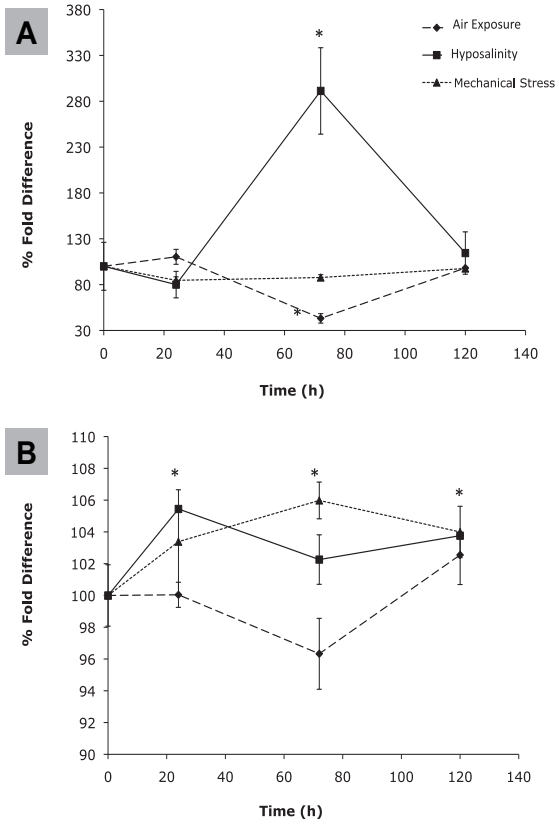


Fig. 2. Fold difference is the ratio between treatment and control values. The graphed values represent how the treatment varies against the control. (A) fold difference in total haemocyte counts. (B) fold difference in total protein concentration of whole haemolymph from stressed oysters. * denotes a significant difference between treatment and control values for that time point (p-value ≤ 0.05). Error bars represent standard error of the mean.

recovered near to control levels for both air exposure and mechanical stress, however, phagocytosis in low saline stressed oysters was still significantly inhibited (T-value = 2.72, df = 12, p = 0.019). Phagocytosis declined again in all three stressors at 120 h (air exposure: T-value = 5.74, df = 10, p = 0.000; mechanical stress: T-value = 2.19, df = 16, p = 0.044; low salinity: T-value = 4.12 df = 12, p = 0.001).

3.3. Total haemocyte counts

3.3.1. Total haemocyte counts; between treatments (ANOVA)

There was no significant difference in total haemocyte counts (THC) between control and treatment values for air exposure, low salinity and mechanical stress.

3.3.2. Total haemocyte counts; between time points (T-tests)

After 72 h, THC in mechanically stressed oysters declined 13%, whilst THC in air-exposed animals decreased by more than half (Fig. 2A). In oysters stressed by low salinity, THC's declined within 24 h, however, at 72 h the THC was 3-fold greater (T-value = −2.99, df = 16, $p < 0.009$). Total haemocyte counts for all three treatments returned to control levels within 120 h.

3.4. Total protein

3.4.1. Total protein; between treatments (ANOVA)

Mechanical stress and low salinity resulted a significant increase in total protein (mechanical stress: F = 6.01, df = 1, p = 0.017; low salinity: F = 4.29, df = 1, p = 0.043). Total protein did not vary significantly in oysters stressed by air exposure.

3.4.2. Total protein; between time points (T-tests)

At 24 h, total protein concentration increased by 6% in low saline stressed oysters, 3% in mechanically stressed oysters and 1% in air-exposed oysters (Fig. 2B). At 72 h protein levels decreased in oysters stressed by air exposure and low salinity in comparison to values at 24 h. Contrasting to this, mechanical agitation led to significant increases in total protein (T-value = −2.50, df = 14, p = 0.025). Protein concentrations returned to near control levels for both air exposure and mechanical agitation at 120 h, whilst it remained significantly elevated in low saline conditions (low salinity: T-value = −2.50, df = 13, p = 0.026).

3.5. Acid phosphatase activity

3.5.1. Acid phosphatase activity; between treatments (ANOVA)

Acid phosphatase activity did not differ significantly when oysters exposed to low salinity or mechanical agitation. Contrasting to this acid phosphatase activity increased significantly in oysters stressed by air exposure (F = 16.75, df = 1, p = 0.000).

3.5.2. Acid phosphatase activity; between time points (T-tests)

At 24 h acid phosphatase activity decreased 11% in low saline conditions, in comparison to air-exposed oysters which increased 7% (Fig. 3A; low salinity: T-value = 2.96, df = 12, p = 0.012; air exposure T-value = −3.75, df = 12, p = 0.003). At 72 h mechanically agitated and low saline stressed haemocytes increased in acid phosphatase activity, whilst air-exposed haemocytes decreased. At 120 h, acid phosphatase activity decreased significantly in low

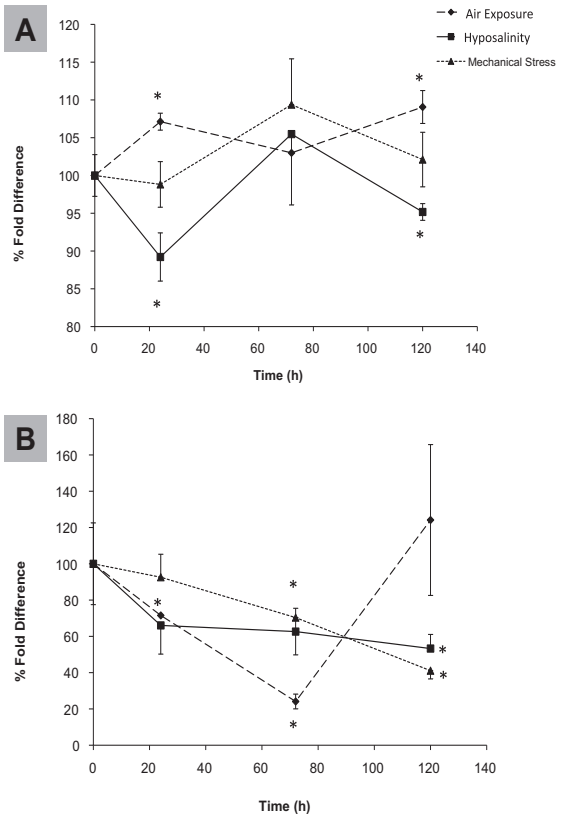


Fig. 3. Fold difference is the ratio between treatment and control values. The graphed values represent how the treatment varies against the control. (A) fold difference in acid phosphatase activity (specific activity) in haemolymph from stressed oysters (B) fold difference in PO activity (specific activity). * denotes a significant difference between treatment and control values for that time point (p-value ≤ 0.05). Error bars represent standard error of the mean.

salinity stressed oysters, whilst it increased significantly in air-exposed oysters (low salinity: T-value = 2.53, $df = 12$, $p = 0.026$; air exposure: T-value = −3.06, $df = 12$, $p = 0.010$). Acid phosphatase activity returned to near control levels at 120 h in mechanically stressed oysters.

3.6. PO activity

3.6.1. PO activity; between treatments (ANOVA)

PO activity decreased significantly in all three treatments (mechanical stress: $F = 5.09$, $df = 1$, $p = 0.028$; low salinity: $F = 11.32$, $df = 1$, $p = 0.002$; air exposure: $F = 8.63$, $df = 1$, $p = 0.005$).

3.6.2. PO activity; between time points (T-tests)

After 24 h PO activity declined for all three stressors, however, exposure to air resulted in significant decreases (Fig. 3B; T-value = 2.30, $df = 10$, $p = 0.044$). At 72 h PO activity in air-exposed oysters had decreased by 76%, whilst PO activity in low salinity stressed oysters declined by 37% and 29% in mechanical agitation. At 120 h PO levels recovered in air-exposed oysters to above pre-treatment conditions, however, low salinity and mechanical agitation remained significantly reduced (low salinity: T-value = 2.84, $df = 10$, $p = 0.018$; mechanical agitation: T-value = 2.31, $df = 14$, $p = 0.037$).

4. Discussion

Through the processes of aquaculture, *P. imbricata* experience repeated perturbations and prolonged periods of stress [35]. The parameters tested in this study represent key immunological responses in bivalve mollusc defence [1]. These data indicate that different environmental stressors have variable effects on the immunological parameters tested. Slight variations in immunological parameters were recorded in control animals. This is inherent of heterogeneous wild-type populations.

Fisher and Newell [16] investigated the effect of salinity (6–30 ppt) on the activity of granular haemocytes in *C. virginica* over a 3–4 week period. These studies identified a decline in the number of circulating granulocytes in low saline conditions. In contrast, Reid et al. [33] found when stressing *R. philippinarum* in low saline conditions (20 ppt), there was a significant increase in granular cells. Similar trends were identified in the current study, with a significant increase in the presence of granulocytes when exposed to low saline conditions. These results vary from those oysters stressed by mechanical agitation. Between 0 and 72 h there was no significant difference between control and stressed oyster populations, although, there was a notable, but not significant decline at 120 h. The physiological effects of this stressor may be cumulative and increase in potency over time. Granulocyte populations did not appear to be affected by air exposure. Malham et al. [25] suggested that fluctuations in haemocyte populations maybe the result of a prioritised redirection of bioenergetic resources toward adaptive physiological functions, i.e. oxygen uptake, glycolysis, lipolysis and respiration.

Phagocytosis represents an important mechanism of host defence [5]. Clearance efficiency is heavily dependent on the ability of haemocytes to recognise, bind to and engulf microbes [4,13,41]. In conjunction with fluctuations in differential and total haemocyte counts, all stressors investigated we found to significantly affect the phagocytic clearance activities of *P. imbricata* haemocytes. The results of the current study suggest that the decline in phagocytosis was not transient. These significant declines may be implicated in the proliferation of disease especially across monocultures such as those in aquaculture. A reduction in phagocytic activity and clearance efficiency may be caused by osmotic effects or increased infiltration of haemocytes into connective tissue and organs [8,9]. In similar studies mechanical stress was found to decrease phagocytic activity of the abalone *Haliotis tuberculata* (*H. tuberculata*; [25], whilst decreases in phagocytic clearance were recorded in low saline conditions (16 ppt) in *Mytilus edulis* [8].

The effects of physiological stress on the phagolysosomal system in *P. imbricata* do not appear to be consistent between stressors. Acid phosphatase is a typical lysosomal enzyme found in the phagocytes of bivalve molluscs. In a recent study, Aladaileh et al. [1] injected *S. glomerata* with the stress hormone noradrenaline. The release of noradrenaline has previously been found to be associated with stressful events such as mechanical agitation [2,22]. These authors showed that noradrenaline injections inhibit acid phosphatase activity. They proposed that a decline in the number of acid phosphatase positive cells maybe related to stress induced apoptosis, a change in lysosomal integrity or enhancement of cellular lysis [4,14,23]. Results from the current study vary from these findings, as acid phosphatase activity increased significantly when haemocytes were stressed by air exposure. Mechanical stress increased acid phosphatase activity, whilst exposure to low saline conditions showed acid phosphatase activity to fluctuate over time.

It has been demonstrated that environmental stressors can drastically alter immunocompetance, which may be implicated in disease persistence and proliferation [21,24]. Reid et al., [33] performed controlled infection experiments with *Vibrio tapetis*, the brown ring disease, in Manila clams. They found that when *Tapes philippinarium* was exposed to stressful conditions, such as low salinity (20 ppt), infection intensity was at its greatest [33]. During

these periods of stress they also found THC to decline significantly [33]. In the present study, THC were found to decline within the first 24 to 72 h after exposure to mechanical stress and within 24 h of low saline conditions. These results also complement the findings of Malham et al. [24], who identified a significant decrease in circulating haemocytes in mechanically agitated abalone, *H. tuberculata*. It has been proposed that during stressful events circulating haemocytes may migrate from the blood stream to the surrounding tissue, that may be prone to injury or infection [24]. Alternatively, haemocytes may respond to stressors by providing nutrients and resources to certain tissues involved in adaptation to stress and survival [24]. Total haemocyte counts increased significantly at 72 h when exposed to low salinity, before returning to control values at 120 h. Exposure of *P. imbricata* to air resulted in a slight increase of THC before a significant decline at 72 h. As the oysters were taken out of the water for 2 h a day over a five-day period, this trend may represent a cumulative response.

Short-term perturbations in environmental stressors elicited variable responses in protein content. Protein concentration increased significantly for both mechanical agitation and low salinity. These results differ from those of Fisher and Newell [16], who found no significant change in haemolymph protein after exposing *C. virginica* to saline conditions ranging from 6 to 30 ppt for one month. Similarly no significant increase or decrease in total protein was found in the shrimp *Penaeus californiensis* cultivated under low saline conditions [36]. In our study, exposure to air resulted in no significant difference in protein between either control or treatment values.

In 2006, Butt et al. demonstrated that the proliferation of the QX disease (*M. sydneyi*) in *S. glomerata* was influenced in part, by altered environmental conditions, which most notably affected the expression of PO. In the current study, one of the most profound effects of all environmental stresses tested was the significant decline in PO activity. Similar studies have also found proPO activity to decline with lowered salinity in aquatic species such as the yellow shrimp *Farfantepenaus californiensis*, the white shrimp *litopenaeus vannmei* and the tiger shrimp *Penaus monodon* [37].

In aquaculture, oysters can experience multiple stressors simultaneously including mechanical agitation associated with general handling, sorting or antifouling as well as long periods of transport (exposure to air; [26,33]. Recent climate change projections predict potential decreases in ambient salinity with the imminent threat of polar ice caps melting and increased magnitude of flood events, effecting estuarine and coastal regions [31]. Immunological responses of circulating haemocyte populations in controlled stress conditions can provide important information about the 'health' of an animal, which is easily assed in the short-term.

In conclusion, this research demonstrates that even moderate periods of stress (when exposed to reduced salinity, mechanical agitation, or exposure to air) induce quantifiable alterations in the immune response of *P. imbricata*. In general, these responses varied depending upon the stressor. Hyposalinity, similar to that which often occurs during periods of prolonged rainfall, greatly affected the cytochemistry and functional responses of haemocytes. Moreover, the negative effects of mechanical agitation and exposure to air, frequently experienced during processing and handling was also confirmed. Future research will focus on the specific effects of stress related hormone release including dopamine and noradrenaline and their impact on immunological cellular responses.

Acknowledgements

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CHAPTER FOUR

IN VITRO EFFECTS OF NORADRENALINE ON AKOYA PEARL OYSTER (*PINCTADA IMBRICATA*) HAEMOCYTES

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Author contributions:

David A. Raftos – Project supervision

Preface

Previous studies investigating the effects of environmental perturbations on invertebrate immune systems have identified increases in the production of stress hormones, namely dopamine and noradrenaline (NA). In addition to these increases, NA has also been shown to induce apoptosis among defensive haemocyte populations. These apoptotic events have been linked to immunosuppression. In the previous Chapter, I showed that environmental stress negatively affects key immunological responses, including phagocytosis and phenoloxidase activity. The aim of the current Chapter was to investigate the effects of NA on defensive haemocytes in *P. imbricata*. Using Terminal dUTP nick-end labelling and Annexin V-FITC staining I showed that there was a significant increase in DNA fragmentation and decrease in membrane stability in haemocytes exposed to NA. The data also demonstrates that NA significantly alters cell structure and morphology as shown by the rearrangement of F-actin (stained with phalliodin-Alexa Fluor 488) and scanning electron microscopy. This change in cytoskeletal morphology could be one explanation for the decline in phagocytosis that occurs after stress, while the induction of apoptosis and subsequent declines in defensive haemocytes may explain the observed decreases in PO activity among stressed oysters. In this way, the cellular responses to NA described here may help to explain the observations made in the previous Chapter.

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In vitro effects of noradrenaline on Akoya pearl oyster (*Pinctada imbricata*) haemocytes

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ABSTRACT

Exposure to fluctuating environmental conditions in bivalve molluscs can lead to physiological stress and up-regulated production of stress-associated hormones, such as noradenaline (NA). Since environmental stressors have been found to have an immunosuppressive effect on *Pinctada imbricata*, we investigated the *in vitro* affects of NA exposure on their defensive haemocytes, focussing specifically on markers of apoptosis. Terminal dUTP nick-end (TUNEL) labelling was used to detect cells displaying DNA fragmentation within tissue exposed to NA. DNA fragmentation was most significant when haemocytes were exposed to 10.0 ng NA/μg protein relative to non-treated controls. Similarly, Annexin V-FITC staining, a marker of early apoptotic events, was evident in cells exposed to 5.0 and 10.0 ng NA/μg protein after 120 min ($p < 0.05$), and haemocyte adhesion to glass slides declined significantly when cells were exposed to 10.0 ng NA/μg protein ($p < 0.05$). A number of morphological and ultrastructural changes in NA-exposed haemocytes were also identified using transmission and scanning electron microscopy. These alterations included chromatin and cytoplasmic condensation, the formation of apoptotic bodies, vacuolisation and blebbing. In NA-treated cells, polymerisation of F-actin was observed around the periphery of the cytoplasm. All of these data suggest that NA induces apoptosis in *P. imbricata* haemocytes.

1. Introduction

Pinctada imbricata, commonly known as the Akoya pearl oyster, is a tropical to semi-temperate epibenthic species that occurs throughout the Indo-Pacific, North America, the Mediterranean, and the Eastern coastlines of Africa and Australia [1,2]. This species is commercially farmed for the production of pearls, predominantly in Japan and China. *P. imbricata* is also being trialled for its commercial viability as a temperate pearling species in eastern Australia. Over the past two decades, Japanese pearl production has declined significantly, from 118 000 kg in 1993, to 63 000 kg in 1996 [2]. This decline in productivity has been linked with the proliferation of diseases that are commonly associated with the overpopulation of monocultures and a range of environmental perturbations [2].

Acute environmental stressors that may affect *P. imbricata*, which are usually cultivated in shallow subtidal zones, include variations in temperature and food availability due to seasonal changes, fluctuations in salinity and dissolved oxygen content, mechanical agitation and exposure to air resulting from tidal oscillations, wave action and farming practices [3]. These environmental stressors have been shown to affect a range of immunological responses in marine bivalves [3–6].

Bivalve immune systems rely upon pattern recognition, in which host pattern recognition receptors detect pathogen associated molecular patterns (PAMPS; 7–9). The recognition of PAMPS in bivalves initiates both humoral and cellular defences [10,11]. Cellular defences are based on haemocyte-mediated responses, including nodule formation, encapsulation, phagocytosis and the production of reactive oxygen species (ROS; superoxide and peroxide; 4,10–13). Humoral responses include the synthesis and secretion of extracellular effector proteins and antimicrobial enzymes, such as lysozyme, and antimicrobial peptides including defensins [10,14].

The immunological parameters that are known to be affected by environmental stressors include the frequency of defensive haemocytes in haemolymph, the production of ROS, phenoloxidase (PO) activity, and phagocytosis [4,6,15]. In the Sydney rock oyster, *Saccostrea glomerata,* PO activity has been shown to be inhibited by low salinity in both laboratory and field trials [16]. In hypo-saline environments, *S. glomerata* were also found to be more susceptible to QX disease, which is caused by the protozoan parasite, *Marteilia sydneyi*. Butt et al. (2006) concluded that the stress induced by low salinity

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causes PO activity to decline, increasing the likelihood of opportunistic infection by *M. sydneyi*. Similar immunosuppression and subsequent increases in disease susceptibility have been demonstrated in other marine bivalves, including *Mytilus edulis* [17] and the oysters, *Crassostrea virginica* [18,19] and *Crassostrea. gigas* [15].

Other studies have this linked stress-associated immuosuppression in bivalves with neuroendocrine responses involving the release of catecholamines (CA), such as noradrenaline (NA) and dopamine (DO; 5,15,20). These endocrine stress responses are mediated by corticotrophin-releasing hormone (CRH) and adrenocorticotropin hormone (ACTH; 5,15). Catecholamines play an essential role in physiological processes within molluscs including the regulation of cillary activity, feeding regimes, locomotion, reproduction and respiration [15]. Exposure to stress and associated increases in neuroendocrine secretions are thought to divert energy resources away from physiological functions such as growth, reproduction and some immunological processes [21]. This response may be deleterious in situations where opportunistic pathogens are present. Recent studies by Lacoste et al. (2002) and Aladaileh et al. (2008) have linked the secretion of CA's with an increased occurrence of apoptotic events in defensive haemocytes from *C. gigas* and *S. glomerata* respectively. Given the key role of haemocytes in immunocompetence, this CA induced apoptosis may be implicated with immunosuppression and disease susceptibility [22].

Apoptosis is an orchestrated series of molecular events commonly associated with morphogenesis, maintenance and the developmental deletion of cells [23]. There are two interconnected pathways that lead to apoptosis, one involving mitochondria (intrinsic) and the other activated by death ligand receptors (extrinsic; 23–26). During apoptosis, cells undergo a series of biochemical and structural changes [23]. A cascade of intracellular events leads to the cleavage activation of caspase-3 resulting in plasma membrane blebbing, combined with the extension of echinoid protrusions, membrane blistering and eventually cell lysis [23].

The current study examines the effects of NA on defensive haemocytes of the Akoya pearl oyster. We investigated the potential relationship between NA and impaired immune function in *P. imbricata* by focusing on hormone-induced apoptosis among defensive haemocytes.

2. Materials and methods

2.1. Oysters

Adult *P. imbricata* (50–80 mm in dorso-ventral height) were sourced from oyster farms in Brisbane Waters, New South Wales (33°27′S 151°14′E). The oysters were housed in aerated aquaria (40 L) containing recirculating seawater at 25 °C. They were left to acclimatize for two weeks prior to experimentation and were fed with Nosan M1 diet every 3 days (Aquasonic Pty Ltd, Wauchope, NSW).

2.2. Haemocyte and tissue sampling

Oysters were removed from aquaria and were placed on absorbent towels to drain excess water from their mantle cavities for 2 min prior to haemolymph extraction. Samples of gill tissue (5 mm^2) were then dissected from the oysters by severing the adductor muscle to expose the flesh. After shucking, haemocytes were withdrawn from the pericardial cavity and the sinus near the adductor muscle using a 27-guage needle fitted to a 1-mL syringe.

2.3. In vitro noradrenaline treatments

Based on concentrations of NA found in a range of molluscs, haemocytes were exposed to 0 (control), 2.5, 5.0 and 10.0 ng of NA per μg of protein [5,20,27–30]. The average protein content of whole haemolymph was determined using a Bradford assay (Bio-Rad protein determination kit). One hundred μL of whole haemolymph were incubated with NA for 0, 30, 60, 90, and 120 min in culture media (20% M199, 10% foetal calf serum, and 70% FSW) before being analysed for Annexin V and phalliodin-Alexa Fluor 488 staining, and DIC microscopy. Each treatment was replicated seven times; with each replicate representing a single oyster. For transmission and scanning electron microscopy 1-mL of whole haemolymph were incubated with NA for 0, 30 and 60 min. Each treatment was replicated three times. Gill tissues explants were exposed to NA for 3 h or 10 h before being analysed for DNA fragmentation. Each of these treatments were replicated six times.

2.4. DNA fragmentation (TUNEL) analysis

Gill tissue explants were embedded in paraffin wax. Thin sections were then cut using an 820 Spencer microtome (American Optical Corporation) and adhered to acid alcohol washed slides. Late stage apoptosis was assessed using a FragEL DNA Fragmentation (TUNEL) kit (Calbiochem, San Diego, CA). Following the manufactures' instructions, thin tissue sections were deparaffinised and rehydrated in 100% Histochoice (Amresco, Ohio, USA), followed by a graded ethanol series, from 100% v/v to 70% v/v. The tissue was then permeabilised using 0.2 mg/mL proteinase K in 10 mM Tris (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 8). Specimens were incubated at room temperature for 20 min, and then rinsed with 1 × Tris buffered saline (TBS). Endogenous peroxidases were inactivated by covering the tissue with 3% H_2O_2 for 5 min. After incubation, the slides were rinsed with TBS and overlaid with 1 × Klenow equilibration buffer, and left for 20 min. The buffer was then drawn from the slides using absorbent paper, before the tissue sections were overlaid with Klenow labelling mixture and incubated in a humid chamber at 37 °C for 1.5 h. The labelling reaction was terminated using the stop buffer provided. The specimens were then immersed in blocking buffer and incubated at room temperature for 10 min. The buffer was aspirated from the slides and 100 μL of 1 × streptavidin-horseradish peroxidase (HRP) conjugate was then overlaid onto the specimens for 30 min. After rinsing with 1 × TBS, 100 μL aliquots of 3, 3′-Diaminobenzidine (DAB) solution were overlaid onto the tissue sections and incubated for 10–15 min. The slides were then counterstained with methyl green, and mounted with Histomount. DNA fragmentation was identified as a black/brown colouration in the nucleus. Ten random fields of view (FOV) were counted per replicate. The percentage of DNA fragmentation was calculated per FOV and the over all average was calculated for the 10 FOVs.

2.5. Annexin V-FITC staining

To assess the early stages of apoptosis haemocytes were stained with anticoagulant Annexin V conjugated to fluorescein isothiocyanate (FITC). Haemocytes were washed with Annexin V binding buffer (0.1 M Hepes, 1.4 M NaCl, 25 mM $CaCl_2$, pH 7.4) before being stained with 150 μL of Annexin V-FITC (1 μg/mL; BD Pharmingen, North Ryde, Australia) and incubated in the dark for 15 min. The cells were then counterstained with 50 mg mL propidium iodide. The slides were viewed using an Olympus Fluoview 300 florescence confocal microscope. Annexin V positive cells fluoresced yellow/green. Ten random fields of view (FOV) were counted. The percentage of Annexin V-positive cells was calculated per FOV and the over all average was calculated for the 10 FOVs.

2.6. Intracellular F-actin staining

To evaluate the effects of NA on F-actin formation, haemocytes were stained with the F-actin selective stain, phalloidin-Alexa Fluor

488 (Molecular Probes). Propidium iodide (Molecular Probes) was used to counter-stain the nuclei. Cell membranes were permeabilized by overlaying the slides with phosphate buffered saline (PBS) containing 0.1% Triton X-100 (Sigma Aldrich) for 2 min at room temperature. The slides were then rinsed with PBS and overlaid with 20 nM phalloidin-Alexa Fluor 488 in a humid chamber omitting all light for 20 min. The slides were then rinsed twice with PBS and counterstained with 70 μL propdium iodide (50 mg mL) for 2 min. After counter-staining, the haemocytes were analysed using an Olympus Fluoview 300 confocal laser-scanning microscope. F-actin was detected by green fluorescence and the counterstained nuclei by red fluorescence.

2.7. Transmission electron microscopy (TEM)

Haemocyte samples were fixed for 20 min in 4% paraformaldehyde, 2.5% glutaraldehyde and 0.3 M sucrose in 0.1 M 1,4 piperazinediethanesulfonic acid (0.1 M PIPES buffer; pH 7.2). The cells were then pelleted by centrifugation ($400 \times g$ for 10 min) and resuspended in fresh fixative overnight. The next day, the haemocytes were embedded in 2% w/v agarose, washed with PIPES (2×1 h), and post-fixed in 1% w/v osmium tetroxide (OsO_4, in 0.1 M PIPES) for 40 min. The pellets were rinsed (2×10 min washes in 0.1 M PIPES) and submerged in 2% w/v filtered aqueous uranyl acetate for 20 min (*en bloc* staining). After *en bloc* staining, the pellets were washed in 0.1 M PIPES 3 times for 3 min each. The pellets were then dehydrated through a graded ethanol series (50%–100%) and embedded in L.R. White resin (80% polyhydroxy substituted bisphenol, dimethacrylate resin 19.6%, C12 methacrylate ester, 0.9% benzoyl peroxide; Proscitech, Kirwan, Australia). Ultrathin sections were cut using a Reichert Ultracut-S ultramicrotome and placed on coated 300 copper mesh grids. The sections were stained with saturated aqueous uranyl acetate for 30 min followed by Reynold's lead citrate for 5 min. Sections were imaged using a Philips CM 10 transmission electron microscope.

2.8. Scanning electron microscopy (SEM)

Haemocytes were fixed using 500 μL of 8% w/v paraformaldehyde and 5% w/v glutaraldehyde in 0.1 M PIPES for 2 h. After fixation, the haemocytes were washed in 0.1 M PIPES and post-fixed with 1% w/v osmium tetroxide in 0.1 M PIPES buffer for 40 min. Stained haemocytes were adhered to acid-alcohol washed coverslips for 30 min using 0.1% ethylene imine polymer solution (PEI; Hampton research, CA, USA). The haemocytes were then dehydrated in a graded series of ethanol (50%–100%) and transferred to a critical point dryer (Emitech K850; Kent, Ashford). Coverslips were mounted on aluminum stubs and coated with gold using a sputter coater (Emitech K550; Kent, Ashford). Samples were examined using a JEOL 6480 LA (Frenches Forrest, NSW) scanning electron microscope.

2.9. Monitoring live haemocytes

Live haemocytes were assessed for their external morphology and ability to adhere to glass microscope slides. The haemocytes were observed under differential interference microscopy (DIC), and the number of adherent cells in ten randomly selected fields of view were counted for each slide. The percentage of adherent cells were calculated per FOV and the over all average was calculated for the 10 FOVs. The data are presented as the mean frequency of adherent haemocytes per field of view ± SEM.

2.10. Statistical analysis

Two-way analysis of variance (ANOVA) followed by Tukey's Post-Hoc multiple comparisons were used to analyse differences between control and NA treated haemocytes from the TUNEL, Annexin V and cell adhesion assays using the statistical package Minitab ver. 16.0 (Minitab Inc., USA, 2011). When statistically significant differences were found, the data were reanalysed using individual one-way ANOVAs to test the difference between individual time points. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. TUNEL – DNA fragmentation

TUNEL-positive haemocytes, indicating double stranded DNA breakage, stained brown in colour (Fig. 1A). Nuclei in TUNEL-positive cells appeared fragmented. There was a dose dependent increase in the frequency of TUNEL-positive cells after NA-exposure (Fig. 1B). The percentage of cells displaying double-strand DNA breakage was greatest in cells treated for 3 h or 10 h with 10.0 ng NA/μg protein when compared to non-exposed control cells ($p < 0.05$; Fig. 1B).

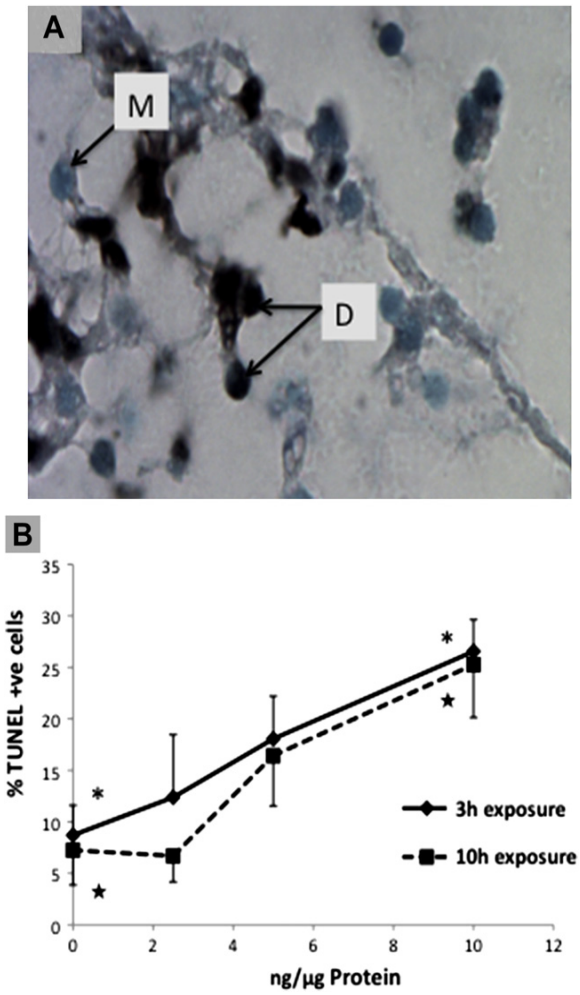

Fig. 1. A, Light micrograph of TUNEL-positive nuclei (D), and methylene blue stained (TUNEL-negative) nuclei (M). B, The percentage of TUNEL-positive cells at 3 h and 10 h after exposure to 0, 2.5, 5.0 and 10 ng NA/μg protein. $N = 7$, bars = standard error of the mean (SEM). Paired * and ★ denote a significant difference between two data points.

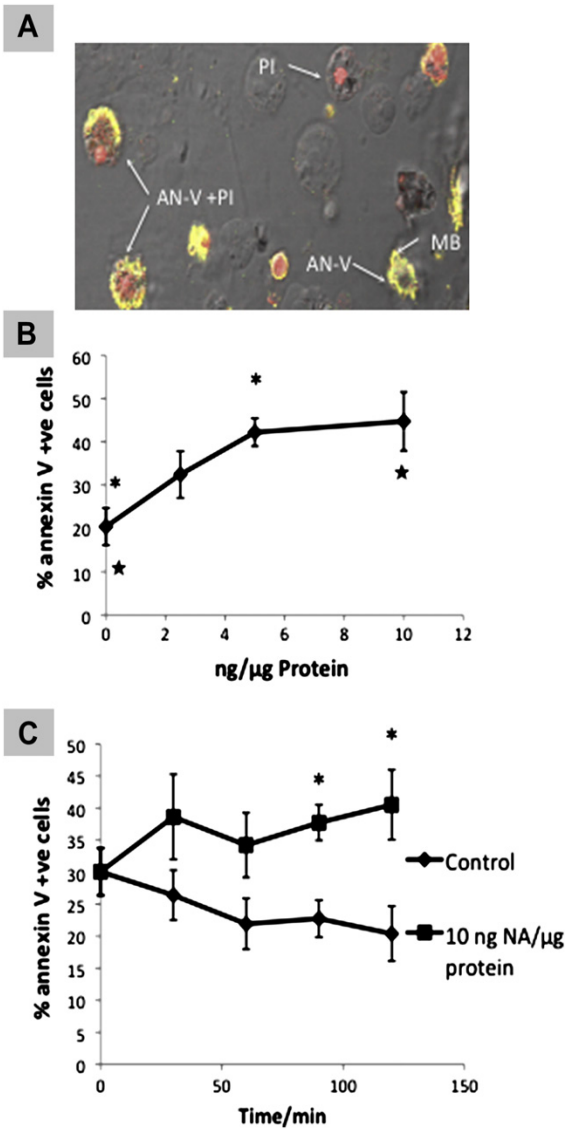

Fig. 2. A, Noradrenaline-exposed haemocytes stained with Annexin V only (AN-V), propidium iodide only (PI), and a combination of Annexin V and PI (AN-V + PI). MB, membrane bleb. B, Percentage of Annexin V-positive haemocytes in cultures exposed to 0 (control), 2.5, 5.0 and 10.0 ng NA/µg protein for 120 min. Paired * and ★ denote a significant difference between two data points. C, Percentage of Annexin V-positive cells in haemocyte cultures exposed to 10.0 ng noradrenaline/µg protein for between 0 and 120 min. $N = 7$, bars = standard error of the mean (SEM). * denotes a significant difference.

Approximately four times more TUNEL-positive haemocytes were evident after treatment with 10 ng NA/µg protein relative to controls.

3.2. Annexin V staining

We examined the extent of phosphatidylserine externalisation (an early apoptotic marker) in NA-exposed haemocytes from *P. imbricata* using an Annexin V-FITC assay. Typically, Annexin V staining was observed around the periphery of cells, and was often associated with blebbing of the membrane (Fig. 2A). A dose dependent increase in the frequency of Annexin-positive haemocytes was evident after 120 min exposure to NA ($p = 0.002$, Fig. 2B). At this time, the percentage of Annexin-positive cells in cultures exposed to both 5.0 and 10.0 ng NA/µg protein was significantly greater than controls ($p < 0.05$). The dose response became asymptotic between 5.0 ng and 10.0 ng NA such that the percentage of Annexin-positive cells at both of these doses was approximately two-fold greater than the controls.

Annexin V staining increased over time when cells were exposed to NA, whilst the frequency of Annexin-positive cells decreased over time in untreated controls. Annexin V staining was consistently greater in cells treated with 10.0 ng NA/µg protein when compared with non-exposed controls over the 120 min exposure period (two-way ANOVA, $p < 0.05$). Both Tukey's post hoc comparisons and one-way ANOVAs identified significant differences between NA-exposed and control cells after both 90 and 120 min ($p < 0.05$).

3.3. F-actin distribution

In untreated haemocytes, F-actin was commonly associated with pseudopodium formation (Fig. 3A). In contrast, polymerisation of F-actin was observed around the entire periphery of haemocytes treated with NA for 10 min, with reduced pseudopodia formation (Fig. 3B). F-actin was also found to coagulate into large globular masses around the outer cytoplasm of NA-treated cells after 30 min exposure (Fig. 3C). Haemocytes exposed to 10 ng NA/µg protein for 30 min exhibited membrane blebbing and the development of apoptotic bodies encased with an F-actin rich membrane (Fig. 3D). Counterstaining with propidium iodide also showed chromatin condensation in NA-treated haemocytes (Fig. 3B–D).

3.4. Transmission electron microscopy

Control haemocytes that were not exposed to NA retained uncondensed nuclei and intact organelles over a 60 min culture period (Fig. 4A). These cells often projected small filopodia. In contrast, haemocytes treated with NA for 60 min displayed acute condensation of the cytoplasm and nuclei (Fig. 4B–D). Condensed chromatin commonly appeared around the periphery of the nuclear membrane in NA-treated cells after 30 min (Fig. 4B). Nuclear fragmentation and the formation of apoptotic bodies were also observed when haemocytes were exposed to (5.0 and 10.0 ng NA/µg protein) for 60 min (Fig. 4C). NA-treated haemocytes were often rounded in shape and commonly lacked filopodia (Fig. 4D). They also displayed both vacuolisation and blebbing of the cytoplasmic contents.

3.5. Scanning electron microscopy

Under SEM, untreated (control 0–60 min) haemocytes typically had a rounded cell body, with numerous filamentous projections (Fig. 5A). In contrast, the plasma membrane of NA-treated cells after 60 min showed characteristic signs of apoptosis with membrane blebbing and they also lacked filopodia (Fig. 5B).

3.6. Effect of NA on cell adhesion

There was a dose dependent decrease in the frequency of adherent haemocytes in cultures exposed to NA for 120 min such that the frequency of adherent haemocytes was significantly lower in cultured haemocytes exposed to 10.0 ng NA/µg protein relative to non-exposed controls ($p < 0.05$).

The frequency of adherent cells decreased over time when cultures were exposed to NA. The percentage of adherent cells was approximately half (35.4%) that of their initial level (62.6%) after 120 min ($p < 0.05$). In contrast, the percentage of adherent cells remained relatively constant in non-exposed controls over the 120 min culture period ($p > 0.05$, 0 min vs. 120 min). As a result,

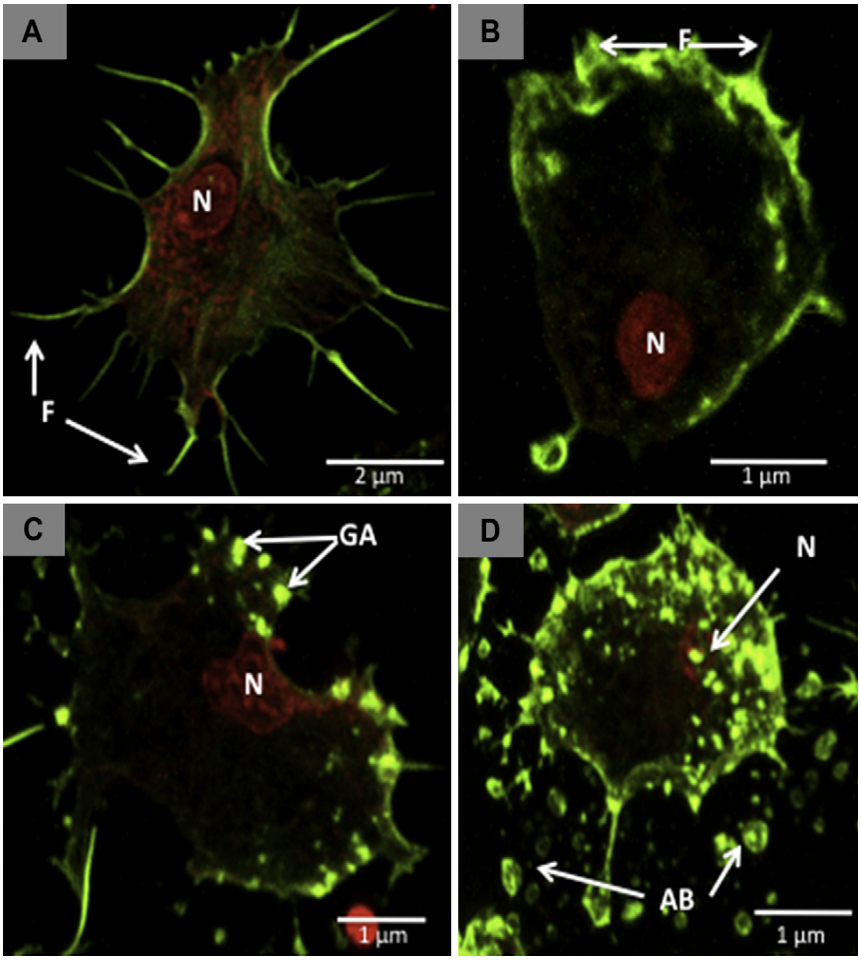

Fig. 3. Confocal fluorescent micrographs of control and NA-treated haemocytes stained with Phalloidin Alexa Fluor 488 (green). Nuclei (N) are counterstained with propidium iodide (red). A Control (non-exposed) haemocyte. B, Haemocyte exposed to 2.5 ng NA/µg protein for 10 min. C, Haemocyte exposed to 5.0 ng NA/µg protein for 30 min. D, Haemocyte exposed to 10 ng NA/µg protein for 30 min. GA; globular actin, AB; apoptotic body; F, filopodia (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

two-way ANOVAs identified significant differences between treatments (control vs. 10 ng NA $p < 0.05$) and over time ($p < 0.05$; Fig. 6B). Tukey's post hoc analysis and a one-way ANOVA identified significant differences ($p < 0.05$) in the frequency of adherent cells between control and NA-treated cells after 90 min of culture.

4. Discussion

Bivalve haemocytes can mount complex immunological responses to invading pathogens [12]. Given the crucial functions of defensive haemocytes, a number of studies have investigated the negative effects of environmental stressors on these cell populations. They have identified reductions in total haemocyte counts, phagocytoic activity, respiratory bursts, and adhesive ability, as well as altered cytoskeletal development, after exposure to environmental stress [28,31–34]. However, the molecular interactions between stress and disease remain poorly understood. A study by Butt et al. (2006) identified a strong correlation between outbreaks of QX disease in *S. glomerata* and environmental stress, whilst Lacoste et al. (2001) found that *C. gigas* were more susceptible to *Vibrio splendidus* infections when exposed to the stress hormone, NA. Lacoste et al. (2001) concluded that NA-induced disease susceptibility was due to impaired defensive activities of haemocytes.

To further resolve the relationship between NA and impaired immune function, the current study investigated the *in vitro* effects of NA exposure on haemocytes from *P. imbricata*. The NA concentrations used were based on studies by Lacoste et al. [15,21,28,29], and Malham et al. (2003). These studies showed that both *C. gigas* and *Haliotis tuberculata* secrete up to 20 ng NA/mL of haemolymph when stressed by mechanical agitation. Lacoste et al. (2001) also investigated the effects of NA doses ranging from 0.01–10.0 µM on luminol-dependent chemiluminescence (CL) activity in *C. gigas* haemocytes. We chose to test NA concentrations between 0 and 10 ng NA/µg protein as these values approximate the range of concentrations that have previously been investigated.

Early (Annexin V) and late (TUNEL) stage apoptotic markers were identified in NA-exposed haemocytes in the current study. Apoptosis can be induced by ß-adrenergic signalling involving caspases, a family of cysteine proteases [22]. The early onset of apoptosis can be identified by a loss of plasma membrane asymmetry. In non-apoptotic cells, membrane phospholipids (i.e. phosphatidylserine; PS) are distributed asymmetrically between the inner and outer leaflets of the plasma membrane [24]. In early apoptosis, PS translocates from the inner to the outer leaflet [23]. We tested this early stage of apoptosis in *P. imbricata* haemocytes using Annexin V, which binds to externalised PS in the presence of calcium. We found NA had a dose and time dependent effect on the

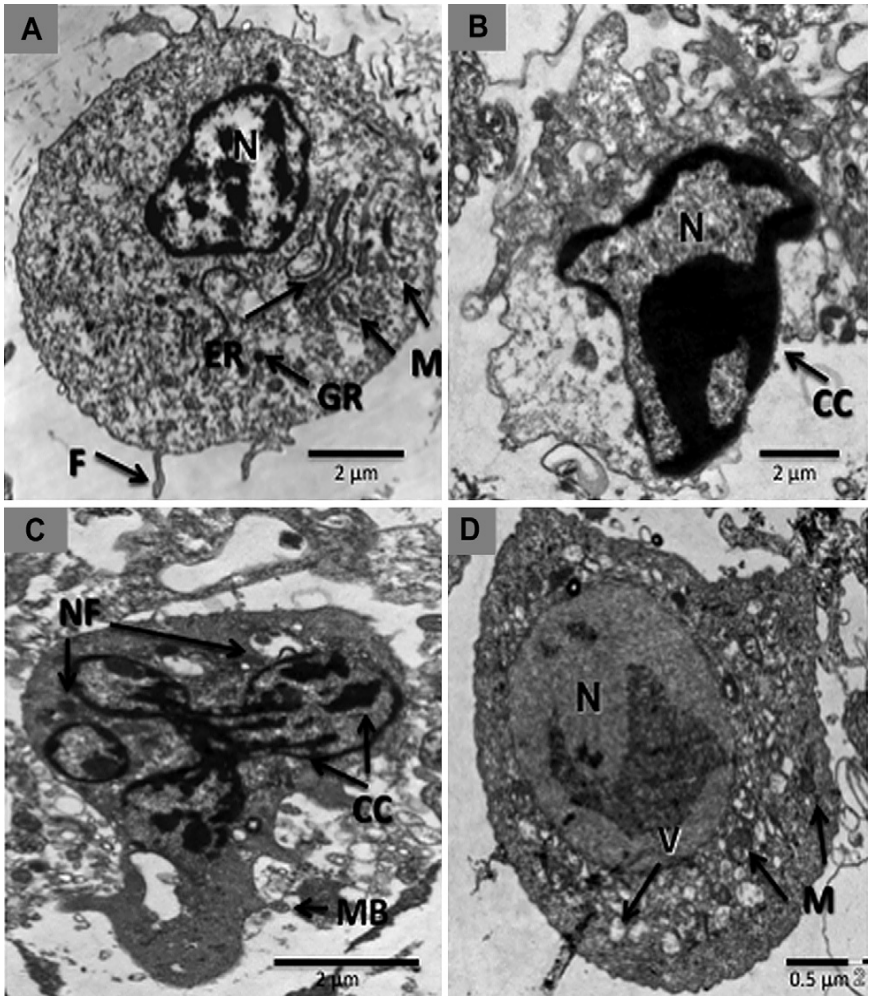

Fig. 4. Transmission electron micrographs of control (non-exposed) and NA-treated haemocytes. A, Control haemocyte after 60 min culture depicting intact organelles: endoplasmic reticulum (ER), mitochondria (M), and nucleus (N). Filopodia (F) are also visible. B, A haemocyte treated with 10 ng NA/µg protein for 30 min displaying condensed chromatin (CC). C, A haemocyte treated with 5 ng NA/µg protein for 30 min undergoing nuclear fragmentation (NF). MB, membrane bleb. D, A haemocyte treated with 10 ng NA/µg protein for 60 min, note the lack of filopodial development. V = vacuole. GR; granule.

frequency of Annexin-positive haemocytes. Annexin-positive haemocytes were approximately two fold more frequent among cells treated with 5.0 ng and 10.0 ng NA compared to non-treated control haemocytes. Annexin V staining was localised around the periphery of cells, which is where PS is typically located when it 'flips' from the inner to the outer membrane leaflet during apoptosis. This suggests that NA initiates PS redistribution in the membranes of *P. imbricata* haemocytes. The distribution of Annexin V within NA-exposed cells

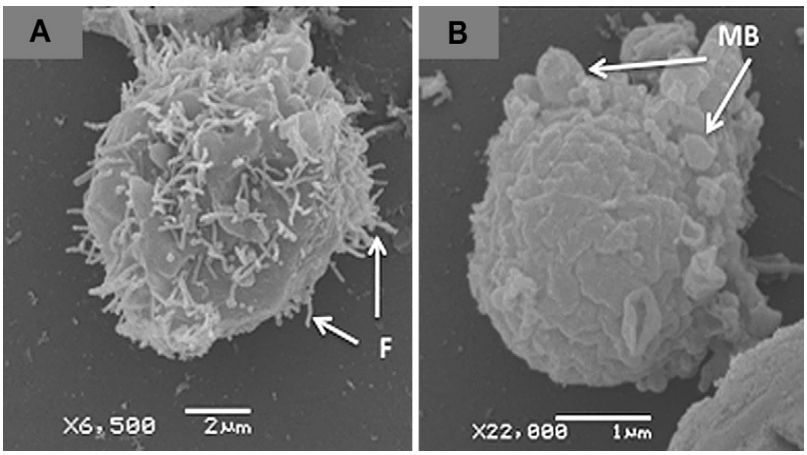

Fig. 5. Scanning electron micrographs of control (non-exposed) and NA-treated haemocytes. A, Control haemocyte after 60 min culture. F, filopodia. B, A haemocyte undergoing membrane blebbing (MB) after exposure to 10 ng NA/µg protein for 60 min.

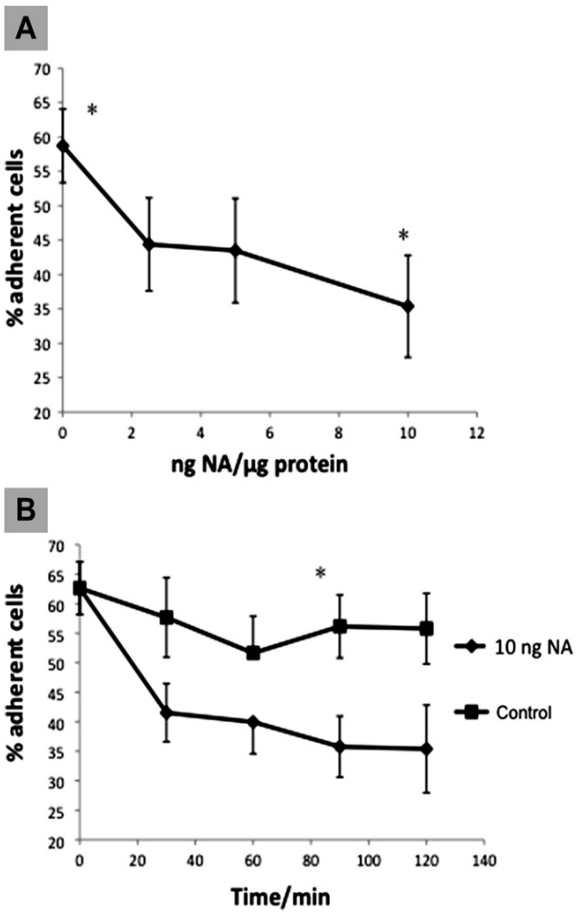

Fig. 6. A, Frequency (%) of adherent haemocytes in cultures exposed to 0, 2.5, 5.0 and 10.0 ng NA/µg protein for 120 min. $N = 7$, bars = SEM. * denotes a significant difference between two data points. B, The percentage of cells adhered to glass slides when exposed to 10.0 ng NA/µg protein. * denotes a significant difference.

also indicated that early apoptotic events affect cell morphology resulting in membrane blebbing. Membrane blebbing was identified as small Annexin V-positive spheres located around the periphery of the membrane. Lacoste et al. (2002) also investigated the redistribution of PS in *C. gigas* exposed to isoproterenol (a substrate used to mimic NA). They showed that NA is able to induce apoptosis via P35-sensitive caspases triggered by ß-adrenergic signalling in *C. gigas* haemocytes.

Late stage apoptosis was identified by the presence of TUNEL-positive haemocytes distributed in *P. imbricata* gill tissue. In the TUNEL assay, nuclear degeneration is detected when Klenow binds to the fragmented ends of DNA. This initiates the addition of biotin-labeled and unlabelled deoxynucleotides, which are detected by a streptavidin horseradish peroxidase (HRP) conjugate. In NA-treated gill tissue significantly more haemocytes were TUNEL-positive than in controls, indicating double stranded DNA breakage. The nuclei of these cells also appeared fragmented. NA had a dose dependent effect on the frequency of TUNEL-positive haemocytes. TUNEL-positive haemocytes were two times more frequent than controls when treated with 5.0 ng NA/µg protein, and four times more frequent in tissue exposed to 10.0 ng NA/µg protein compared to untreated controls. These data suggests that NA induces DNA fragmentation in *P. imbricata* haemocytes, which occurs within 3 h of exposure to NA.

The apoptotic events reflected by Annexin V and TUNEL staining seemed to coincide with alteration of the cytoskeleton associated with redistribution of F-actin. When haemocytes were exposed to 5.0 and 10.0 ng NA/µg protein, F-actin became concentrated in coagulated masses within the plasmalemma. These masses were commonly associated with regions of membrane blebbing and the production of F-actin encased apoptotic bodies. Aladaileh et al. (2008), observed a similar phenomena after exposing *S. glomerata* haemocytes to 150 nM NA. In other studies, the rearrangement of F-actin within the cytoskeleton has been associated with the early onset of apoptotic signal transduction [35,36].

Changes observed in the F-actin cytoskeleton and the DNA fragmentation detected by TUNEL may account for the alteration of haemocyte ultrastructure after *P. imbricata* haemocytes were exposed to NA. The onset of apoptosis after 30 min exposure to NA was evident under TEM as chromatin condensation in the nucleus, which was segregated into sharply delineated masses. In conjunction with these nuclear changes, the cytoplasm was also found to be condensed. Exposure to NA for 60 min resulted in budding of cytoplasmic contents into apoptotic bodies that were closely associated with the redistribution of F-actin. Similar morphological changes have been observed in apoptotic haemocytes associated with branchial lesions in the European flat oyster, *Ostrea edulis* [37]. NA has also been found to induce apoptotic changes in both plasma membrane morphology and nuclear chromatin in *C. gigas* and *S. glomerata* [5,22].

It is likely that NA-induced changes in the cytoskeleton and morphology of haemocytes affect their defensive activities. In this context, *in vitro* experiments in the current study showed that NA negatively affects the ability of haemocytes to adhere to glass slides in both a dose and time dependent fashion. This response to NA is likely to affect cellular defence mechanisms such as phagocytosis, which is dependent on cell adhesion and actin-mediated cytoskeletal activity. Similar effects are also evident in oysters subjected to environmental stress. Lacoste et al. (2002) found that phagocytic activity decreases when *C. gigas* are stressed by mechanical agitation [20,29]. Similarly, we have identified a reduction in phagocytic activity in *P. imbricata* haemocytes exposed to hyposalinity (25ppt), mechanical stress (300 rpm for 10 min) and desiccation (2 h aerial exposure every 24 h; 6). These data suggest that the secretion of biogenic amines in association with stress, which results in cellular dysfunction and apoptosis, provides one explanation for the link between stress, impaired immunocompetence and increased disease susceptibility.

Despite this, the physiological role of NA induced apoptosis is unclear. Haemocytes themselves are known to secrete NA, and so NA mediated apoptosis of haemocytes may represent a regulatory feedback mechanism. Apoptosis is also known to be an integral component of bivalve immunity, initiated by the production of ROS in haemocytes [38,39]. The secretion of ROS is thought to mediate apoptosis via the intrinsic pathway involving mitochondria [39]. Previous studies have shown resistant *C. gigas* can overcome *Perkinsus marinus* and *Haplosporidium nelsoni* infection better than non-resistant strains due to enhanced phagocytosis, respiratory bursts and apoptosis [40]. In this context, NA may enhance the contribution of defensive apoptosis in the short term whilst, leading to longer-term immunosuppresion through the loss of defensive haemocytes.

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CHAPTER FIVE

PHENOLOXIDASE ACTIVITY AS AN INDICATOR OF STRESS IN THE SILVER-LIP PEARL OYSTER, *PINCTADA MAXIMA*

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Author contributions:

David A. Raftos – Project supervision

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Preface

In this Chapter the relationship between phenoloxidase (PO) activity and the effects of stress was further investigated using field-based experiments. It was previously shown in Chapter Three that mechanical agitation, hypo-salinity and exposure to air can significantly reduce PO activity. To further explore this relationship, we exposed the sliver-lip pearl oyster, *P. maxima*, in the field to typical stressors associated with husbandry and handling, including; high power cleaning jets, being struck with a chisel, a combination of the jets and chisel, and finally exposure to air. We also exposed juvenile oysters to air. Results from these field experiments demonstrate that PO activity in mature oysters was initially inhibited after exposure to stress, followed by a period of increased activity, while PO activity was significantly up-regulated in juvenile oysters for the duration of the experiment. The findings in mature oysters agree with those from Chapter Three and suggest that PO activity may be a viable gauge of stress in the field.

The use of phenoloxidase activity as an indicator for stress in the silver-lip pearl oyster, *Pinctada maxmia*

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Abstract

Aquaculture necessitates a range of husbandry and handling procedures that induce or exacerbate stress. The relationship between environmental stress and suppression of immune responses has been defined in a range of invertebrate models. In the current study, we investigated the effects of antifouling practices on PO (phenoloxidase activity), which is associated with host defense in mature pearl oysters. The stressors included the use of a cleaning machine (high pressure water jets), being struck with a chisel to remove fouling, a combination of the cleaning machine and chiseling, and exposure to air. We also investigated the effects of exposure to air on PO activity in juvenile pearl oysters. PO activity declined significantly (by 56%, relative to the control) 48h after oysters were chiseled ($p < 0.05$). Similarly, PO activity was inhibited in oysters exposed to jets and oysters treated with a combination of the jets and chisel (by 43% and 40% decrease, respectively). Ninety-six hours after treatment PO activity increased significantly ($p < 0.05$) in oysters exposed to the combination treatment (2.61 fold increase) and to jets alone (2.06 fold increase). Exposure to air alone initially resulted in a significant decline (by 43%) in PO activity after 48h ($p < 0.05$), and then a subsequent increase (2.61 fold increase) after 96h ($p < 0.05$). In contrast, PO activity in juvenile oysters remained significantly elevated relative to controls at both 24 and 96h after exposure to air ($p < 0.05$). These data indicate that modulation of PO activity maybe a useful indication of stress in pearl oysters.

1. Introduction

Large-scale pearl production through pearl oyster aquaculture is now a highly lucrative industry, grossing around $500 million per annum in Australia alone (Kuchel et al., 2011b). The silver-lip pearl oyster, *Pinctada maxima,* forms the foundation of the Australian pearl production. This species is also cultivated throughout south-east Asia and the Pacific. Pearl culture necessitates handling, sorting and cleaning processes that involve physiological stressors such as emersion and byssal thread excision. In conjunction with natural environmental perturbations, the stressors associated with intensive culture conditions and farming practices have been associated with outbreaks of opportunistic diseases. In 1992, Davis et al. investigated the collapse of the black abalone industry, *Haliotis cracherodii*, due to severe outbreaks of withered foot syndrome (*Xenohaliotis americanus*) in southern California. Results from their study indicated that a combination of external stressors, including resource competition with sea urchins, as well as changes in physico-chemical parameters induced by pollution and water temperature, increased the incidence of disease (Davis et al., 1992). In addition to these findings, recent studies have demonstrated that overcrowding of both *P. fucata* and *P. margaritifera* spat results in the stress-associated spread of Vibrio sp. (FAO, 2007; Subhash, 2009).

The techniques used in pearl oyster cultivation vary based on species type and the country that they are farmed in. However, until recently, the Australian *P. maxima* industry has been considered to be relatively disease-free. However, in October 2006, outbreaks of oyster oedema disease (OOD) resulted in mass mortalities of up to 2.8 million *P. maxima* in the Exmouth Gulf, Western Australia (WA; Humphrey and Barton, 2009; Jones et al., 2010). This disease only infects *P. maxima* and not the closely related *P. margaratifera* (Jones et al., 2010). Morphologically, infected

oysters were found to have mild oedema, with retracted mantles, weak adductor function and oedematous separation of epithelial tissues from underlying stroma (Madin, 2007; Humphrey and Barton, 2009). Transmission experiments between healthy and infected oysters were unsuccessful, and attempts to identify the etiological agents have so far have been futile (Humphrey and Barton, 2009). Since 2009, OOD has been reported further north at other *P. maxima* farms.

Haplosporidium infections also pose a considerable threat to *P. maxima* cultivation. Three outbreaks of *Haplosporidium sp.* infections have been reported in recent years, all of which have been concentrated in the north of WA (Hine and Thorne, 1998; Jones and Creeper, 2006; FAO, 2007). Outbreaks of the haplosporidium *Minchinia occulta* have also been reported in the cupped oyster, *Saccostrea cucullata,* resulting in up to 80% mortality (Bearham et al., 2009). This has caused considerable alarm throughout the pearling industry because the epizootics in *S. cucullata* have occurred in close proximity to pearl oyster leases (Bearham et al., 2009). DNA-based diagnostic assays of *P. maxima* spat from the area have revealed infection with both *M. occulta* and *H. hinei* (Bearham et al., 2009).

Although these infections have not yet been linked to environmental changes, stress has been shown to alter immune function in a number of bivalve molluscs increasing disease susceptibility (Lacoste et al., 2001a). In a recent study by Kuchel et al. (2010), the effects of environmental stressors including; exposure to air, mechanical agitation (MA), and hypo-salinity, were investigated in *P. imbricata*. Both PO activity and phagocytosis, key mediators of immune responses, were significantly reduced in all three treatments (Kuchel et al., 2010). Similarly, total haemocyte counts, phagocytosis and the production of reactive oxygen species (ROS; superoxides and peroxides) were found to be significantly reduced in the Pacific

oyster, *Crassostrea gigas,* after exposure to MA (Lacoste et al., 2002). These studies provide evidence that stress can cause immuno-suppression, which may influence the spread of opportunistic pathogens.

Oysters possess a form of innate non-adaptive immunity (Nell and Perkins, 2006). Current evidence suggests that they lack both long-term immunological memory and precise antigen specificity. Interestingly, they can detect conserved patterns of pathogen-associated molecular patterns (PAMPS) using pattern recognition receptors (PRRs; Peters, 2004; Jiravanichpaisal et al., 2006; Aladaileih et al., 2007). These systems induce cellular defences based on haemocyte-mediated responses, including nodule formation, encapsulation, phagocytosis, and the production of ROS (Pipe, 1992; Hegaret et al., 2003; Aladaileih et al., 2007).

In addition to hydrolytic intracellular killing systems, PO is an integral component of subcellular antimicrobial activity in oysters (Munoz et al., 2006). The production of active PO is dependent upon a proPO-activating system, which is initiated by the presence of small quantities of PAMPS, such as LPS, peptidoglycans, laminarin and β-1,3-glucans (Asokan et al., 1997; Cerenius and Söderhall, 2004). Phenoloxidase is considered to be biologically "sticky" and adheres to the surface of foreign particles to form aggregates (Marmaras et al., 1996; Ling and Yu, 2005). The enzymatic end product of PO is melanin, a pigment that retards microbial growth (Cerenius and Söderhall, 2004; Nappi and Christensen, 2005). Intermediates of the PO cascade, such as quinones, are also highly reactive antimicrobial compounds (Marmaras et al., 1996; Söderhall and Cerenius, 1998).

Changes in environmental parameters can alter crucial biological functions such as PO activity (Pankhurst & Van Der Kraak 2000). Such stress responses are coordinated by a series of metabolic events, resulting in the secretion of hormones,

including dopamine and noradrenaline (NA; Ottaviani et al. 1998). In 2001a, Lacoste et al. investigated the effects of MA on the hormonal stress response in the Pacific oyster, *C. gigas*. After exposure to MA, NA was found to increase significantly in the circulating haemolymph (Lacoste et al., 2001a). Following these studies, Aladaileh et al. 2008, showed that exposing Sydney rock oysters (*Saccostrea glomerata*), to NA resulted in significant declines in immunological parameters, including PO activity and phagocytosis, as well as differential and total haemocyte counts. In addition to immunosuppression, the secretion of NA has been shown to induce apoptosis among defensive haemocytes in *P. imbricata* (Kuchel and Raftos 2011a).

Sensitivity of PO activity to environmental fluctuations suggests that this enzyme might be a reliable marker of "stress". Currently, there are few such markers. In 1972, Jeffries used taurine/glycine ratios in both the gill and mantle tissues of the hard clam, *Mercenaria mercenaria,* to indicate stress levels and associated infection with the polychaete worm, *Polydora*. Molar ratios less than 3 indicated a 'physiologically normal' population, while any ratio exceeding 5 was found to be associated with acute stress resulting in increased disease susceptibility (Jeffries, 1972; Sindermann, 1979). Other indices that have been used for the identification and analysis of stress include "scope for growth" (measuring the potential for somatic growth and gamete production) and oxygen/nitrogen ratios (Bayne and Scullard, 1977; Sindermann, 1979). Since these early studies, research has focused on the bio-molecular responses of organisms to stress. The regulation and expression of specific proteins, such as heat shock proteins, metallothioneins, and antioxidants are valuable indicators of physiological states in marine bivalves (Clegg et al., 1998; Gestal et al., 2007; Fabbri et al., 2008). Their expression provides the potential to understand the mechanisms by which marine organisms respond to environmental and anthropogenic

stressors (Dahlhoff, 2004). However, for the purpose of rapidly identifying stress "on farm", such analyses are often time-consuming and expensive. Hence, the aquaculture industry requires a more feasible 'litmus test' to identify the early onset of stress, providing the potential to alter farming conditions accordingly. To that end, the current study investigated whether common husbandry and handling practices associated with pearl farming and anti-fouling affect PO activity in *P. maxima*.

2. Materials and Methods

2.1 Oysters

Silver-lip pearl oysters (*P. maxima*), with dorso-ventral height of 150-180mm (mature) and 45-60mm (juvenile), were kindly supplied by Cygnet Bay Pearls, (Broome, Australia, 16° 27' 5" S, 123° 0' 31" E). The oysters were housed at Kimberly Marine Research Station (KMRS) on long lines in panels in the open ocean. The oysters were acclimated for one month prior to experimentation.

2.2 Experimental design

2.2.1 Mature oysters

Four stressors commonly associated with pearl oyster husbandry were tested; exposure to air, antifouling with a high-powered jet, chiseling to remove fouling organisms, and a combination of both the jet and chisel treatments. The chiselling, jets and combined treatments also involved emersion. High-pressure jet (cleaning) machines (M&J Engineering, Perth) are typically used as a routine part of the antifouling process at Cygnet Bay Pearls. They comprise a set of pipes within a large metal housing that directs pressurized seawater from both above and below onto a central conveyor belt. The panels containing oysters are laid on the conveyor and are fed through the machine. Both the pressure of the water and the speed of the conveyor belt can be adjusted manually to suit the degree and type of fouling on panels for effective cleaning. In addition to water jets, chisels are also used to "chip" oysters once they have passed through the cleaning machine. Chipping involves the removal of mostly hard (and resilient soft) fouling that has not been removed by the jets. Barnacles and fouling bivalves are tapped from a 45° angle (or greater) at the point of contact with the oyster shell. In the current study, to replicate the effects of antifouling

stress, oysters were either struck with a chisel for 20s, exposed to a high-powered jet for 45s, or a combination of both treatments. Another set of oysters were exposed to air only for 3h. After these treatments, the oysters were returned to the water. For each time point after treatment (1, 24, 48, and 96h), 9 oysters from each treatment were randomly selected and haemolymph was extracted from each (Fig. 1A). Control oysters that remained untouched in the water were removed from the water at the same time points (9 oysters per time-point) and at the beginning of the treatment (0h).

2.2.1 Juvenile oysters

To test the effects of emersion, juvenile oysters were exposed to air for 3h. The treated oysters were then returned to the water and sampled 24 and 96h post treatment (Fig. 1B). Control oysters were removed from the water and sampled immediately at 0, 24, and 96h.

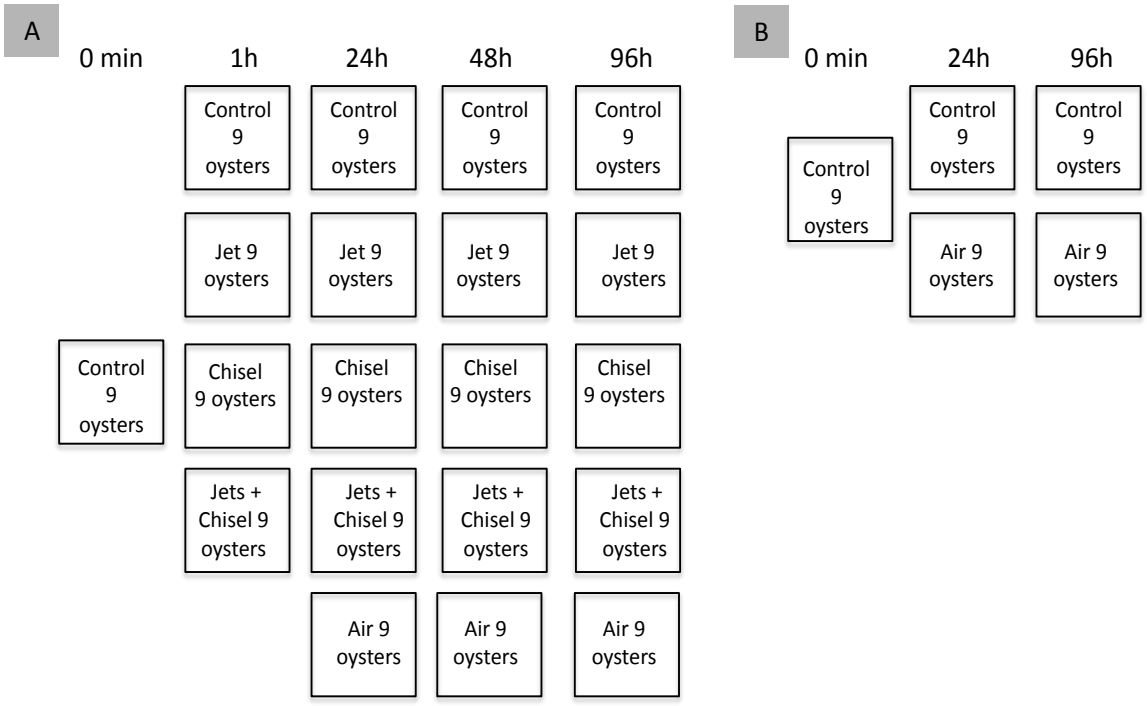

Figure 1. Schematic diagram of the experimental design for (**A**), mature oysters, (**B**), juvenile oysters.

2.3 Haemolymph collection

Oysters were shucked open by severing the adductor muscle. Haemolymph was collected by penetrating the pericardial cavity located near the adductor muscle, using a 21-gauge needle fitted to a 1 mL syringe. Whole haemolymph was then transferred into sterilized 1.5 mL microcentrifuge tubes and stored in liquid nitrogen at – 80 ºC. Haemocyte lysates (HL) were produced by freeze-thawing the haemolymph (– 80 ºC/ room temperature) three times. The lysed samples were centrifuged at 5,000 x *g* for 30 min (4 ºC) to remove cellular debris.

2.4 Protein quantification

Total protein content of HL was quantified using a Bio-Rad Bradford protein assay kit according to the manufacturer's instructions (Bio-Rad, Regents Park, NSW, Australia). Absorbance was measured at 595 nm using a Bio-Rad model 550-microplate spectrophotometer (Bio-Rad, Regents Park, NSW, Australia) and protein content was interpolated from a standard curve generated with bovine serum albumin (BSA).

2.5 Phenoloxidase assays

Phenoloxidase activity was assayed spectrophotometrically in 96-well flat-bottomed microtitre plates (Sarstadt, Technology Park, SA). Hydroquinine monomethyl ether (4HA; Sigma Aldrich, Castle Hill, Australia) was used to measure monophenolase activity. This assay was adapted from Aladaileh et al. (2007). One hundred microlitres of HL were added per well in triplicate for each sample. The HL was then incubated with 100 μL 4HA (5 mM in FSW), containing 1 mM 3-methyl-2-benzothiazoline hydrazone (MBTH; Sigma Aldrich, Castle Hill, Australia). The

samples were held at room temperature (in the dark) and the absorbance was measured at 490 nm at 0 and 60 min after addition of the substrate. Phenoloxidase activity was calculated as a rate over time. Data were adjusted for the total protein content (Bio-Rad Bradford assay) of each sample and the absorbance of blanks (wells containing PO substrates without HL). Data at each time-point are presented as fold differences relative to the mean PO activity of the 0 min controls (no stress treatment).

2.6 Data analysis

Data analysis was performed using Minitab ver. 16.0 (Minitab Inc., USA, 2011). Two-way analysis of variance (ANOVA) were used to assess changes over time and treatment. One-way ANOVAS followed by Tukey's Post-Hoc multiple comparisons were used to analyse differences between control and treated oysters from the four treatments; chiselling, jets, chiselling and jets, and air alone. When statistically significant differences were found, the data were reanalysed using individual one-way ANOVAs to test the difference between time-points. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1 PO activity in mature oysters exposed to jets, chisel, and jets + chisel

Two-way ANOVA comparing all three treatments against the control over time showed there were significant differences in PO activity relative to the controls over time ($p = 0.001$) but not between treatments ($p = 0.391$). One-way ANOVAs showed that PO activity was significantly lower than controls (by 56%) in oysters treated by chiselling after 48h (Fig. 2A; $p = 0.016$). Phenoloxidase activity in oysters treated with jets and chisel + jets was also lower than controls (43% and 40% respectively). However, one-way ANOVAs showed that these responses were not statistically significant when compared to controls (jets, $p = 0.059$ and jets + chisel, $p = 0.060$). Ninety-six hours after treatment, PO activity was significantly greater than controls in oysters subjected to jets + chisel ($p = 0.001$; 2.61 fold increase), as well as in oysters treated with jets alone ($p = 0.004$, 2.06 fold increase). Oysters treated by chiselling only also showed elevated levels of PO activity (1.74 fold increase). However, this increase was not statistically significant when compared to controls ($p = 0.056$). One-way ANOVAS also showed that PO activity in controls did not differ significantly over time, while treatment with the jets, and jets + chisel, had significantly higher PO activity at 96h relative to the 0h time-point ($p = 0.006$ and $p = 0.004$ respectively).

3.2 PO activity in mature oysters stressed air exposure

Phenoloxidase activity declined significantly 48h after mature oysters were exposed to air ($p = 0.046$; Fig. 3B; by 43%). Following this decline, PO levels increased significantly (by 2.60 fold) relative to the controls after 96h ($p = 0.023$). Overall, PO activity increased significantly over time in air-treated oysters ($p =$

0.004), while PO activity in control oysters did not differ significantly over time (p = 0.489).

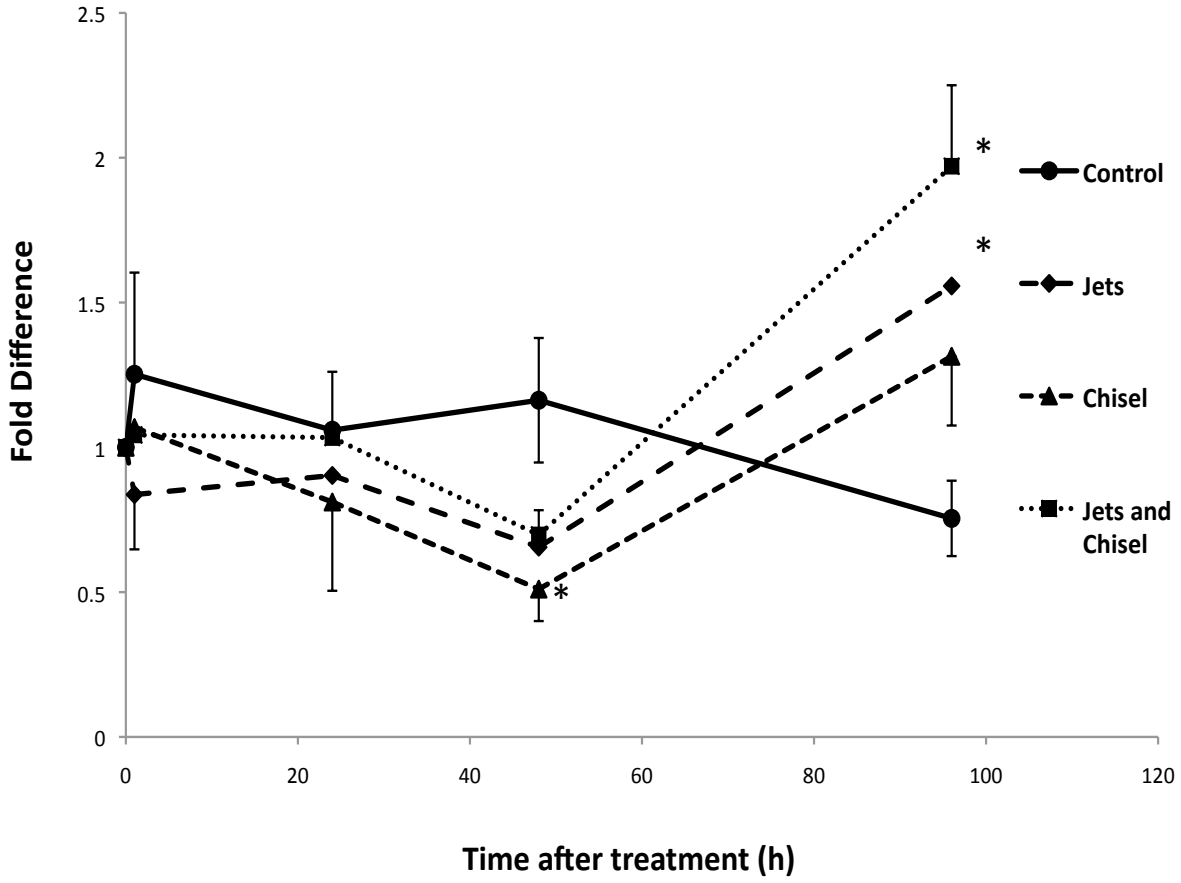

Figure 2. Fold differences (relative to the 0 time-point) of PO activity in haemolymph from mature oysters stressed by jets, chisel, and jets + chisel. * Denotes a significant difference between treatment and control values for that time point (p-value > 0.05). Error bars represent standard error of the mean.

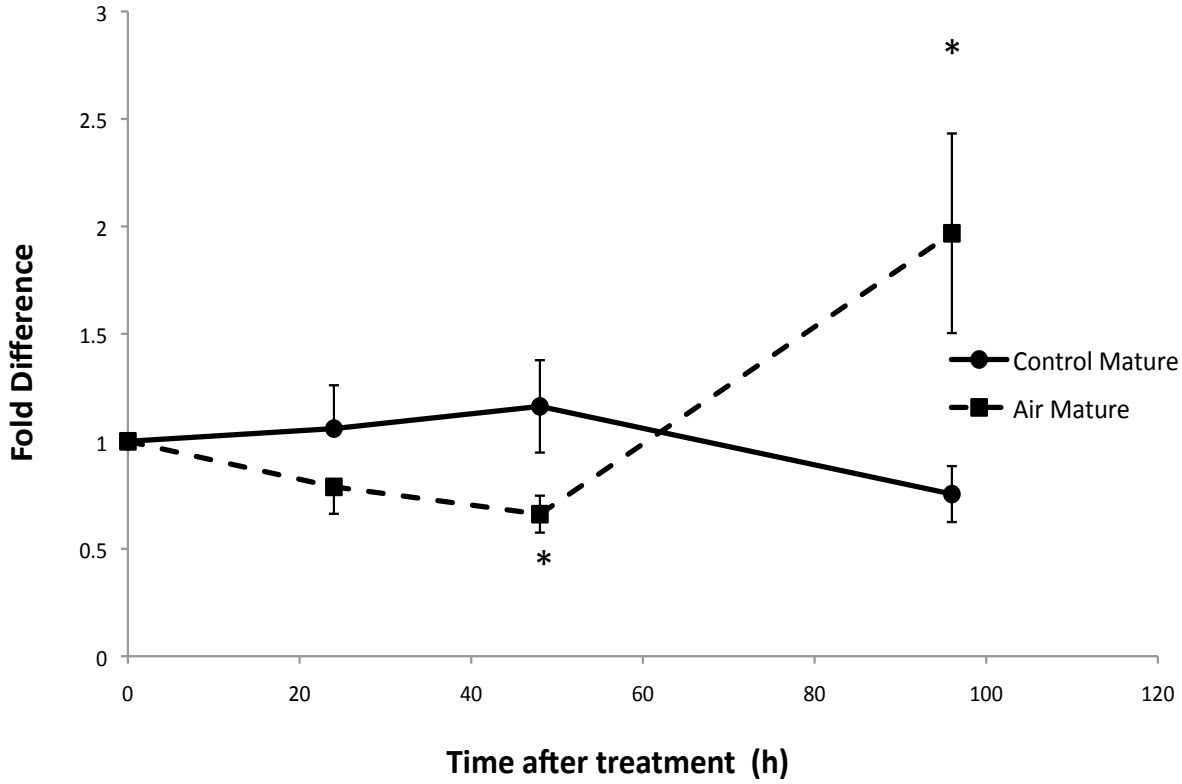

Figure 3. Fold difference (relative to the 0 time-point) in PO activity of mature oysters stressed by air. * Denotes a significant difference between treatment and control values for that time point (p-value > 0.05). Error bars represent standard error of the mean.

3.3 PO activity in juvenile oysters exposed to air

Two-way ANOVA showed there to be a significant difference between controls and treatments ($p = 0.004$), but not over time ($p = 0.126$). Subsequent one-way ANOVAs showed that, 24h after juvenile oysters were exposed to air, PO activity was significantly greater (by 2.07 fold) than non-treated controls ($p = 0.000$; Fig. 4). After 96h, PO levels in air-exposed juveniles were 5.56 fold higher than those of non-treated controls ($p = 0.027$).

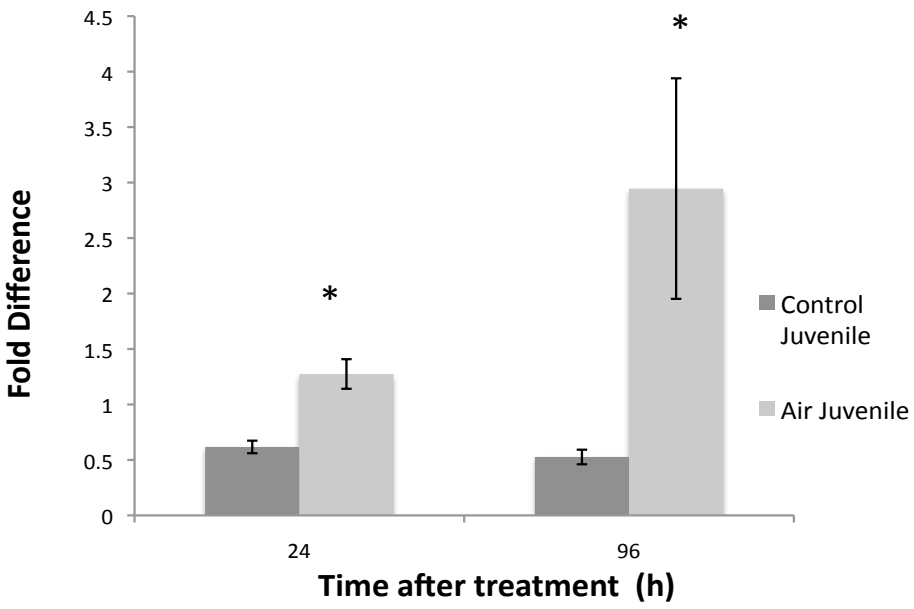

Figure 4. Phenoloxidase activity in juvenile oysters stressed by air. * denotes a significant difference between treatment and control values for that time point (p-value > 0.05). Error bars represent standard error of the mean.

4. Discussion

Aquaculture necessitates husbandry and handing practices that can introduce new, and/or exacerbate existing, stressors. The current study indicates that common stressors associated with anti-fouling (exposure to air, treatment with high-powered water jets, or being struck with a chisel) alter PO activity in the economically important pearling species, *P. maxima*.

Pearl oyster cultivation is a highly lucrative industry. However, it relies upon healthy oysters to produce quality products. In order to maximize growth and yield, it is important to understand the interactions of introduced stress and its implications on oyster health and the spread of opportunistic pathogens. Fouling biota have been shown to negatively affect the development and growth of commercially cultivated oysters (Mohammad, 1976; Chellam, 1978, Taylor et al., 1997). A study by Jakob and Wang (1994), found that bi-weekly antifouling promoted growth of *C. virginica*, while regular net changes in the giant scallop, *Placopecten magellanicus*, resulted in a 68% increase in muscle weight (Claereboudt et al., 1994). Hence, it has been considered necessary to remove fouling organisms periodically during farming. However, the anti-fouling processes may themself exacerbate stress (Parsons and Dadswell, 1992). Previous studies have shown that stress (exposure to air, MA and hypo-salinity) can adversely affect both physiological and immunological functions in pearl oysters (Kuchel et al., 2010), while repeated manual handing of juvenile scallops can result in mortality (Ventilla, 1982).

Stressors such as low salinity and starvation are known to affect the immune responses of many marine invertebrates. Lacoste et al. (2001a) identified a specific physiological mechanism behind such stress-related immuno-deficiency in *C. gigas*. Haemolymph from stressed oysters was found to have elevated levels of the hormone,

noradrenaline (NA; Lacoste et al., 2001a). Increased NA concentrations were shown to inhibit immune responses, such as phagocytosis and the production of ROS (Lacoste et al., 2001a). In other studies, *C. gigas* were exposed to different grading techniques; rotary, inside/out and flatbed (Qu et al., 2009). During the 48h test period, significant increases in dopamine and NA were recorded in oysters exposed to both the flat bed and inside/out graders. The rotary grader was found to induce the least amount of stress (Qu et al., 2009). These findings are comparable to those more recently with *P. imbricata,* which suggested that immunosuppression may be part of a NA-mediated physiological stress response (Kuchel and Raftos, 2011a).

Changes in PO activity resulting from stress have been investigated as a potential explanation for the onset and proliferation of QX disease in Sydney rock oysters. Butt et al. (2006) performed *in vitro* experiments comparing the effects of salinity on immunocompetence, including the PO cascade. Oysters were exposed to water obtained from areas suffering severe QX outbreaks, and with artificially diluted oceanic seawater. The seawater collected from QX affected sites was known to have reduced salinity due to heavy rainfall. Both of these treatments elicited a dramatic decline in PO activity, suggesting that decreased salinity suppresses PO activity (Butt et al., 2006). This conclusion was supported by field trials that exploited the natural salinity gradient in the Georges River, Sydney (Butt et al., 2006). Oysters situated up-stream in less saline conditions had lower PO activities than those closer to the ocean. Oysters at upstream sites were also far more susceptible to QX disease. Butt et al. (2006) concluded that the stress of low salinity causes PO activity to decline, resulting in opportunistic infection. In other studies, decreases in salinity have also been shown to negatively affect PO expression or activity in the yellow leg shrimp, *Farfantepenaeus californiensis* (Vargas-Albores et al., 1998). Similar immuno-

suppression associated with disease susceptibility has been illustrated in other marine bivalves, including *Mytilus edulis* (Grundy et al., 1996; Butt et al., 2006) and the oysters, *C. virginica* (Anderson et al., 1996: Fisher et al., 1999; Butt et al., 2006) and *C. gigas* (Lacoste et al., 2001b, 2001c).

Butt et al. (2006, 2007) also identified a number of additional environmental parameters, including starvation, that suppress the oyster immune system. The frequency of haemocytes and PO activity declined significantly when the dietary intake of oysters was halved over a 2-week period (Butt et al., 2007). These results are comparable to those obtained for other marine invertebrates, such as the Eastern oyster (*C. virginica*; Hegaret et al., 2004) and the Pacific oyster (*C. gigas*; Zhang and Li 2006). Tanner et al. (2006) investigated the effect of hypoxia hypercapnia and pH on PO activity in the Atlantic blue crab, *Callinectes sapidus*. Phenoloxidase activity declined significantly with decreases in pH and oxygen (Tanner et al., 2006). Previous studies have investigated the effects of hypoxia hypercapnia on immunocompetence by injecting sub-lethal doses of *Vibrio campbellii* into *C. sapidus* (Holman et al., 2004). Blue crabs that were stressed by hypoxia hypercapnia had much higher bacterial loads than control crabs (Holman et al., 2004).

Recent studies investigating the effects of environmental stressors (MA, exposure to air, and hypo-salinity) on immunological activity in *P. imbricata* have also shown that these stressors significantly reduce both phagocytosis and PO activity (Kuchel et al., 2010). In addition, Aladaileh et al. (2008) found that the stress hormone, NA, significantly retards PO activity in *S. glomerata*. This depression in PO activity may be attributed to a decline in the frequency of defensive haemocytes. Kuchel and Raftos (2011a) showed that NA induced both early and late stage

apoptosis among *P. imbricata* haemocytes. This response may be one explanation for a decline in immunological activity amongst stressed oyster populations.

Following on from these laboratory-based experiments, the current study shows that the husbandry and handling associated with pearl oyster antifouling in the field can alter PO activity. After 48h, PO activity declined by ≥ 40% in mature, treated oysters relative to the controls. These declines maybe the result of a rapid decrease in the frequency of haemocytes from the circulating haemolymph, through apoptotic processes similar to those described by Kuchel and Raftos (2011a) and Aladaileh et al. (2008). Following the initial decline, PO activity increased significantly in mature oysters exposed to air, jets, and jets + chisel such that PO activity was 2.61, 2.06, and 2.61 fold greater than controls after 96h. Similar increases in PO activity after acute stress has been documented in the shrimp, *Fenneropenaeus chinensis* by Liu et al. (2006). These authors propose that after an initial decline in PO activity, subsequent significant increases in PO expression resulted as a compensatory mechanism (Liu et al., 2006).

Comparatively, juvenile oysters exposed to air were found to have significantly elevated PO activity for the duration of the experiment. Few studies have compared the immunological responses of juvenile and mature bivalves. In 2006, Guenther et al. showed that juvenile *P. fucata* fouled significantly less than mature oysters. These authors suggested that juvenile oysters should be subject to cleaning less frequently than adults. More recently, Schleder et al. (2008) compared the immunological activity of both mature and juvenile scallops, (*Nodipecten nodosus*). Their results showed that PO activity was significantly higher in juvenile scallops stressed by alkaline conditions than mature scallops. These results compliment our current findings.

In conclusion, our study has shown that stress induced by pearl oyster cultivation/antifouling can alter the short-term activity of PO and so may affect its associated immunological functions. Currently, the industry lacks a commercially useful assay for stress in oysters. Due to its apparent changes in activity, we posit that PO activity might be a useful stress biomarker with industry-related applications for both pearl oysters and other aquaculture species.

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CHAPTER SIX

CHANGES IN THE TRANSCRIPTIONAL EXPRESSION OF OXIDATIVE STRESS RESPONSE GENES IN AKOYA PEARL OYSTERS (*PINCTADA IMBRICATA*) EXPOSED TO AIR AND MECHANICAL AGITATION

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Preface

Molecular biomarkers provide the potential to understand the mechanisms by which marine organisms respond to environmental and anthropogenic stressors. The expression of these genes are valuable indicators of physiological state. The aim of this Chapter was to investigate the effects of stress on antioxidant activity (peroxiredoxin, PRx; glutathione-S-transferase, GST; glutathione peroxidase, GPx) and the generic stress biomarker heat shock protein (HSP-70) induced by husbandry and handling. We exposed to oysters to either air or mechanical agitation (MA). Both PRx and GPx declined by up to 50% 60 min after treatment with both MA and air, while GPx transcription declined significantly 60 min after exposure to MA. HSP-70 expression did not vary between 60 min and 6 h, this result was possibly due to inter-animal variability. In Chapter Three we showed that the stress hormone, noradrenaline (NA), can induce apoptosis in defensive haemocytes of *P. imbricata.* Based on previous findings, we hypothesised that these responses to environmental stress may be due to; 1, cells that produce reactive oxygen species (ROS) dying due to apoptosis, which then causes a decline in the production of oxidative stress genes; or 2, increases in stress-induced NA levels cause a decrease in the expression of antioxidant genes, which results in an increase in ROS leading to apoptosis.

Changes in the transcriptional expression of oxidative stress response genes in Akoya pearl oysters (*Pinctada imbricata*) exposed to air and mechanical agitation

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Abstract

The Akoya pearl oyster is an economically important species farmed throughout Japan and China, and on a small scale in Australia. Recent proliferation of Akoya viral disease (AVD) has dramatically reduced both wild and farmed populations in Asia. It is thought that AVD is exacerbated by anthropogenic pressures associated with over-crowding and stress caused by fluctuations in the environment, as well as by husbandry and handling. To test the effects of these stressors, the differential expression of genes encoding three antioxidant enzymes (peroxiredoxin, PRx; glutathione-S-transferase, GST; glutathione peroxidase, GPx) and the generic stress biomarker heat shock protein, HSP-70, was investigated using quantitative real-time PCR on mRNA extracted from gill tissues of *P. imbricata* that had been exposed to either mechanical agitation (MA) or air. We observed a significant decline (up to 50%) in the expression of both GST and PRx 60 min after oysters were exposed to air and MA. GPx transcription declined significantly 60 min after exposure to MA. Hierarchical cluster analysis and multidimensional scaling (MDS) demonstrated that even though no significant differences were identified in the expression of these genes 6 h after exposure to stress, there was still a clear differentiation between the controls and the two stress treatments. After 6 h, MDS plots showed antioxidant expression returning to near control levels. MDS plots also demonstrated that there was no clear distinction between exposure to air or MA, suggesting that air exposure is the causative agent of stress, not MA. HSP-70 expression also declined when oysters were exposed to both stressors, but these decreases were not statistically significant. Overall, the results suggest that the antioxidant systems of oysters are affected by exposure to air. This contributes to a growing understanding of short-term stress responses in bivalve molluscs and their potential effects on cellular homeostasis.

1. Introduction

The Akoya pearl oyster, *Pinctada imbricata*, is a commercially important aquaculture species cultivated throughout Japan and China for both its meat and pearls. Since 1996, productivity in Asia has declined significantly due to the proliferation of Akoya viral disease (AVD), from which 150 million oysters have perished (Beard and Wade, 2002; Pit, 2004). The outbreak of disease and its continuing proliferation has been linked to environmental stress associated with increasing urbanisation and industrialisation (O'Connor et al., 2003). The decline in Asian cultured Akoya pearls has created an opportunity for other pearling nations, including Australia, to enter the market. In 2003, the New South Wales Department of Primary Industries (NSW DPI) and Radiata Pty Ltd began to assess the viability of Akoya pearl culture in Port Stephens, Australia (O'Connor et al., 2003). Results from initial studies on the viability of this industry were encouraging and a commercial Akoya pearl oyster venture is now in operation.

Intertidal marine invertebrates, such as oysters, are exposed to a range of stressors including fluctuations in water temperature, salinity, tidal/wave action, food availability, and predation, all of which can impact on their physiology (Lacoste et al., 2002a; Malham et al., 2003; Butt and Raftos, 2006; de Almeida et al., 2007; Kuchel et al., 2010). Research investigating environmental stress has led to the development of a number of molecular indicators of environmental stress, most notably, stress-associated genes, such as heat shock protein (HSP-70), glutathione-S-transerferase (GST), peroxiredoxin (PRx), and glutathione peroxidase (GPx; Clegg et al., 1998; Gestal et al., 2007; Fabbri et al., 2008). The regulation and expression of these genes are valuable indicators of physiological states in marine bivalves and they provide the potential to understand the mechanisms by which marine organisms respond to

environmental and anthropogenic stressors (Dahlhoff, 2004).

Pearl oyster farming involves a range of practices that either introduce new, or exacerbate existing stressful conditions. These include antifouling and sorting (often associated with emersion), starvation prior to nucleation, and byssal thread excision. In addition, Australian *P. imbricata* are typically farmed in estuaries that are influenced by freshwater inflows at the southern extent of the species range. Sub-lethal periods of stress associated with aquaculture practices have been shown to affect normal physiological functions including the effectiveness of cellular stress and immune responses, involving the production of reactive oxygen species (ROS; superoxides and peroxides; Lee and Söderhall, 2002). Low concentrations of ROS are essential for normal physiological functions. However, excess accumulation can result in the production of hydroxyl radicals (OH), or hydroxylchlorous acid, leading to oxidative stress (Wang et al., 2010). Oxidative stress typically causes lipid peroxidation, protein oxidation, DNA breakage and point mutations, and apoptosis (Nikapitiya et al., 2009). To combat the accumulation of ROS, antioxidant enzymes such as PRx, GPx, and GST reduce levels of H_2O_2, as well as organic peroxides and peroxynitrite (Diet et al., 2007; Cong et al., 2009).

Understanding these molecular responses could provide important new insights into the cellular stress responses that influence the limits of *P. imbricata's* tolerance. In this study, we used real-time polymerase chain reaction (qRT-PCR) to measure the expression of genes encoding PRx, GPx, GST and HSP-70 in *P. imbricata* that had been subjected to mechanical agitation (MA) and/or exposure to air.

2. Materials and Methods

2.1 Animals

Oysters were kindly supplied by W. O'Connor and K. Johnston (NSW Department of Primary Industries), who obtained them from Broken Bay Pearls Pty Ltd. (Tuncurry, NSW; 33º27'S 151º14'E). Oysters were housed in 45 L aquaria with a flow-through sea water system maintained at 22 °C. The oysters were fed every two days with Nosan M-1 diet (Aquasonic Pty Ltd, Wauchope, NSW).

2.2 Experimental design

To test the effects of stress on gene expression in *P. imbricata*, oysters were exposed to two treatments; mechanical agitation (MA) or exposure to air (Figure. 1). A total of 3 aquaria housed 21 oysters each. For MA, 3 oysters from each aquaria were placed dry on an MS1 Minishaker (Crown, Scientific, NSW, Australia) and shaken for 10 min at 300 rpm. The oysters were then left exposed to air for a further 50 min before gill tissue was harvested (60 min time-point). To investigate gene expression at a later time-point, the above treatment was repeated with another set of 9 oysters (3 selected from each aquaria) that were returned to the aquaria at the end of the 60 min MA treatment for a further 5 hours before their gill tissues were harvested (6 h time-point). To investigate the effects of air exposure alone, 3 oysters from each of the 3 aquaria were removed and left on the bench for 60 min before their gill tissues were harvested. Another group of nine oysters, were returned to the aquaria at the end of the 60 min air exposure for a further 5 hours before their gill tissues were harvested (6 h time-point).

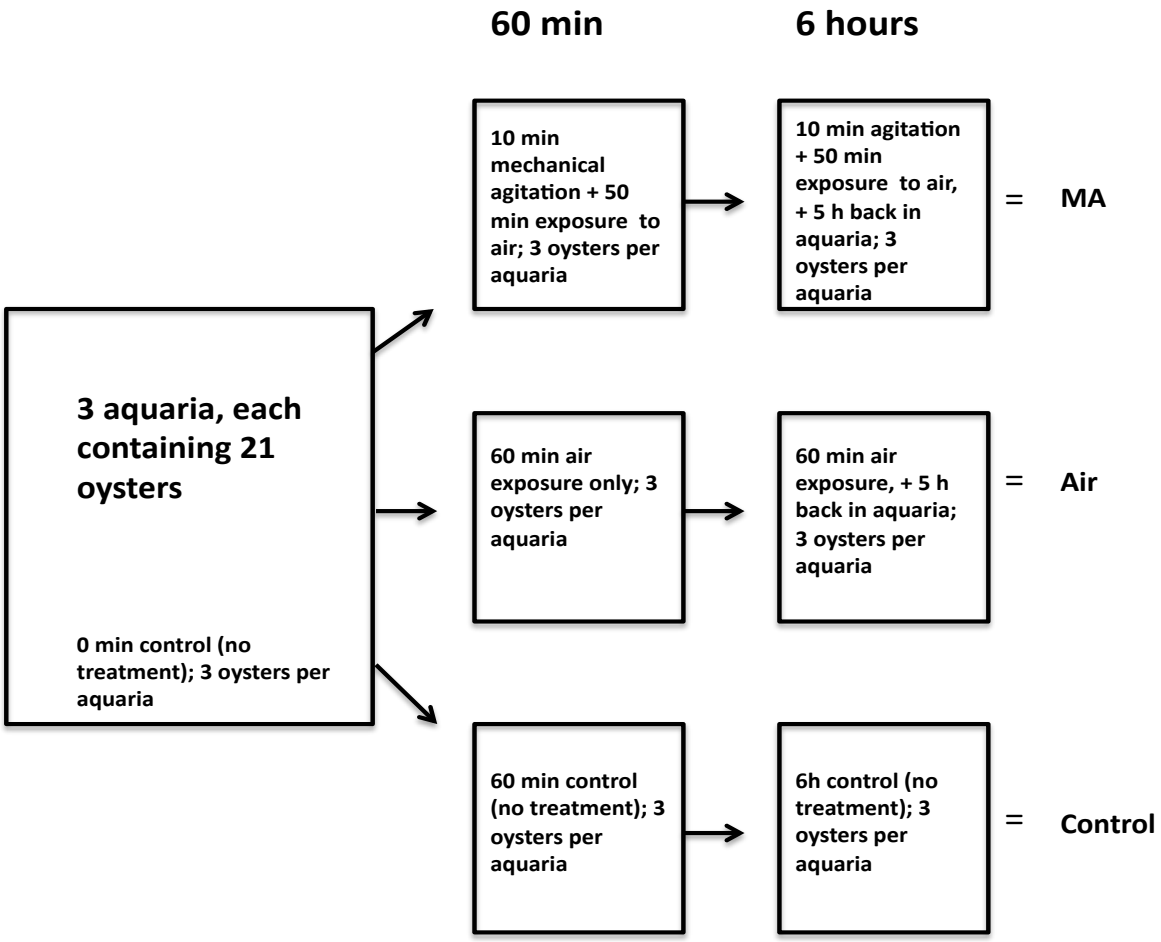

Figure 1. Schematic representation of the experimental design.

2.3 Tissue sampling and RNA extraction

Gill tissue was extracted from each oyster by severing the adductor muscle and excising gill tissue. The extracted tissue was immediately immersed in 500 μL RNA-later (Invitrogen, Vic, Australia). Samples were stored at -80 ºC. When required, gill tissue was removed from RNA-later and pulverized in liquid nitrogen with a sterile mortar and pestle. Total RNA was then extracted from the pulverized tissue using Tri Reagent according to the manufacturer's instructions (Sigma-Aldrich, Castle Hill, NSW, Australia). The RNA was re-suspended in 50 μL of DNAse/RNAse free water and quantified using a Nanodrop spectrophotometer (Nd-1000, NanoDrop Technologies, DE, USA) at 230 nm, 260 nm, 280 nm and 320 nm to obtain estimates

of RNA quantity and quality. Total RNA was stored at -80 ºC.

2.4 Reverse transcription

Three μg of template RNA from each oyster, 1 μl of Oligo dT Primer (SPAncRT; 50 μM), 1 μl of dNTP mix (10 mM) and RNase free water (made up to 13 μl) were mixed and heated to 65 ºC for 10 min before being cooled on ice for 1min. Reverse transcription was undertaken using Super Script-III (SS-III) Reverse Transcription kits (Invitrogen, Vic, Australia) with working solutions of 4 μl of first strand buffer (5 X concentration), 1 μl of DTT (0.1 mM), 1 μl of RNaseOut and 1 μl of SS-III RT. Reverse transcription was performed in a Bio-Rad thermocycler at 46 ºC for 1.5 h, followed by 70 ºC for 15 min, and then cooled to 4 ºC. cDNA quality was assessed using a 1 % formaldehyde gels (20 mM MOPS, 5 mM CH_3COONa, 1 mM EDTA, 7.4 % formaldehyde). cDNA samples were stored at -20 ºC.

2.5 Primer design and semi quantitative polymerase chain reactions

The expression of antioxidant genes (glutathione-s-transferase (GST), glutathione peroxidase (GPx), peroxiredoxin (PRx), the generic stress response marker heat shock protein-70 (HSP-70), and the putative housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), elongation factor-1 (EF-1) and 18S was analysed using real-time PCR (qPCR). Primers were designed from nucleotide sequences identified using NCBI BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi) with Primer 3 software (frodo.wi.mit.edu/primer3/). Table 1 summarizes the primers designed. To confirm that the primers amplified single products, semi-quantitative PCR amplifications were performed using 3 μg of each cDNA sample in a 50 μL reaction containing 10 mM of each dNTP, 25 mM

$MgCl_2$, 5 x Taq buffer, Taq polymerase (Invitrogen), and primer pairs made up to 50 μL using RNase free water. The cycling conditions consisted of a 2 min denaturation at 94 °C followed by 34 cycles of heat denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and polymerization at 72 °C for 1 min, and a final extension of 5 min at 72 °C. For each primer set, a negative control without cDNA was performed. Products were run on 0.7 % agarose gels to confirm the presence of a single product for each primer pair.

Table 1. Primer nucleotide sequences.

Primer	Forward (sense)	Reverse (antisense)	Source
peroxiredoxin	CGTTAAAGGCAGCCAATCAT	TGGACAGATGGACGGACATA	GU362543, *P. fucata* (Jiang et al., 2009)
glutathione peroxidase	TCCTGGAGACGGATACGAAC	TCCCTTTGTTTGGCTGTACC	GU362541, *P. fucata* (Jiang et al., 2009)
glutathione-s-transferase	AGCTGGGCGATATAGTGGTG	CCATGGCCATAAAGCAAAAT	GU362542, *P. fucata* (Jiang et al., 2009)
heat shock protein - 70	CGAAAACAAACGAGCAGTGA	ACAAAATGGCTGCCTGTACC	EU822509, *P. fucata* (Wang et al., 2009)
GAPDH	GGGGTGAATGAGGACAGCTA	AGATACAGGCACACGGAAGG	AB205404, *P. fucata* (Takeuchi and Endo 2006)
18S	AGCCCTTTGCTTGTCTCAAA	CGTTTCTCATGCTCCCTCTC	AB205404, *P. fucata* (Zhang et al., 2001)
EF-1	GCGCCTGTCCTTGATTGCCACA	ACGGTCTGCCTCATGTCACGGA	EF502000, *Crassostrea ariakensis* (Yang and Wu 2007)

2.6 Qualitative Real-Time PCR

After confirming that the primers amplified single products, quantitative expression was analyzed using qRT-PCR. Three serial dilutions (1/5, 1/25 and 1/125) were performed for each cDNA sample. qRT-PCR reactions were performed in triplicate using KAPA SYBR FAST qPCR system (KAPA Biosystems Inc. Woburn, MA, USA) on a Mastercycler EP Realplex thermal cycler (Eppendorf, Hamburg,

Germany). One microliter of template was mixed with 0.1 μL of forward and reverse primers and made up to a final volume of 20 μL with KAPA SYBR FAST master mix. qPCR reaction conditions were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 63 °C for 30 s and elongation for 30 s at 72 °C. At the completion of the qPCR reaction, melting curve analysis was performed to detect any non-specific amplification.

2.7 Analysis of qPCR data

Raw Ct values from the serial dilutions for each sample were averaged and fitted to a linear regression. The linear regression was used to calculate the amplification efficiency (E) of each qRT-PCR reaction using the following formula:

$$E = 10 + 1^{(-1/slope)}$$

GAPDH, 18s and EF-1 were tested as potential housekeeping genes. The most stable housekeeping gene was determined using the NormFinder applet for Microsoft Excel (www.mdl.dk/publicationsnormfinder.htm; Andersen et al., 2004). In all cases, GAPDH proved to be the most suitable gene, and was therefore selected as the house keeper (reference) gene for subsequent analyses. For each of the target genes tested, GPx, PRx, GST, HSP-70, GAPDH, 18s and EF-1, individual Ct values were transformed to relative ΔCT values using the following formula:

$$\Delta Ct = Ct_{(target/stress)} - Ct_{(control),}$$

where target/stress represents the average (3 oysters per aquaria) 1/5 dilution Ct value

from treated (air or MA) oysters, subtracted from the average 1/5 dilution Ct value from control oysters from the corresponding aquaria (Pfaffl 2001).
The relative expression/fold difference (ΔΔCt) was then calculated as:

$$\Delta\Delta Ct = E^{\Delta Ct(target)} / E^{\Delta Ct(house\ keeper/GAPDH)}$$

2.8 Statistical analysis

Two-way analysis of variance (ANOVA) followed by Tukey's Post-Hoc multiple comparisons tests were used to analyze differences between untreated controls and air or MA treated oysters using Minitab ver. 16.0 (Minitab Inc., USA, 2011). When statistically significant differences were found, the data were re-analyzed using one-way ANOVAs to test differences between individual time-points. Results were considered significant when $P < 0.05$. Data were also subjected to multivariate analysis using Primer 6 (Primer-6 Ltd., Lvybridge, UK, 2011). The average 1/5-dilution CT values of GPx, PRx and GST for stress treatments were transformed (square root) and plotted against the control at the 60 min and 6 h time-points using Bray-Curtis matrices with multidimensional scaling and hierarchical cluster analysis.

3. Results

3.1 Variation in the expression of individual genes

Exposure to both MA and air after 60 min resulted in a significant ($p < 0.05$) transcriptional down regulation of PRx (by 30.7 % and 28.8 % respectively) and GST (by 57.1 % and 35.9 % respectively) in comparison to controls (Figure 2. A and C). GPx expression declined significantly in oysters exposed to MA (32.2 %) but not air 60 min after exposure (Figure 2. B). HSP-70 expression did not differ significantly in oysters exposed to MA or air after 60 min (Figure 2. D). None of the target genes differed significantly in expression relative to controls 6 h after treatment (Figure 2. A - D).

3.2 Multivariate analysis

Both MDS and hierarchical cluster analysis of combined GPx, PRx, and GST expression data revealed substantial differences between controls and both treatments at both time-points (Figure 3. A and B). Controls tested at 60 min and 6 h clustered together, whereas time after treatment had the greatest effect on oysters subjected to MA and air. Hence, the MA and air treatments clustered together at the 60 min and 6 h time-points. There was a clear distinction between 60 min and 6 h in the combined expression data, with the 6 h treatments lying intermediate between controls and the 60 min time-point.

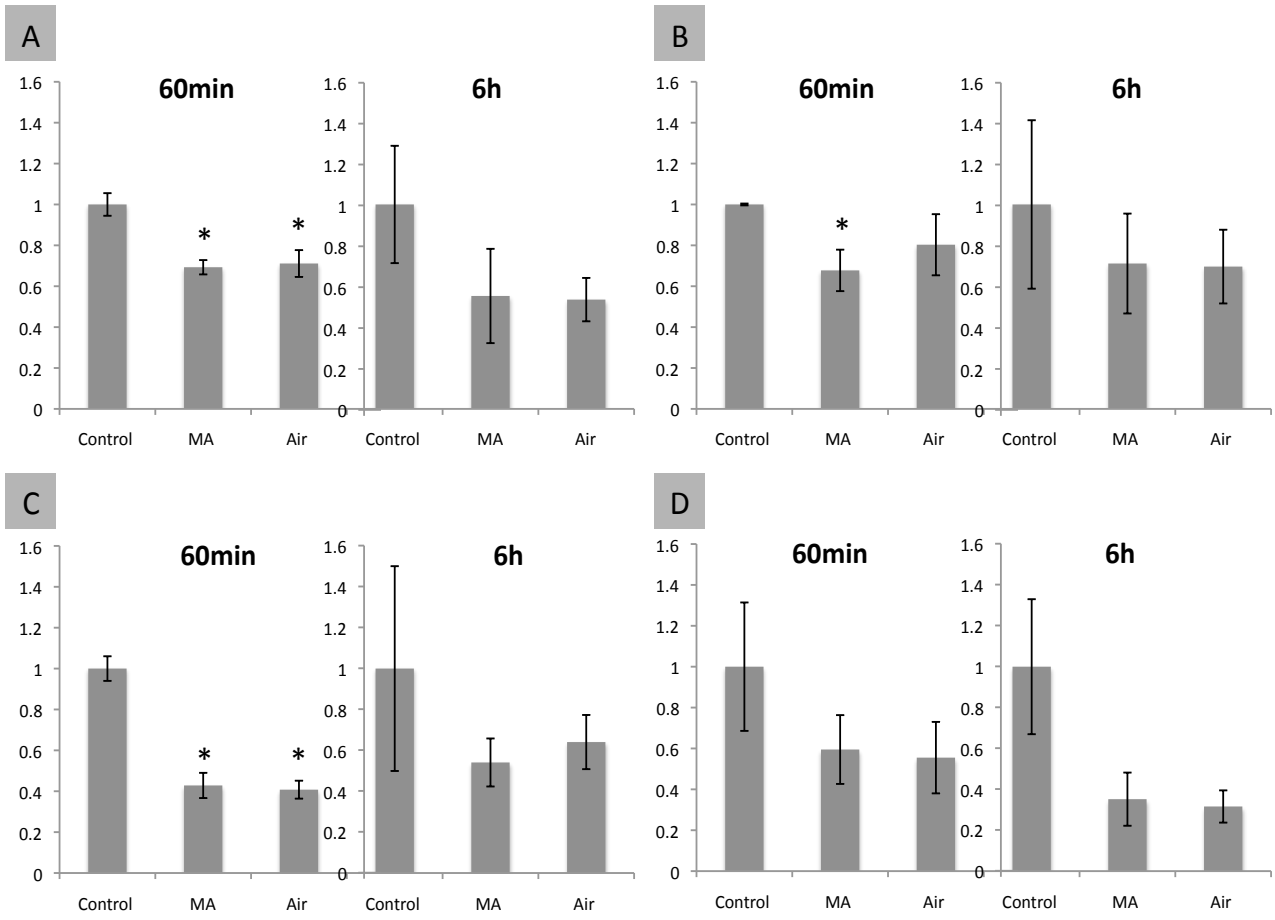

Figure 2. (**A**) Fold change in the expression of peroxiredoxin, (**B**) glutathione peroxidase, (**C**) glutathine-S-transferase, and (**D**) heat shock protein-70. MA; mechanical agitation, * denotes a significant difference relative to the untreated control ($p < 0.05$).

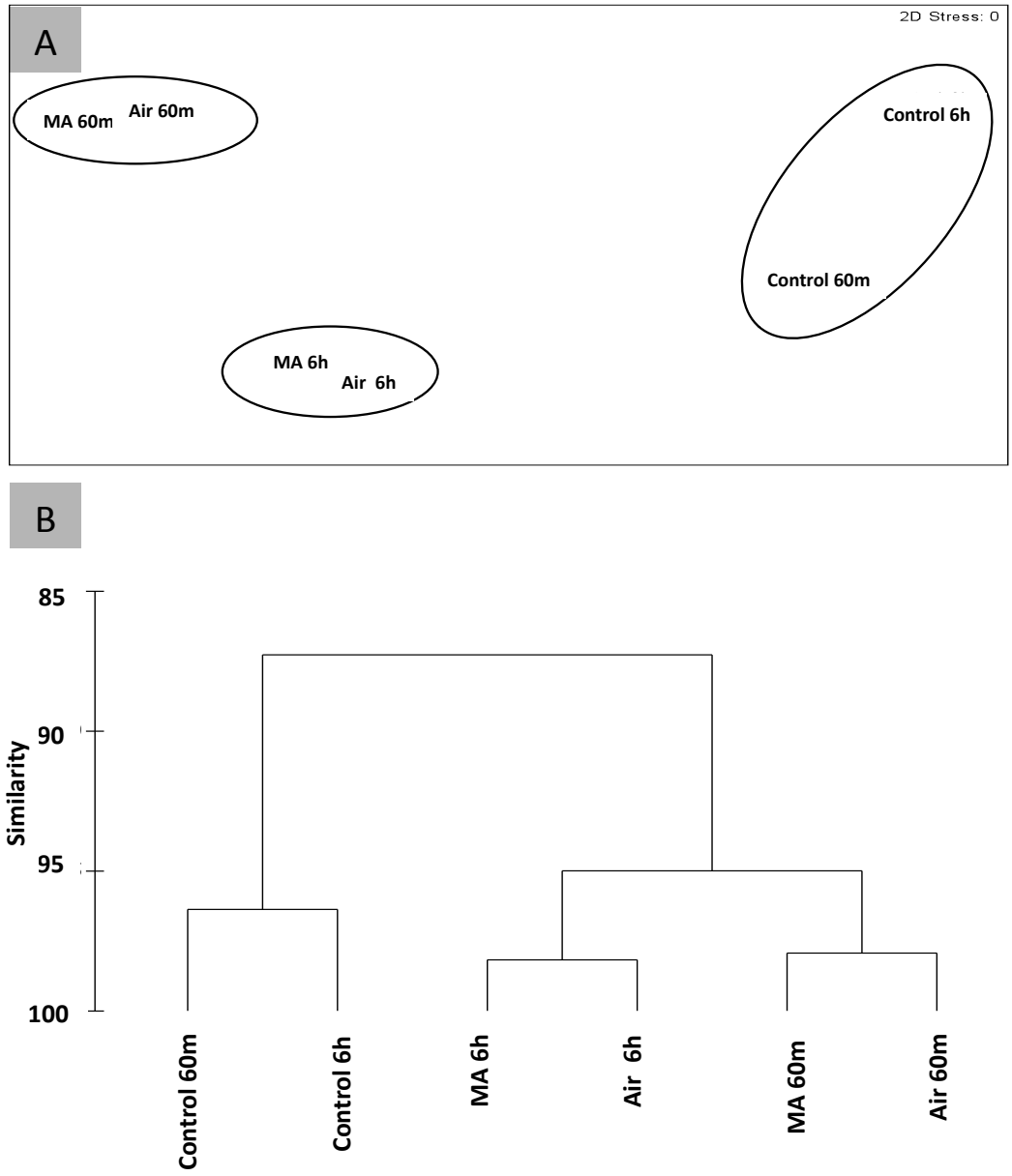

Figure 3. (**A**) multidimensional scaling plot based upon Bray-Curtis matrices of square root transformed data and, (**B**) cluster analysis of the average CT values of glutathione-S-transferase, glutathione peroxidase, peroxiredoxin at 60 min and 6 h**.**

4. Discussion

This study demonstrates that both exposure to air and MA lead to the short-term inhibition of the transcription of antioxidant enzymes in *P. imbricata*. Husbandry and handling commonly associated with pearl oyster aquaculture often introduces these acute forms of stress (Lacoste et al., 2002a). Molluscs possess a range of enzymes involved in antioxidant defence. Among these, GPx (a selenium-dependent enzyme) actively catalyses the reduction of hydroperoxides (ROOH) and H_2O_2 using glutathione as the reducing substrate (Zhang et al., 2011). Similarly, PRx reduces H_2O_2 to $H_2O + O_2$. GPx and PRx are typically distributed throughout the cytoplasm and protect against membrane degradation resulting from oxidative stress. GPx also plays an important role in the immune system and its activity has been documented in a range of invertebrates including in the freshwater bivalve, *Unio tumidus* (Doyen et al., 2006), and the Zhikong scallop, *Chlamys farreri* (Mu et al., 2010; Zhang et al., 2011). GSTs are a ubiquitous group of multifunctional phase II detoxifying enzymes present in all animals, plants, and microorganisms (Kim et al., 2009; Park et al., 2009; Jena et al., 2009). They detoxify potentially harmful electrophillic substrates, such as hydrocarbons, by attaching glutathione (Ketterer et al., 1983; Willett et al., 2000). They also play an important role in the removal of ROS, the regulation of redox balances, as well as stress-mediated cell signaling pathways and apoptosis (Konings and Penninga, 1985; Cho et al., 2001; Wang et al., 2001; Siritantikorn et al., 2007; Kim et al., 2009). Given the crucial function of these enzymes, monitoring their expression in response to stress is rapidly becoming an important component of environmental and ecotoxicological studies (Williams et al., 2003).

Other common molecular biomarkers of environmental stress include HSPs, which are a family of highly conserved molecular chaperones (Clegg et al., 1998).

HSPs maintain protein structure and function in cells by preventing proteins from unfolding or assist in refolding denatured proteins (Morimoto et al., 1994; Hartl, 1996; Clegg et al., 1998). These proteins are synthesized under 'stressful' conditions (not exclusively temperature) or physiological perturbation and have been identified in a range of bivalve molluscs including; *C. gigas* (Farcy et al., 2009), *Mytilus galloprovincialis* (Anestis et al., 2007), *M. edulis* (Chapple et al., 1997), *Bathymodiolus childressi* (Berger and Young 2006), *Ostrea edulis* (Boutet et al., 2003).

We used qRT-PCR to examine the expression of PRx, GPx, GST and HSP-70 in Akoya oysters that had been exposed to air and MA under controlled laboratory conditions. Glutathione-S-transerfase and PRx transcription declined significantly 60 min after oysters were exposed to both air and MA, whilst GPx expression decreased significantly 60 min after oysters were exposed to MA. Similar significant decreases in the expression of antioxidant genes were not evident after 6 h. However, hierarchical cluster and MDS analysis showed that gene expression 6 hrs after stress was clearly distinct from controls, and was intermediate between the controls and the 60 min time-point. In addition, the MDS analysis showed that combined expression of the antioxidant genes after MA and exposure to air did not differ from each other at either time-point. This suggests that exposure to air, which was a component of both treatments, is the main causative agent of stress and that agitation does not have an additional synergistic effect.

Given these observations, responses of antioxidant gene expression to environmental stress indentified in the current study may be due to the death of cells that produce antioxidant enzymes due to NA-induced apoptosis. Alternatively, increases in stress induced NA may directly cause a decrease in the expression of

antioxidant genes, which results in an increase of intracellular ROS, causing oxidative stress that stimulates apoptosis. A number of studies have shown that stress can decrease the expression of antioxidant genes and circulating ROS. In a study by Ahmad et al. (2000) GPx activity was found to be reduced in the catfish, *Channa punctatus,* after exposure to paper mill effluent (1%), while PRx was found to be down-regulated in *Haliotis discus discus* (*H. discus*) upon exposure to haemorrhagic septicaemia virus (VHSV; Nikapitiya et al., 2009). This decline in PRx is of significant immunological interest, as VHSV is a salmonid virus that does not infect abalone.

It has also been shown that the transcriptional down-regulation of antioxidant genes can result in short-term oxidative stress leading to increased apoptotic events decreasing defensive haemocyte populations. In 2002a, Lacoste et al. showed there was a decrease in phagocytosis among *C. gigas* haemocytes exposed to MA. Further studies showed that the stress hormone, noradrenaline (NA), to be implicated with the decrease in defensive cell populations due to apoptosis (Lacoste et al., 2002b). Kuchel and Raftos (2011) also found exposure to NA induced apoptosis among defensive haemocytes in *P. imbricata*. Similarly, Nikapitiya et al. (2009) found that oxidative stress induced by the presence of H_2O_2 in disk abalone, *H. discus*, resulted in apoptotic events, including the relocation of phosphatidylserines from the inner to the outer membrane leaflet as detected by Annexin-V. In cells with up-regulated PRx VI, the amount of H_2O_2 was reportedly reduced, thus protecting against DNA fragmentation.

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CHAPTER SEVEN

THE AKOYA PEARL OYSTER: ENVIRONMENTAL STRESS AND DISEASE

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Preface

There have been numerous disease outbreaks in farmed marine invertebrates over the past few decades. In 1996, the proliferation of Akoya viral disease (AVD) dramatically reduced both wild and farmed populations of *P. imbricata* throughout Asia. It is thought that AVD and the spread of many other marine pathogens is exacerbated by anthropogenic pressures associated with over-crowding and stress caused by fluctuations in the environment, as well as by husbandry and handling. In these epizootics a number of studies have been conducted to investigate the etiological agents and environmental conditions that may influence their relationship. This Chapter synthesises the findings of others, as well as from my research, focusing on how the environment may influence immunological status and the associated spread of opportunistic pathogens.

Chapter 7 of this thesis has been removed due to copyright reasons

CHAPTER EIGHT

GENERAL DISCUSSION

GENERAL DISCUSSION

The relationship between the environment, stress and disease is dynamic and often unpredictable (Le Mollac and Haffner, 2000). The growth of a robust and economically successful pearl industry relies upon favourable conditions that maintain and promote oyster health. The aim of this thesis was to characterize the effects of environmental stress on immunological activity in pearl oysters, in part by studying the relationship between hormonal stress responses and the oyster immune system.

To meet this aim, Chapter Two focused on defining the functional morphology and cytochemisty of *P. imbricata* haemocytes. The characterisation of haemocytes is an imperative first step in defining immunological responses to disease and infection because invertebrate blood cells are primary mediators of host defence. Three types of haemocytes were characterized; granulocytes, hyalinocytes and serous cells, based on the presence/absence of granules, and nucleus to cytoplasm ratios. Granulocytes were the most common cell (62 ± 2.81%), followed by hyalinocytes (36 ± 2.35%), and serous cells (2 ± 0.90%). Both granulocytes and hyalinocytes were involved in phagocytic clearance activities. Granulocytes were more phagocytically active than hyalinocytes, engulfing up to four times the amount of Congo red-stained yeast. Cytochemical stains for phenoloxidase (PO), peroxidase, superoxide, melanin, and neutral red (acidic vesicles), showed that enzymes and metabolites associated with phagocytic activity and intracellular killing were localised in granules within granulocytes (Pipe, 1992; Carballal et al., 1997; Arumugam et al., 2000). Granule size was used to characterize three distinct granulocyte sub-types; small granulocytes (4.00–5.00 μm in diameter) with small granules (0.05–0.5 μm in diameter), large

granulocytes (5.00–9.00 μm in diameter) with large granules (0.50–2.50 μm in diameter), and a combination form (5.00–9.00 μm in diameter) with both large and small granules.

Hyalinocytes were characterized as possessing few or no granules and had a 'silky hyaline' appearance. Although hyalinocytes were involved in phagocytic activities, they lacked many of the hydrolytic enzymes or metabolites associated with intracellular killing. There has been much conjecture associated with the developmental state of these cells (Cheng, 1984). A number of studies have suggested hyalinocytes are a precursor to granulocytes (Mix, 1976). However, the developmental state of hyalinocytes from *P. imbricata* does not appear to reflect the models proposed by other authors. Wen et al. (1994) suggested that the extensive presence of Golgi apparatuses is associated with cellular differentiation, which results from the progressive formation of membrane-bound granules. In our current study, Golgi apparatuses were observed in both granulocytes and hyalinocytes. Hence, the simple presence or absence of Golgi apparatuses may not reflect the developmental state of *P. imbricata* haemocytes.

The third cell type identified was the serous cell, also known as "brown cells" or "rhogocytes". These cells comprised 2.0 ± 0.9% of the total haemocyte population and measured between 5 and 12 μm in diameter in suspension. These cells were densely packed with large, non-fluorescent eosinophilic granules (up to 3.5 μm diameter), that in other species are thought to be composed of a chemical complex similar to that of lipofuscin, mucoprotein, glycoprotein, glycolipid and acid mucopolysaccharides (Haigler, 1964; Cheng and Burton, 1966). It is not clear whether serous cells perform any direct immunological function in *P. imbricata*. They did not stain for defensive enzymes or products, such as PO, peroxidase, melanin or

superoxide, and did not phagocytose Congo red-stained yeast. Serous cells are poorly understood. However, they are thought to function either in waste sequestratoration, or as storage compartments, and are produced by the Keber's gland in *Tapes philippinarum* (Haigler, 1964).

Perturbation in the different haemocyte types of oysters has been associated with environmental stress (Fisher and Newell, 1986). Husbandry and handling associated with aquaculture exposes farmed oysters to repeated and prolonged periods of stress that may be implicated in a range of disease outbreaks and altered immunological activity. For instance, proliferation of the Akoya viral disease (AVD) throughout Japan is thought to be the result of overcrowding and industrialization (O'Connor et al., 2003). Hence, in Chapter Three I investigated the effects of three typical stressors, mechanical agitation (MA), low salinity, and exposure to air on the immunocompetence of *P. imbricata* haemocytes.

The data demonstrated that different environmental stressors had variable effects on total haemocyte counts, total protein content of haemolymph, granulocyte to hyalinocyte ratio, acid phosphatase activity, PO activity and phagocytosis. Both phagocytosis and PO activity decreased significantly when oysters were exposed to all three stressors. Phenoloxidase is a key immunological enzyme involved in the recognition and melanization of invading pathogens, while phagocytosis is the foundation of cell-mediated defense (Cerenius and Söderhall, 2004). In a study by Lacoste et al. (2002a), *C. gigas* exposed to mechanical agitation for 15 min showed a significant decline in total haemocyte counts, phagocytosis and the production of reactive oxygen species (ROS; superoxides and peroxides).

In my study, there were also transient decreases in total haemocyte counts after MA and exposure to air, while significant increases in total haemocyte counts

were evident after exposure to low salinity. The total protein content of haemolymph increased significantly when oysters were stressed by MA and low salinity, but not by exposure to air.

It is possible that these changes in haemocyte frequency and function represent adaptive responses by different haemocyte populations. Previous studies have proposed that during stressful events, circulating haemocytes may migrate from the blood stream to the surrounding tissue responding to mechanical injury or infection (Malham et al., 2003). Alternatively, haemocytes may respond to stressors by providing nutrients and metabolic resources to certain tissues involved in adaptation to stress and survival (Malham et al., 2003).

The effects of physiological stress on the phagolysosomal system identified in Chapter Three did not appear to be consistent between stressors. Acid phosphatase activity increased significantly when oysters were exposed to air, while MA and low salinity caused no significant effects. Also, the numbers of granulocytes in the haemolymph increased significantly when oysters were stressed by hypo-saline conditions, while the relative abundance of granulocytes did not differ significantly after MA or exposure to air. These differences may have differential effects on immunocompetence. In 2006, Butt et al. demonstrated that hyposalinity, associated with estuarine conditions, is directly correlated with QX disease outbreaks in *Saccostrea glomerata*. These authors showed there is a natural infection gradient associated with decreasing salinity in the Hawkesbury River, NSW, Australia, and that low salinity affects a range of immune functions.

Environmental perturbations of the type presented in Chapter Three have also been found to up-regulate the synthesis of stress hormones, such as noradrenaline (NA) and dopamine (DO) in *C. gigas,* and that these hormones alter immune function

(Lacoste et al., 2002a; Malham et al., 2003). Immunological parameters that are known to be affected by these catecholamines (CA's) include the frequency of defensive haemocytes in haemolymph, the production of ROS, PO activity, and phagocytosis (Lacoste et al., 2001b, 2001d, 2002b; Hegaret et al., 2003). Lacoste et al. (2002a) and Aladaileh et al. (2008) also linked the secretion of CA's with an increase in apoptotic events among defensive haemocytes in *C. gigas* and *S. glomerata*, respectively.

Given the key role that haemocytes play in immunocompetence, CA-induced apoptosis of defensive haemocytes may be implicated with immunosuppression and disease susceptibility, and could explain changes in haemocyte populations evident after stress (Lacoste et al., 2002a). For this reason, in Chapter Four, I investigated the effects of NA (0.0, 2.5, 5.0 and 10.0 ng NA/µg protein) on apoptosis in *P. imbricata* haemocytes. Both early and late stage apoptotic markers were used to investigate the effects of NA on haemocytes *in vitro*. The early onset of apoptosis can be identified by a loss of plasma membrane phospholipid asymmetry, whereby membrane phospholipids (i.e., phosphatidylserine; PS) translocate from the inner to the outer membrane leaflet (Watanabe et al., 2002). Late stage apoptosis was identified by DNA fragmentation (TUNEL assay). In my experiments, plasma membrane integrity was found to decrease, while the frequency of TUNEL-positive cells increased with increasing NA concentrations and over time. Noradrenaline-treated cells stained with Annexin-V or TUNEL also displayed characteristic signs of apoptosis, including membrane blebbing and DNA fragmentation, respectively.

Previous studies have implicated cytoskeletal rearrangement with the early onset of apoptotic signal transduction (Van de Water et al., 1996; Ho et al., 2006). Correspondingly, F-actin staining in NA-treated *P. imbricata* haemocytes

demonstrated a disruption in cytoskeletal morphology, which was associated with membrane blebbing and the production of F-actin encased apoptotic bodies. These findings were reinforced by both TEM and SEM. Ultrastructural changes documented in NA-treated haemocytes included condensation of both nuclear chromatin and the cytoplasm, as well as the formation of apoptotic bodies, vacuolization and blebbing. Noradrenaline has also been found to induce similar apoptotic changes in both plasma membrane morphology and nuclear chromatin in *C. gigas* and *S. glomerata* (Lacoste et al., 2002a; Aladaileh et al., 2008).

These changes in both the cytoskeleton and internal morphology were matched by altered immunological function of *P. imbricata* haemocytes. Noradrenaline was found to decrease the ability of haemocytes to adhere to glass slides in both time and dose dependent fashions. Phagocytosis is a process that is dependent on both cytoskeletal rearrangement and the ability of a haemocyte to adhere to pathogens. For this reason, NA synthesis associated with environmental perturbations may affect the defensive function of *P. imbricata* haemocytes *in vivo*.

To elaborate on the findings of Chapters Two to Four in a 'real world' context, in Chapter Five I investigated the effect of husbandry and handling practices in the field on PO activity in both juvenile and adult silver-lip pearl oyster, *P. maxmia*. This work was conducted on a pearl oyster farm in Broome, Western Australia. Due to the technical difficulty of operating in this extremely remote location with limited laboratory resources, phagocytic activity could not be accurately assessed in this experiment. Instead, whole haemolymph samples were preserved and shipped to Macquarie University in order to quantify PO activity.

Adult *P. maxima* were exposed to air, high powered cleaning jets, struck with a steel chisel, or a combination of treatments involving both the chisel and cleaning

jets. The data indicated that there is an initial period of immunosuppression (a decline in PO activity of ≥ 40% in all treatments) among mature oysters up to 48 h after exposure to the stressors. After 48 h, PO levels recovered to almost twice those of the controls in all stress treatments. This response appeared to be a compensatory mechanism, possibly reflecting a recovery of granulocytes in the haemolymph.

Juvenile *P. maxima* were also studied in the field trial. However, they were only exposed to air, because the other treatments were not applicable due to their small size. Phenoloxidase activity of juveniles was significantly up-regulated after both 24 and 96 h in comparison to the controls. This suggests that juvenile oysters mount different responses to stress than adult oysters. Few studies have compared the immunological responses of juvenile and mature bivalves. In 2008, Schleder et al. compared the immunological responses of both mature and juvenile scallops (*Nodipecten nodosus*), exposed to alkaline conditions. Their results showed that PO activity was significantly higher in juvenile *N. nodosus* than mature scallops.

The data in Chapter Five and the previous chapters imply that changes in the immunological parameters of haemolymph after stress could, in part, result from altered gene expression in haemolypmh. To test this, in Chapter Six I measured the expression of genes involved in oxidative and generic stress responses. It was not possible to directly test the expression of PO because gene sequences for this enzyme in oysters are not yet known. Oxidative cellular stress responses are involved in protecting organisms from irreversible damage caused by the excess accumulation of reactive oxygen species (ROS: superoxides and peroxides; Lacoste et al., 2002a). They are also closely associated with phagocytosis. Research on oxidative stress has led to the development and use of a number of common biochemical indicators, most notably stress-associated gene products such as glutathione peroxidase (GPx),

peroxiredoxin (PRx), and glutathione-S-transferase (GST). Heat shock protein-70 is also a common generic biomarker of stress (HSP-70; Clegg et al., 1998; Gestal et al., 2007; Fabbri et al., 2008).

To test expression of these genes, *P. imbricata* were exposed to either MA or air for 60 min and 6 h. After 60 min, both PRx and GST expression declined significantly in both treatments, while GPx transcription declined significantly after exposure to MA, but not air. HSP-70 expression also declined when oysters were exposed to both stressors, but these decreases were not statistically significant. Hierarchical cluster and multidimensional scaling analyses (of GST, GPx and PRx) indicated that the transcriptional inhibition of the antioxidant enzymes resulting from stress was transient and had begun to return to control (unperturbed) levels within 6 h. The data also suggested that exposure to air, which was the common component in both treatments, was the main causative agent of the stress responses, and that agitation (shaking) did not have an additional synergistic effect on the expression of these genes. Given that the expression of HSP-70 was not significantly affected when oysters exposed to MA and air, monitoring antioxidant genes in pearl oysters may yield a more informative suite of biomarkers in response to husbandry and handling stressors.

I propose two models for the decline in antioxidant transcription. First, NA may be secreted in response to stress, resulting in the apoptosis of specific haemocyte populations that produce antioxidants. The decline in the relative frequency of these cells would then decrease the overall expression of GST, PRx and GPx. Alternatively, NA may directly inhibit the expression of the genes that encode for antioxidant enzymes. This could result in the accumulation of ROS/oxidation within cells triggering subsequent apoptosis. Given these scenarios, the regulation and expression

of antioxidant genes may be especially valuable indicators of physiological status in marine bivalves, and could hold great potential for understanding the mechanisms by which oysters respond to environmental and anthropogenic stressors (Dahlhoff, 2004).

The final Chapter of my thesis (Chapter Seven) reviewed the Akoya pearl industry and its development within Australia in order to provide a context for the data in Chapters Two to Six. It details the importance of stress/disease relationships and the potential obstacles that *P. imbricata* industry may face based on experiences with other *Pinctada* species. Most pearl oyster research has focussed on investigating pearl production and on improving the overall pearl quality. However, as my research has implied, eliminating or reducing the incidence of physiological dysfunction by limiting stress, and a better understanding of stress/disease aetiology may also greatly improve the yield of pearls. The industry requires better practices for the identification and elimination of stressful factors that affect bivalve health. In this context, pearl oyster farming could benefit from the development of a rapid 'litmus-like' test that could identify stress levels in oysters. Based on results from these tests, farmers could better interpret the health status of their oysters and alter farming practices accordingly. The data presented in this thesis suggests that simple assays of oxidative enzymes, such as PO, may provide one option to assess stress in the field.

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The following articles were written during my PhD candidature but are not included in the body of my thesis for examination

CHAPTER NINE

SUPPLEMENTARY DATA PART A;

CYTOSKELETAL REARRANGEMENTS IN HUMAN ERYTHROCYTES INDUCED BY SNAKE VENOMS: LIGHT MICROSCOPY OF SHAPES AND NUCLEAR MAGNETIC RESONANCE STUDIES OF MEMBRANE FUNCTION

Cell Biology International. 36 (2012); 87-97.

Preface

As shown in Chapter Four, the stress hormone noradrenaline (NA) was found to alter membrane structure and function in *P. imbricata* haemocytes. In addition to apoptosis, NA exposure resulted in the relocation of phosphatidyl serines (a characteristic event of early stage apoptosis) from the inner to the outer membrane leaflet as well as relocation of intracellular F-actin. In the current Chapter, we investigated the effects of four snake venoms and their impact on the structure and function of erythrocyte membranes. Exposing, erythrocytes to snake venom resulted in the rearrangement of F-actin into highly condensed aggregates. The snake venoms were also found to reduce the anion-exchange function of capnophorin (Band 3), affecting cell morphology.

Chapter 9 of this thesis has been removed due to copyright reasons

CHAPTER TEN

SUPPLENENTARY DATA PART B;

THE ENVIRONMENT, IMMUNITY AND DISEASE IN OYSTERS AND OTHER MARINE INVERTEBRATES.

Preface

As demonstrated in Chapters Three to Six, external influences such as environmental stress, in addition to husbandry and handling can alter the immunological state of farmed marine invertebrates. This article further investigates this relationship in a range of marine species, using the Sydney rock oyster (*Saccostrea glomerata*) and the proliferation of QX disease (*Marteilia sydneyi*) as a model of these interactions.

The Environment, Immunity and Disease in Oysters and other Marine Invertebrates

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Key words: *stress, husbandry and handling, Saccostrea glomerata*

1. Introduction

Aquaculture (the farming of marine organisms) is an increasingly important component of world fisheries output. Worldwide, aquaculture industries had revenues of US$ 106 billion in 2008 (FAO 2010). Despite this, there is an increasing trend for infectious diseases to limit production, and there is evidence that problems with emerging diseases are becoming more frequent. This is particularly true of the edible oyster industry, where a range of protozoan, bacterial and viral infections are having significant impacts on productivity. Whilst there may be many factors involved in the emergence of new disease in oyster aquaculture, a growing body of evidence suggests that environmental stress resulting in the inhibition of oyster immune systems may be at least partially responsible for the trend toward more severe disease impacts on the industry. This chapter discusses the relationship between environmental stress, the oyster immune system and disease, focusing on one well-studied host/parasite model, QX disease in Sydney rock oysters. Below, we describe the Sydney rock oyster industry, the impacts of disease on that industry, evidence that environmental factors influence disease susceptibility, and attempts to counteract these effects through selective breeding programs.

2.The Sydney rock oyster industry

The Sydney rock oyster, *Saccostrea glomerata* (previously *S. commercialis*), has been cultivated in Australian coastal waters for the last 120 years. A decline in natural oyster stocks following European settlement led to the establishment of the first cultivation techniques in the eastern Australian state of New South Wales (NSW) in the 1870's (NSW Fisheries 2000). Currently, Sydney rock oysters are produced on the eastern Australian seaboard from the NSW/Victorian border in the south, to

Moreton Bay in subtropical Queensland (Nell 1993). There has also been some success in farming the species in Western Australia (Nell 2002) (Fig. 1).

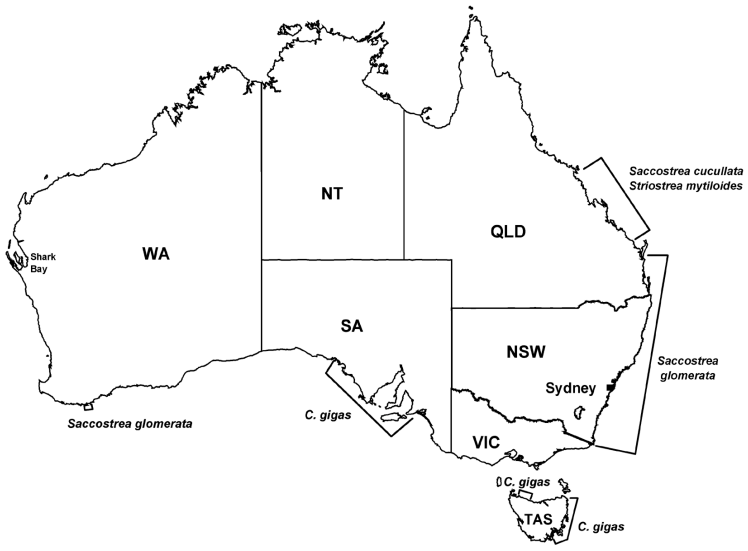

Figure 1. Major oyster growing areas of Australia (Nell 2001a).

Sydney rock oyster farming is the fourth biggest oyster fishery in the world and remains one of Australia's largest aquaculture industries despite production levels having fallen by over 40% since the 1970's (Fig 2; Heasman et al., 2000). This decline has been due in large part to outbreaks of infectious diseases, predominantly Winter Mortality and QX disease. Approximately 7 million dozen Sydney rock oysters were produced nationally during 2004-2005 with production valued at AUD $34 million at the farm gate (DII 2005). This translates into AUD $150-200 million in retail sales. In comparison, the production of Pacific oysters (*Crassostrea gigas*) in NSW during the same period was valued at approximately AUD $2 million (DII 2005). The majority of Pacific oysters are produced in southern Australian waters, with Tasmania and South Australia each producing over 3 million dozen annually. The sale of Sydney rock oysters is almost solely confined to domestic markets. In the

NSW industry, 83% of the oysters produced are sold locally, 17% are sold interstate and the remainder (<1%) are exported to overseas markets (Nell 2001b).

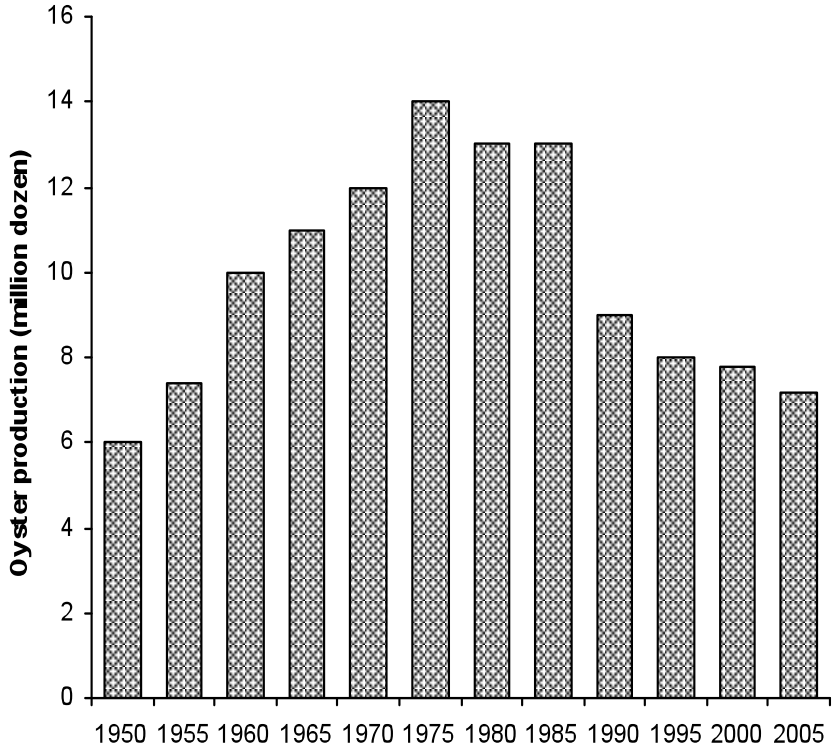

Figure 2. Sydney rock oyster production grew steadily until the mid 1970's when pressure from infectious disease epizootics caused a major decline in the industry. Production levels have stabilized over the past decade (ABARE 2004; DII 2005).

3.Oyster diseases

Since the early 1800's, when overfishing and destruction of natural oyster beds led to the development of oyster farming throughout Europe, disease has been the prominent controlling factor in oyster population dynamics (Roch 1999). Global oyster production is based almost entirely on only five species. Hence, oyster industries are usually local monocultures that are subject to inherent disease

epizootics. Many oyster diseases are caused by protozoan parasites, possibly because estuarine environments where oysters are cultured provide an ideal medium for the dispersal and survival of parasitic sporonts due to their natural currents and stable temperature. Viral disease, primarily mediated by Ostreid herpes viruses, are also becoming an increasing threat to oyster aquaculture worldwide.

Their susceptibility to disease means that the dynamics of the Sydney rock oyster industry are partly controlled by outbreaks of infectious diseases. Currently, oyster production in eastern Australia is afflicted by two major infectious diseases, Winter Mortality and QX disease. Both of these diseases occur only in *S. glomerata.* The transfer of oysters between estuaries for on-growing during the 1960's was originally thought to have aided the spread of both diseases (Nell 2003).

The etiological agent of Winter Mortality was previously known as *Mikrocytos roughleyi*. However, it has now been reclassified as *Bonamia roughleyi* (Cochennec-Laureau et al., 2003). This includes the parasite in a genus that also commonly afflicts European flat oysters. As its name suggests, Winter Mortality predominantly occurs during the colder months from June to August and is restricted to the cooler southern range of Sydney rock oysters. In affected areas, mortalities of up to 80% are common. Oysters in their third winter, just prior to reaching market size, are the most susceptible (Smith et al., 2000). Current methods used to manage the disease include transferring oysters to upstream areas where lower salinities are thought to decrease susceptibility to *M. roughleyi* infection. Many farmers also sell their oysters prior to their third winter when they are most at risk of infection. However, this option is rarely available to growers in the more southerly regions, because oysters in these colder waters do not reach market size until after their fourth year (Smith et al., 2000).

4. Marteilia sydneyi and QX Disease

4.1 *Marteilia sydneyi*

M. sydneyi are protozoans from the phylum *Paramyxea* (Berthe et al., 2004). The genus *Marteilia* is composed of two species; *M. sydneyi* and *M. refringens* (Berthe et al., 2004). *M. sydneyi* is reproductively distinct from *M. refringens*. Their sporangiosori (primary cells, or reproductive plasmodial cells) contain 8-16 sporonts (secondary cells), each of which contain at least two, and sometimes three, mature spores (tertiary cells) (Fig. 3; Berthe et al*.,* 2004). Comparatively, *M. refringens* often produce less sporonts, and therefore, less spores than *M. sydneyi* (Perkins and Wolf, 1976). Aside from this, histological studies have shown that there is no marked difference in the size of mature sporonts between the two species (Perkins and Wolf, 1976).

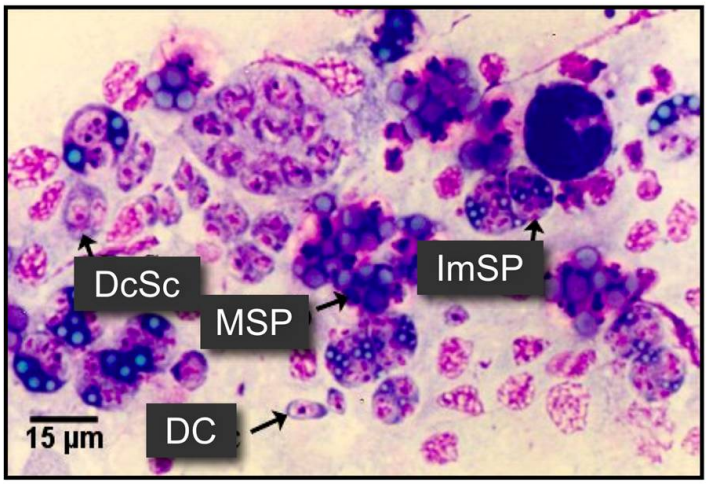

Figure 3. Light micrograph depicting the developmental stages of *M. sydneyi* in the gut of *S. glomerata.* DC, daughter cell; DcSc, daughter cell housing secondary cells; ImSP, immature sporont; MSP, mature sporonts (Kleeman et al*.,* 2002).

4.2 Geographic spread of QX disease

M. sydneyi was initially detected in Sydney rock oysters from the Pumicestone Straight on Moreton Bay, Queensland in 1968 (Roubal et al., 1989). However, the parasite was not classified until 1972, when comparisons were made between it and spores from oysters infected with *M. refringens* (Wolf, 1972; Balouet, 1979). Outbreaks of QX disease were first reported during the late 1970's. These early outbreaks were restricted to a small number of estuaries from The Great Sandy Straight of southern Queensland to the Macleay River in northern NSW (Adlard and Ernst 1995).

After the initial outbreaks in Queensland and northern NSW, QX disease was later discovered in the Georges River, Sydney, in 1994. Examination of *S. glomerata* digestive tract samples taken from Lime Kiln Bar in the Georges River identified tricellular sporonts that were typical of *M. sydneyi* infections (Adlard and Ernst 1995). During 1993/94, over one million dozen oysters were produced in the Georges River. However, consistent seasonal outbreaks of QX disease have caused production to steadily decline in that river so that only 62,000 dozen oysters were produced in 2000/01. This constitutes a fall in production of 94% over 6 years (Nell and Hand 2003). Current production in the Georges River is restricted to the importation of oysters for maturation after the end of the *M. sydneyi* infective season and experimental deployment of *M. sydneyi* resistant strains. Pacific oysters, which are unaffected by QX disease, now reportedly make up 80% of the oysters on the Georges River foreshore (Nell and Hand 2003). This is causing additional problems for the Sydney rock oyster growers remaining on the estuary through increased competition and by catch of Pacific oyster spat.

The Hawkesbury River, approximately 50 km north of the Georges River in NSW, is the most recent growing area to experience an outbreak of QX disease. This estuary had previously been the second largest producer of Sydney rock oysters in Australia. The disease was first detected in the Hawkesbury River during 2004, when an examination of oysters from upstream oyster leases uncovered sporulating *M. sydneyi* in oyster guts. By the end of 2005, Sydney rock oysters in most growing areas of the estuary had suffered mortality rates of up to 90% (Butt and Raftos 2007b).

The development of a sensitive, polymerase chain reaction (PCR) diagnostic assay for *M. sydneyi* has recently been used to test for the presence of the parasite in numerous estuaries along the NSW coastline. Initial tests showed that *M. sydneyi* was present in at least seven estuaries previously thought to be parasite free, including some of the most productive Sydney rock oyster growing areas on the eastern seaboard (Adlard and Wesche 2004) (Fig. 4). Further work by Adlard et al. (pers. comm. 2007) used PCR-based diagnostics to identify *M. sydneyi* in 31 of the 38 oyster growing estuaries in NSW. Even though the parasite is present in these areas, many have not suffered QX disease outbreaks. Based on these data, it is now widely believed that *M. sydneyi* is an endemic parasite of Sydney rock oysters throughout Australia (R. Adlard, pers. com.), and that QX is an opportunistic disease that occurs only when oysters are subjected to environmental stress.

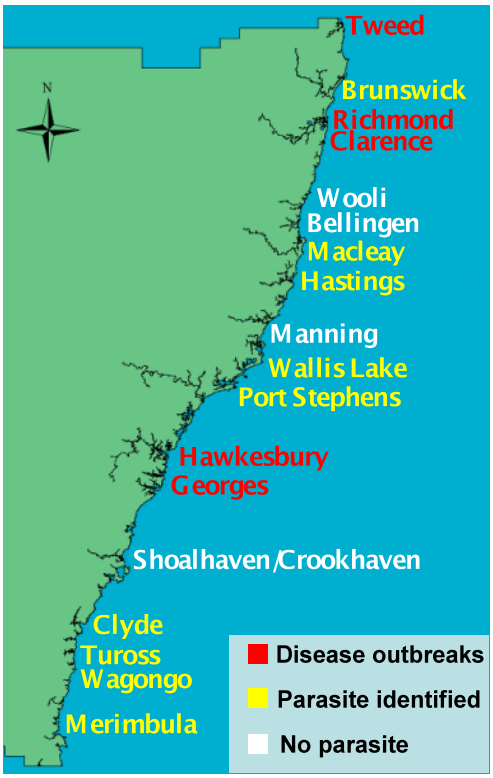

Figure 4. Testing for the presence of *M. sydneyi* in estuaries along the coast of NSW has shown that the parasite is present in a number of oyster growing areas that have not been subjected to QX disease outbreaks. A number of estuaries remain untested (Adlard and Wesche 2004).

4.3 QX disease management

Until recently, a limited understanding of the factors that result in QX disease outbreaks has hindered the development of management strategies to eradicate or control QX disease. Management of *M. sydneyi* is an obvious concern for farming of *S. glomerata*, as outbreaks are a significant threat to long term cultivation and the industry's viability (Berthe et al., 2004).

Grizel (1979) trialled the use of chemicals to control *M. refringens* in *C. gigas* using methylene blue and malachite green. Results from these exposures showed that neither of these chemicals had adverse effects on the parasite or its ability to

proliferate (Grizel, 1979). More recently, Lester (1986) investigated the effects of pyrimethamine and sulphadoxine injections, as well as immersion in trimethoprim, sulphamethoxazole, chloroquine phosphate and pyrimethamine on *M. sydneyi*. Again, none of the treatments were found to be toxic to the parasite.

There have also been suggestions that biological control could be used to eliminate parasite populations. However, a suitable candidate for biological control of *M. sydneyi* has yet to be identified. A hyper-parasite (*Nosema ormiersi*) was reported as a potential control agent for *M. refringens* (Berthe et al*.,* 2004). *N. ormiersi* initiates necrotic changes within the parasite, such as primary cell and sporangial cell degeneration, membrane alterations, cytoplasmic condensation and reduction in spore numbers (Berthe et al., 2004). Unfortunately, further investigations revealed that *N. ormiersi* is not a feasible biological control agent because it cannot be cultured *in vitro*.

In the absence of more effective control measures for either QX or Winter Mortality, Sydney rock oyster stocks are often moved to the upper reaches of estuaries just prior to Winter Mortality outbreaks, because lower salinities in these areas seemed to limit pathogen development (Nell 2003). Similar methods have been implemented to control MSX and Dermo disease in the United States, where low salinity has also been found to limit parasite activity (Hine 1996). Ford et al. (1999) showed that the survival of Eastern oysters infected with Dermo disease is improved in populations grown in warmer waters with high food availability. These environmental factors allow oysters to increase body mass at a faster rate than the pathogen responsible for Dermo disease, *Perkinsus marinus*, can proliferate. This prevents pathogen densities from reaching lethal levels. No such management practices are currently available for the control of QX disease. As a result, research

efforts have focused upon decreasing host susceptibility to the parasite rather than eliminating the parasite from the environment.

In 1997, NSW Fisheries developed a selective breeding program to combat QX disease (Newton et al., 2004). This program focused on interbreeding oysters that had survived QX epizootics (Nell et al., 2000; Newton et al., 2004). Comparisons between non-selected (wild type) controls and selected lines showed that, after two generations, QX-associated mortalities had decreased by 22% in selected oysters (Nell and Hand 2003). Survivorship has continually improved with each subsequent generation of selection. By the fourth generation, mortalities in selected lines from Lime Kiln Bar, the site most heavily affected by QX disease in the Georges River, had decreased to 63% after exposure to consecutive QX seasons. This was an improvement of over 35% compared to the mortality rates of 99% in non-selected controls (Nell and Perkins 2006). Large numbers of hatchery bred QX disease resistant oysters are now being sold to industry.

Similar success in improving disease survivorship through natural selection has been achieved in other oyster species. Eastern oysters have been successfully selected for resistance to MSX disease in the USA (Ford and Haskin 1987; Allen 1998). A similar breeding program was also established to select European flat oysters for resistance to Bonamia infections. Improved survivorship in resistant oysters was purportedly related to the delayed onset of infection compared to susceptible controls (Naciri-Graven et al., 1998). Bonamia resistance in the *O. edulis* was correlated with an increase in the number of haemocytes circulating in the haemolymph. In contrast, a decline in hyalinocytes assisted in MSX resistance in the American Eastern oyster (Naciri-Graven et al., 1998). Naciri-Graven et al. (1998)

suggested that hyalinocytes are targeted by *Bonamia ostreae* infection and that their decline increased the immune competence of *O. edulis* against the parasite.

QX disease resistant (QXR) oysters bred selectivity by the NSW Department of Primary Industries are now know to carry valuable genetic traits that confer disease resistance. Breeding for QX resistance has negatively selected against a specific isoform of the defenisive enzyme, phenoloxidase (PO). That form of the enzyme is designated PO^b (Bezemer et al., 2006). Bezemer et al. (2006) found that over the course of a QX outbreak the frequency of PO^b decreased significantly in both QXR and unselected wild-type oysters. Further analysis showed that PO^b was substantially less frequent in QXR than wild-type oysters (Bezemer et al., 2006). It was concluded that the presence of PO^b increases susceptibility of *S. glomerata* to QX disease, and that other PO isoforms provide resistance against the parasite. This implies that PO is an effective defence system capable of controlling *M. sydneyi* and preventing QX disease.

To test this hypothesis, Butt et al. (2007c) studied the phagocytosis of *M. sydneyi* and the presence of PO in *S. glomerata* haemocytes. They found that PO was concentrated within the granules of haemocytes. Morphometric analysis of haemocytes revealed that QXR haemocytes are larger and have more intense PO staining than haemocytes from wild-type oysters (Butt and Raftos 2007c). Similarly, Kuchel et al. (2010a) compared the intracellular killing activities of both wild-type and QXR haemocytes when exposed to *M. sydneyi*. They found phagolysosomal activity to be significantly increased in selectively bred oysters.

In a recent study by Simonian et al. (2009), proteomic comparisons were made between QXR and wild-type oysters. By using both 2-dimensional electrophoresis and mass spectrometry they identified 6 proteins associated with QX resistance. Two

of the proteins characterized were found to be homologous with superoxide dismutase molecules found in *C. gigas* and *C. virginica*. Similar results were obtained by Green et al. (2010), who used suppression subtraction hybridization to show that superoxide dismutase and other molecules involved in antioxidant defense are upregulated in QXR oysters.

5. Invertebrate immunology

The implication of PO in QX disease resistance suggests that the control of *M. sydneyi* and its progression to cause QX disease is influenced by the host oyster's immune system (Butt et al., 2007b, 2007c). Oysters with intact, functioning immune systems may be able to suppress maturation of *M. sydneyi* from its infective plasmodial form, preventing the development of QX disease. Despite this, there is still relatively little information on oyster immune systems (Nell and Hand, 2003).

Historically, invertebrates have been described as lacking the sophisticated acquired immunological responses present in vertebrates. The absence of specific immunoglobulins means they are incapable of anticipatory immune reactions or of generating pathogen-specific memory (Smith and Söderhäll 1991). However, there is currently some conjecture over these conclusions, with recent research claiming to have discovered forms of specific and adaptive immunity in invertebrate species (Arala-Chaves and Sequeira 2000; Kurtz 2005; Kurtz and Armitage 2006). Work on a range of invertebrates, including sea urchins, insects, and snails has demonstrated that a much greater range of proteins are elicited in response to immunological challenges than would be expected from an innate defence response (Zhang and Loker 2003; Zhang et al., 2004; Watson et al., 2005; Smith et al., 2006). Initial evidence suggests that many of these proteins are members of hyper-variable gene families and may be

part of pathogen-specific recognition systems (Nair et al., 2005; Terwilliger et al., 2006). Such systems might diversify and improve an individual's response to immunological challenges. Most importantly, none of the new hyper-variable defence systems identified so far are closely related to each other.

This recent work has also shown that 'invertebrate immunity' is no longer a sufficient descriptor for immunological defence encompassing various taxa. Previously, immunological function in one invertebrate phylum was inferred from studies of another. However, it is now clear that major differences may occur between immunological function, even within phyla. This highlights the importance of investigating immunology ad hoc in individual species, and stresses that care must be taken when comparing between taxanomic groups (Hooper et al., 2007).

Despite these observations, it is still widely held that most invertebrates rely on 'innate' immune systems. Innate immunity includes cellular responses, whereby a range of circulating cells are involved in phagocytic, cytotoxic and inflammatory activities, and humoral components (Roch 1999). In bivalve molluscs, humoral activity occurs in the haemolymph and results in the extra-haemocytic destruction of invading pathogens. Pattern recognition receptors (PRRs), such as lectins, are primarily responsible for extracellular non-self recognition (Anderson 1996). Lectins recognize carbohydrate moieties on the surface of foreign material, which they can opsonize, promoting phagocytic clearance by circulating haemocytes (Fisher 1986). Lysosomal hydrolases are another important functional group of humoral factors (Anderson and Beaven 2001). These molecules are thought to be released into the haemolymph following the degranulation of haemocytes. Lysosomal hydrolases include lysozyme, acid phosphatase, aminopeptidase and lipase. All of these

molecules are responsible for controlling infection and for the degradation of cell surface integrity among invasive microorganisms (Pipe 1990; Xia et al., 2000).

The cellular component of innate immunity is based on haemocyte function. In a recent study by Aladaileh et al. (2007a), *S. glomerata* was found to have three major haemocyte types; haemoblast-like cells, hyalinocytes and granulocytes. Phagocytosis, by which foreign particulate matter is ingested and destroyed, is one important cellular component of bivalve immunity. Phagocytic haemocytes are often granulocytes, with their degree of granularity correlating with phagocytic efficiency (Anderson 1996; Aladaileh et al., 2007a). When a pathogen is encountered by a blood cell (haemocyte or coelomocyte), it is recognised as "non-self" by a process of molecular "pattern recognition" (Söderhäll and Cerenius, 1998). Most bacterial cell walls contain a rigid component of peptidoglycan. In gram-negative bacteria this is covered by an external layer of lipopolysaccharides (LPS) (Peters and Raftos 2003). Both peptidoglycans and LPS represent pathogen-associated molecular patterns (PAMPs) that are detected by PRRs of the mollusc immune system (Tiscar and Mosca, 2004). Eukaryotic pathogens also express PAMPs in the form of plasmalemma-associated carbohydrates, such as ß-glucans (Fryer and Bayne, 1995). PAMPS are not expressed by invertebrate haemocytes, and so are an ideal target for discriminatory recognition by host PRRs (Jiravanichpaisal et al., 2006). PRRs include lectins, prophenoloxidase (proPO) activating molecules, fibrinogen-related proteins (FREPs) and encapsulation-promoting peptides that are either situated on haemocyte surfaces or are dissolved in the haemolymph (Peters and Raftos 2003).

Phagocytosis also promotes the expression of inducible nitric oxide synthase (NOS), which causes the liberation of antimicrobial nitric oxide from arginine (Lee and Söderhall, 2002). Intracellular killing by oxidative products is assisted by specific

antimicrobial agents localized in lysosomes, including lysozyme, acid phosphatase, and proteases (Maramaras et al., 1996). These enzymes contribute to the final hydrolytic digestion of engulfed microbes (Bugge et al., 2007). Lysosomes also contain pathogen-specific defensins, which disrupt the cellular membrane of phagocytosed microbes. Defensins self-assemble as pores in microbial membranes, thus preventing the microbe from regulating its intracellular environment (Pipe, 1992). All of these processes (oxidative killing, enzymatic degradation and pore formation) occur in the low pH of 2-5 of the phagolysosomes generated by membrane ion pumps (Asokan et al., 1997).

Particles too large to be phagocytosed are often encapsulated by adhesive haemocytes that attach to the surface of the particle. PO produces melanin and its immediate precursors, which are deposited directly onto the encapsulated particles' surface. Once encapsulated, invasive organisms are sequestered and soon die (Sorrentino et al., 2002). Recent evidence suggests that, in addition to hydrolytic intracellular killing systems, PO makes a substantial contribution to intracellular antimicrobial activity in oysters (Munoz et al., 2006). The formation of melanin during encapsulation is the end result of the proPO activating system (Söderhäll 1999). The active enzyme in this system, PO, has been most thoroughly described in arthropods. However, it has also been identified in many other invertebrate taxa, including marine bivalves (Hellio et al., 2006; Munoz et al., 2006). In many organisms, PO is associated with pigmentation. However, it is the enzyme's role in non-self recognition systems and the subsequent synthesis of cytotoxic melanin precursors that has attracted the attention of invertebrate immunologists (Asokan et al., 1997).

ProPO, the inactive proenzyme of PO, occurs in both the haemocytes and haemolymph of bivalve molluscs (Deaton et al., 1999). In arthropods, limited proteolysis cleaves proPO to form active PO. This cleavage occurs in the haemolymph as a result of serine proteolysis (Marmaras et al., 1996). The serine proteases that activate proPO also act as PRR's. They respond to microbial intrusion by detecting signature molecules located on the surface of microorganisms. This system is so sensitive that only a few picograms per litre of LPS from bacteria or β-1,3-glucans from fungi are required to induce proPO activation (Söderhäll and Cerenius 1998; Sung et al., 1998). Even though they are extremely sensitive, the PRR's that activate proPO are also highly specific. β-1,3-glucans from pathogenic fungi can elicit a response, whilst other glucans, that are not associated with fungal pathogens do not (Söderhäll et al., 1994).

Once activated, PO is responsible for catalyzing a cascade of proteolytic reactions, finally resulting in the synthesis of melanin. The reaction series mediated by PO is known to follow two distinct stages (Fig. 5). The first stage involves monophenoloxidase activity where the *o*-hydroxylation of tyrosine produces 3,4-dihydroxyphenylalanine (L-DOPA). In the second stage, diphenols are oxidised to their respective quinones via diphenoloxidase activity (Bai et al., 1997; Yamamoto et al., 2000). The subsequent steps of the cascade involve the binding of free amino acid groups to the newly formed quinones. This creates protein cross-linkages, which eventually result in the formation of stable protein polymers (Sritunyalucksana and Söderhäll 2000). The most significant of these polymers is melanin.

From an immunological perspective, it is important to note that many toxic metabolites with proven fungistatic and antibacterial activity are formed as by-products of melanization. Many of the intermediaries of the PO cascade are also

involved in cellular defensive responses including phagocytosis, clotting and nodule formation (Söderhäll et al., 1994; Peters and Raftos 2003). Recently, it has been discovered that PO activation and subsequent melanisation can be associated with individual phagocytic cells. Hillyer et al. (2003) found that granular haemocytes in mosquitoes were involved in the phagocytosis of bacteria. Once ingested, both the bacteria and the granulocyte became completely melanised. Similar results have also been reported in the tobacco hornworm, *Manduca sexta* and *S. glomerata*, where phagocytic haemocytes were associated with active PO and melanisation (Ling and Yu 2005; Aladaileh et al., 2007b).

The toxicity of PO metabolites means that precise systems exist to regulate the activation and amplification of the PO cascade to avoid deleterious effects on host cells. Serine protease inhibitors in various invertebrates are thought to be the most common regulatory mechanism in the PO cascade (Lanz et al., 1993; Söderhäll and Thornqvist 1997). By preventing serine proteolysis, these inhibitors can stop the partial cleavage of proPO to form active PO. Daquinag et al. (1995) have also identified another enzyme that acts as a competitive inhibitor of PO in the housefly, *Musca domestica*.

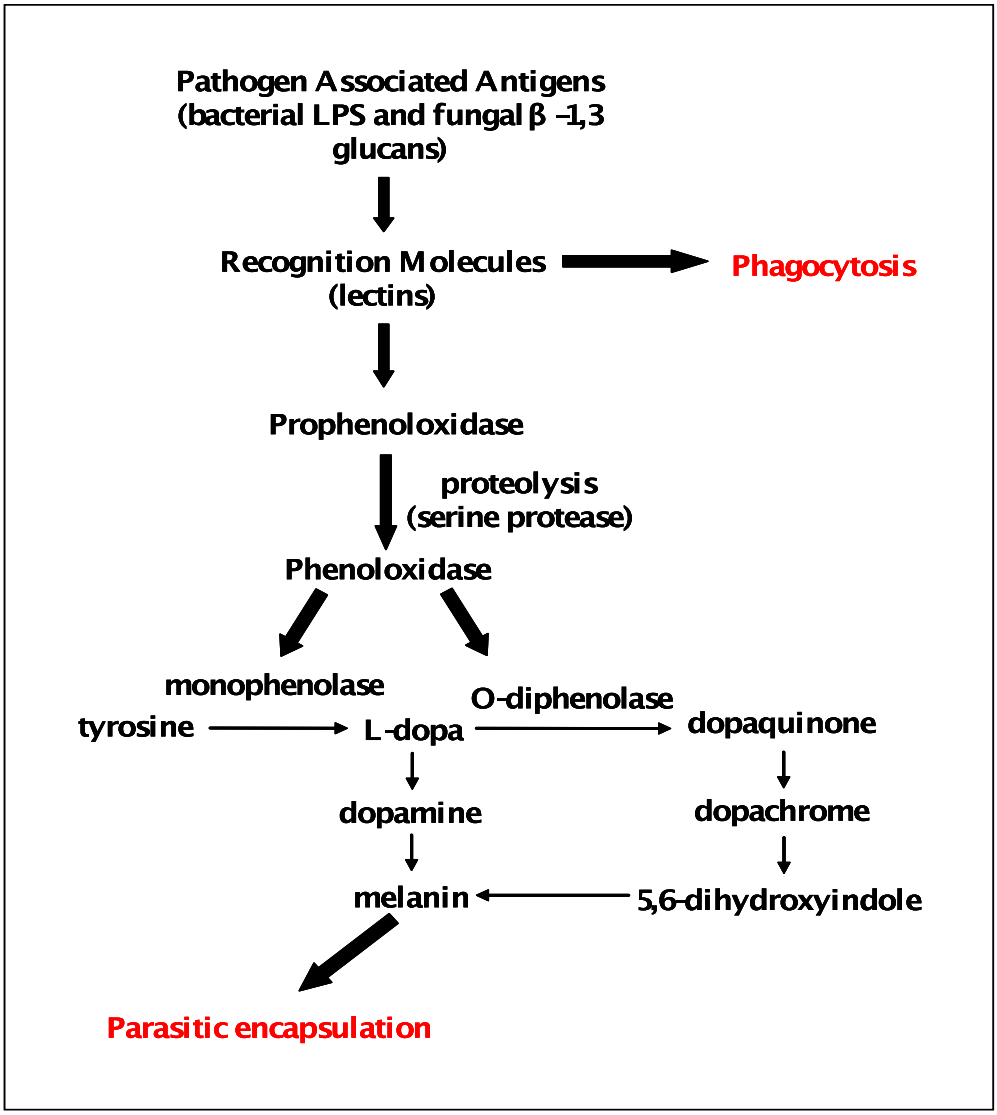

Figure 5. A simplified, schematic diagram of the phenoloxidase cascade (Söderhäll et al., 1994). Inactive prophenoloxidase is cleaved by serine proteases to form the active enzyme, phenoloxidase. Phenoloxidase then catalyses the formation of the pigment melanin and its cytotoxic precursors.

6. The Role of PO in QX Disease

Numerous studies have focused on the role of PO in disease resistance and susceptibility (Sorrentino et al., 2002). A study by Newton et al. (2004) on third generation *S. glomerata* bred for QX disease resistance (QXR oysters) demonstrated that QXR oysters have a marked increase in PO activity compared with unselected

wild-type broodstock, and susceptible oysters are known to carry a particular form of the enzyme (PO^b). Changes in PO activity resulting from environmental stress have also been investigated as a potential explanation for the onset and proliferation of QX disease. Butt et al. (2006a) performed *in vitro* experiments comparing the effects of salinity on immune competence, with specific reference to the PO cascade of Sydney rock oysters. Oysters were exposed to water obtained from regions with severe QX outbreaks, and with artificially diluted oceanic seawater. The seawater collected from infected sites was known to have been affected by heavy rain prior to epizootics and had very low salinities. Both of these treatments elicited a dramatic decline in PO activity (Butt et al., 2006a). The data suggested that a decline in salinity suppresses PO activity and may be associated with the onset of QX epizootics (Butt et al., 2006a).

This conclusion was supported by field trials that exploited the natural salinity gradient in the Georges River, NSW (Butt et al., 2006b). Oysters situated up-stream in less saline conditions had lower PO activity than those closer to the ocean. Oysters at upstream sites were also far more susceptible to QX disease. Butt et al. (2006b) concluded that the stress of low salinity causes PO activity to decline, resulting in opportunistic infection. Similar immunosuppression associated with disease susceptibility has been demonstrated in other marine bivalves, including *M. edulis* (Grundy et al., 1996; Butt et al., 2006b) and the oysters, *C. virginica* (Anderson et al., 1996: Fisher et al., 1999; Butt et al., 2006b) and *C. gigas* (Lacoste et al., 2001a, 2002).

Butt et al., (2007a) also identified a number of additional environmental parameters, including starvation, that can suppress the oyster immune system. The abundance of haemocytes and PO activity declined significantly when the dietary

intake of oysters was halved over a 2-week period (Butt et al., 2007a). These results are comparable to those obtained for other marine invertebrates, such as *C. virginica*) (Hegaret et al., 2004) and *C. gigas* (Zhang and Li, 2006).

7. Environmental stress and immunological activity in oysters

The survival of all organisms depends upon their ability to maintain homeostasis. This balance is perhaps most difficult to maintain in sessile poikilothermic osmoconformers, such as bivalve mollusc. External stressors constantly threaten the physiological steady state of these organisms (Lacoste et al., 2002). Stressors vary widely but include temperature, salinity, pH and other anthropogenic or environmental variables that act at the level of individuals or populations. The estuarine environment, which is home to commercial and wild oyster populations, is prone to extreme environmental changes. These changes are associated with tidal fluctuations and rainfall events, including effluent and sediment loads resulting from upstream runoff. Marine invertebrates require a range of adaptive responses to counteract these stressors. Such responses, or the stressors themselves, often affect the normal function of physiological mechanisms, including the immune system. Immunological suppression, which often results from environmental stress, leaves organisms more susceptible to disease epizootics (Chu et al., 2002).

External stressors may also increase disease susceptibility in invertebrates by elevating the virulence of infectious agents. In Dermo disease, which affects the Eastern oyster, the pathogen (*Perkinsus marinus*), is more virulent at high temperatures and salinities (Chu and Hale 1994). Ironically, this can also provide scope for the management of potential diseases. Hosts can be grown under conditions

that are less suited to pathogen survival, but remain within the physiological tolerance of the host to minimise epizootic disease outbreaks (Hauton et al., 2000).

This link between hosts, pathogens and the environment among aquatic organisms has been acknowledged for some time (Fig. 6). As long ago as 1974, it was recognised that disease outbreaks in fish only occurred when environmental conditions were suitable (Snieszko 1974). However, the mechanistic link between host immunological defence and environmental change has only been established far more recently. Lacoste et al. (2002) have shown that oysters possess a form of neuroendocrine response similar to that activated in vertebrates responding to stress. The principal hormone involved is noradrenaline. Once released into the haemolymph, noradrenaline has been shown to inhibit immunological function.

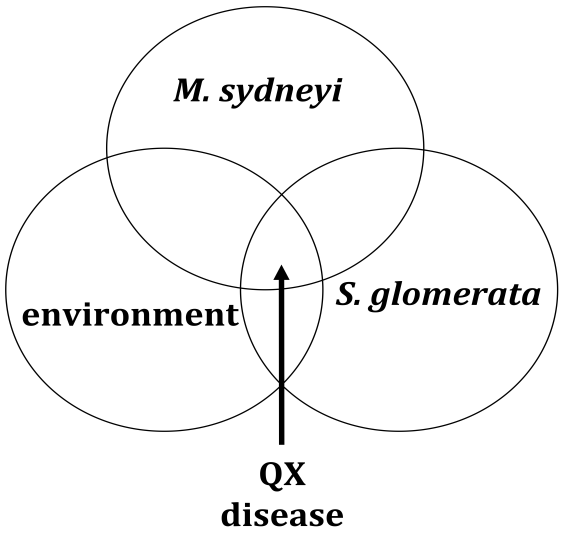

Figure 7. A Venn diagram of the relationship between environmental stress, hosts and disease, using the example of QX disease in Sydney rock oyster hosts. The diagram demonstrates the inter-relationship between host (*S. glomerata*), pathogen (*M. sydneyi*) and the environment that is required before epizootic (QX) disease outbreaks can occur.

Many studies have set out to identify the precise environmental or anthropogenic stressors that affect immunological activity in molluscs (Table 1). Pipe et al. (1999) examined the effects of copper on various immunological parameters in the marine mussel, *M. edulis*. Initial results revealed dose dependant changes in haemocyte numbers after copper exposure. The number of circulating eosinophilic granulocytes decreased when mussels were exposed to elevated copper concentrations. These eosinophilic cells are responsible for most peroxidase, PO and phagocytic activity (Pipe et al., 1997). However, contrary to expectations, decreases in PO and peroxidase activity in copper affected mussels were not statistically significant. This could be explained in part by the large variability in the activity of these enzymes between individual mussels (Pipe et al., 1999).

Further work on the effects of anthropogenic pollutants on the *M. edulis* immune system showed that polycyclic aromatic hydrocarbons (PAH), such as fluoranthene and phenanthrene, inhibit phagocytic activity and damage lysosomes. PAH's accumulate within lysosomes so that damage is caused by direct physical disturbance of the lysosomal membrane. Similar disruption inhibits phagocytosis (Grundy et al., 1996). The combined effects of high temperature and copper exposure also had deleterious effects on *M. edulis* haemocytes. Both total and differential haemocyte counts were affected, as were superoxide and phagocytic activities (Parry and Pipe 2004).

Although evidence suggests that estrogens directly influence gonadal development, gametogenesis and reproductive function in molluscs, information relating to the effects of estrogens and xeno-estrogens on immunological function is limited (Porte et al., 2006). Despite this, evidence from mammalian taxa suggests that estrogens are capable of affecting the function of neutrophil granulocytes, monocytes,

and macrophages. In mammalian systems, these cell types have been reported to express both nuclear and membrane associated estrogen receptors, and their responses to estrogens may also be mediated by rapid non-genomic pathways (Gou et al., 2002). In molluscan models (*Mytilus* spp.), the presence of endogenous 17 β-estradiol has been established in hemolymph and circulating haemocytes, which represent the functional equivalent of the mammalian monocyte/macrophage lineage (Canesi et al., 2004a). These findings, taken together, suggest estrogens/xeno-estrogens may also play a functional role in modulating immune function in molluscs.

Recent studies of mussel (*M. galloprovincialis*) haemocytes have shown that exposure to environmental estrogens cause the extra-cellular release of hydrolytic enzymes, destabilisation of lysosomal membranes and increases in bactericidal activity (Canesi et al., 2004b; Canesi et al., 2004a). Subsequent *in vivo* and *in vitro* studies have also shown significant effects of various estrogenic chemicals on phagocytic and lysozyme activities in *Mytilus* haemocytes (Canesi et al., 2007). Studies on the effects of 17 β-estradiol in a number of clam species have demonstrated that estrogens have the capacity to affect immunological competence, particularly phagocytic activity (Champeau and Narbonne, 2006; Gauthier-Clerc et al., 2006). Overall, it seems that alternative pathways of estrogenic action are also present in molluscs, and that like mammalian immunocytes, mussel hemocytes respond to estrogenic compounds with modulation of immune function.

The Pacific oyster has been investigated to determine the effects of mechanical stress on immunological function. Mechanical agitation was investigated because oyster culture techniques require individuals to be continually sorted and redistributed throughout their development. Lacoste et al. (2002) tested whether such a disturbance made oysters more susceptible to disease outbreaks. They found that

haemocyte migration, phagocytosis and ROI production were all inhibited after continuous shaking for fifteen minutes. These immunological parameters rapidly recovered in the 60-90 min after shaking, possibly as a compensatory or wound healing response. Similar results were reported when abalone (*Haliotus tuberculata)* were exposed to mechanical disturbance. Immediately after agitation, haemocyte numbers, as well as migratory, phagocytic and superoxide activities, were significantly decreased. This was followed by a compensatory increase in most parameters four hours after the stress (Malham et al., 2003). In both of these studies, noradrenaline and dopamine concentrations were measured as stress indicators. Both hormones increased significantly immediately after the onset of the mechanical disturbance. These results suggest a direct link between physical stress, hormonal responses and the manifestation of immunological impairment (Lacoste et al., 2002; Malham et al., 2003).

Table 1. Studies on the effects of various stressors on molluscan immunological parameters (effects cited when statistically significant, $p < 0.05$).

ORGANISM	STRESSOR	IMMUNOLOGICAL EFFECT	REFERENCE
Akoya pearl oyster	Mechanical disturbance, hypo-salinity, air exposure	▪ Inhibited phagocytic and phenoloxidase activity	(Kuchel et al., 2010b)
Clam	Anoxia	▪ Inhibited phagocytic and lysozyme activity ▪ Reduced total haemocyte counts	(Matozzo et al., 2005)
Pacific Oyster	Mechanical disturbance Improved diet Acute temperature and salinity changes Gametogenesis	▪ Reduced phagocytosis ▪ Inhibited reactive oxygen intermediates ▪ Increased oxidative activity and phagocytosis ▪ Increased haemocyte mortalities and reduced aminopeptidase activity ▪ Increased haemocyte mortalities ▪ Impaired phagocytic activity and haemocyte adhesive	(Lacoste et al., 2002) (Delaporte et al., 2003) (Gagnaire et al., 2006) (Delaporte et al., 2006)

		capacity	
	Starvation	▪ Decreased condition index and lysosomal membrane integrity	(Zhang and Li 2006)
Eastern Oyster	Sediment derived pollutants	▪ Increase in infection susceptibility ▪ Enhanced existing infection	(Chu and Hale 1994)
	Tributyltin (TBT)	▪ No effect on phagocytic activity, serum lysozyme or ROI activity	(Anderson 1996)
	Copper	▪ Adverse effects on lysosomal destabilisation	(Ringwood et al., 1998)
	Other contaminants	▪ Increased susceptibility to *Perkinsus marinus* infection ▪ Reduced phagocytosis and hemocyte viability	(Fisher et al., 1999)
Taiwan Abalone	Ammonia	▪ Decreased THC's, PO and phagocytic activity and clearance efficiency ▪ Increased susceptibility to *Vibrio* infection	(Cheng et al., 2004a)
	Heat tolerance	▪ Decreased PO and phagocytic activity ▪ Increase in susceptibility to *Vibrio parahaemolyticus* infection	(Cheng et al., 2004b)
	Low dissolved oxygen concentrations	▪ Decreased THC's, respiratory burst, phagocytic activity and clearance efficiency ▪ Increased susceptibility to *Vibrio* infection	(Cheng et al., 2004c)
Red Abalone	Pentachlorophenol and salinity	▪ Reduced haemocyte adhesive capacity, chemotaxis and phagocytic activity	(Martello et al., 2000)
Common Abalone	Mechanical disturbance	▪ Initial inhibition of haemocyte numbers, migratory activity, phagocytosis and superoxide production ▪ Increase in most parameters post stress	(Malham et al., 2003)
Mussel	Fluoranthene exposure	▪ Increased THC but no affect on proportions of different cells ▪ increase in ROI activity, % of hemocytes exhibiting PO and peroxidase activity	(Coles et al., 1994)
	PAHs	▪ Inhibited phagocytosis ▪ Damaged lysozymes	(Grundy et al., 1996)
	Copper	▪ No change to PO and	(Pipe et al.,

	Temperature and copper	peroxidase activity ▪ Decrease in % of eosinophilic cells ▪ Phagocytosis stimulated ▪ effects on total and differential haemocyte counts, superoxide and phagocytic activity	1999) (Parry and Pipe 2004)
European flat oyster	Salinity	▪ No difference between lysozyme levels ▪ No effect on respiratory burst	(Hauton et al., 2000)
Manila Clam	Improved dietary nutrition	▪ Increased THC's, granulocyte percentage, phagocytic rate and oxidative activity	(Delaporte et al., 2003)

Immunological stress was also investigated in a recent study by Kuchel et al. (2010b), who examined the effects of stressors (mechanical agitation, hypo-saline conditions, and exposure to the air) on haemocytes of Akoya pearl oysters. Oysters were stressed and tested over a five-day period examining; PO activity, total and differential haemocyte counts, phagocytosis, acid phosphatase activity, and total haemolymph protein content. Interestingly, all three stressors induced significant decreases in phagocytic and PO activities (Kuchel et al., 2010b).

Water temperature not only influences oyster physiology, but it also plays a significant role in pathogenic load densities. Outbreaks of bacterial Vibrio infections in pearl oysters are typically associated with tropical conditions. This is because, at water temperatures > 29ºC, bacteria thrive. Vibrios are typically opportunistic pathogens, and so they tend to proliferate when oysters are subjected to stress. In 2009, mass mortalities of *P. fucata* larvae were reported in Indian hatcheries, while similar outbreaks were also recorded on the Cook Islands in *P. margaritifera* (FAO, 2007). In both of these cases, it was concluded that a combination of overstocking and associated stress resulted in the proliferation of a Vibrio sp. (Subhash 2009).

Other studies carried out on oysters have demonstrated very different effects on immunological function results from the type of environmental stress that is invoked. A study of the Eastern oyster, *C. virginica*, found that exposure to various concentrations of Tri-butyl-tin had negligible affects on immunological activity (Anderson et al., 1996). Similarly, exposure to different salinity levels had no detectable affect on lysozyme or respiratory burst activities in the European flat oyster, *O. edulis* (Hauton et al., 2000). This contrasted with results from studies looking at dietary effects on immunological activity in *C. gigas*. Zhang and Li (2006) found that starving oysters for 42 days reduced condition indices and lysosomal membrane integrity. Similarly, a study by Green et al. (2010) investigated the effects of acid sulfate, Al^{3+} and reduced salinity on immunocompetence in *S. glomerata*. They found the expression of the immunological gene, peroxiredoxin, to decrease by 1.7 fold when oysters were exposed to reduced salinity (15ppt). Interestingly, neither acid sulfate, nor Al^{3+} elicited any significant changes in gene expression. In contrast, improving dietary nutrition in Pacific oysters increased oxidative activity and phagocytic clearance rates. These results were also replicated in similar trials using the Manila clam, *Ruditapes philippinarum* (Delaporte et al., 2003).

Internal physiological stresses can also impair immunological activity. Gametogenesis in Pacific oysters has been shown to reduce phagocytic activity and haemocyte function (Delaporte et al., 2006). It is thought that these physiological stresses, associated with broadcast spawning, could explain some of the seasonal variability in immunological activity observed in many molluscan species (Duchemin et al., 2007).

8. The neuroendocrine system and stress

Homeostasis is maintained by both behavioural and physiological responses and it is regulated by the endocrine system (Oehlmann and Schulte-Oehlmann 2003). Invertebrate endocrine systems might lack the complexity of those in vertebrates, but invertebrates do possess discrete endocrine organs that consist of neurosecretory cells (Oehlmann and Schulte-Oehlmann 2003).

Neuroendocrine stress responses in molluscs occur as a coordinated series of metabolic events (Goedken et al., 2005). The corticotrophin releasing hormone (CRH) and adrenocorticotropin hormone (ACTH) are the main mediators of physiological stress responses (Ottaviani et al., 1998). Both CRH and ACTH control the secretion of catecholamines (biogenic amines; CA), such as dopamine (DO) and noradrenaline (NA), which act primarily as neuroregulators (i.e. neurotransmitters and neuromodulators; Lacoste et al., 2001b, 2001c, 2001d). Hormonal messengers, such as DO and NA, have been detected in a range of invertebrates, such as cnidarians (*Renilla koellikeri*), and bivalve mollucs (*Placopecten magellanicus*, *S. glomerata*, *C. gigas and P. imbricata*; Aladaileh et al., 2008; Kuchel et al., 2011). In these species, catecholamines have been found to control respiration, feeding activity, metamorphosis, and reproduction (Sakharov and Salanki 1982; Teyke et al., 1993; Beiras and Widdows 1995; Wang et al., 2006, Aladaleih 2007b).

Lacoste et al. (2001b) conducted a series of experiments to determine the effects of NA on *C. gigas* haemocytes (Lacoste et al., 2001a, 2001b, 2001c, 2001d). Results from their studies indicated that NA has a dose-dependent inhibitory effect on phagocytosis and modulates the production of ROS. Recent studies by Aladaileh et al. (2008) showed that NA injections in *S. glomerata* cause a decrease in PO activity, as well as declines in total haemocyte counts and overall phagocytic activity

(Aladaileh et al., 2008). Kuchel et al. (2011) performed *in vitro* studies investigating the effects of NA on pearl oysters (*Pinctada imbricata)*. They found that NA induces hemocyte apoptosis using the early apoptotic marker, Annexin-V. They also showed that NA disrupts cytoskeletal architecture and results in F-actin rearrangement. Terminal dUTP nick-end labelling (TUNEL) revealed a significant increase in DNA fragmentation in NA-exposed gill tissue, while ultrastructural changes were documented using transmission electron microscopy. These studies support the hypothesis that neuroendocrine responses can compromise immunological function.

It is equally clear that a range of environmental stressors induce the secretion of NA in oysters. Mechanical disturbance in *C. gigas* causes an increase in NA levels, and a corresponding decrease in immunological activity (Lacoste et al., 2001e; Lacoste et al., 2002). Similarly, Aladaileh et al. (2007) showed that NA secretion was stimulated in Sydney rock oysters by altered salinity, temperature fluxes and physical agitation. Assays of the abalone, *H. turberculata*, also identified the presence of both dopamine and noradrenaline within their circulatory systems after a 15-min period of mechanical disturbance (Cheng et al., 2006; Malham et al., 2003). Immune parameters such as total haemocyte counts, migratory activity, phagocytic and respiratory bursts were all found to be compromised by the same treatments (Cheng et al., 2006).

9. Immunological suppression and disease susceptibility

The role and range of environmental stressors involved in the impairment of immunological activity in invertebrates has been established in a variety of species. However, the effects that immunological suppression has on disease susceptibility have only been investigated in a small number of host-pathogen relationships

(Lafferty and Kuris 1999). One study investigated the collapse of the Black abalone (*H. cracherodii*) fishery on the Californian coast. Field studies concluded that the combination of numerous stressors, including increased water temperatures, water pollutants, over-fishing and competition from sea urchins left abalone susceptible to the etiological agent of withered foot syndrome, *Xenohaliotis americanus* (Davis et al., 1992; Lafferty and Kuris 1993). Other laboratory studies using the abalone, *H. diversicolor supertexta*, found that separate exposures to ammonia, high temperatures and low dissolved oxygen concentrations all impaired cellular immunological responses (Table 1). As a result, the abalone in all treatments showed increased susceptibility to infection with *Vibrio parahaemolyticus* (Cheng et al., 2004a; Cheng et al., 2004b; Cheng et al., 2004c).

Disease susceptibility in the Eastern oyster, *C. virginica,* has also been shown to increase in response to introduced pollutants (Chu and Hale 1994; Fisher et al., 1999). However, Chu et al. (2002) concluded that this increased susceptibility may not have been caused by immunological inhibition. Despite testing a range of cellular and humoral immunological parameters, no differences could be detected between control oysters and those exposed to contaminated sediments.

However, in other investigations, many anthropogenic stressors have also been found to induce immunological suppression (Fig. 7). For example, stress induced by husbandry and handling in salmonid fish has been shown to increase the secretion of cortisol (Pickering, 1993). Stress in the Chinook salmon (*Oncorhynchus tshawytscha*) similarly resulted in the secretion of cortisol which was found to decrease immunological activity (Maule et al., 1989).

There is also a link between hormonal stress responses and apoptotic events (Lacoste et al., 2002; Aladaileh et al., 2008). Sokolova et al. 2004 found heavy metals

to alter haemocyte morphology and induce apoptosis in the Eastern oyster, *C. gigas*. Similarly studies investigating the effects of tri-n-butyltin (TBT) and herbicide 2,4-dichlorophenoxy acetic acid on the mussel, *M. galloprovincialis*, were shown to affect haemocyte proliferation and increase the incidence of apoptosis.

Given these findings, a number of studies have examined 'stress indices' as potential indicators of disease susceptibility. Jeffries (1972) found that stressed clams (*Mercenaria mercenaria*) are more likely to be invaded by the pathogen, *Polydora* (Sindermann, 1984). This study focused specifically on amino acid ratios, in particular taurine/glycine ratios of the gill and mantle tissues (Jeffries, 1972). Ratios above 3:1 indicated stress, whilst anything exceeding 5 was an indication of acute stress and increased disease susceptibility (Jeffries, 1972; Sindermann, 1984). Similarly, Feng et al. (1970) used taurine/glycine ratios to examine stress responses and infection in *C. virginica*. Other indices that have been used for the identification and analysis of stress include "scope for growth" (measuring for the potential for somatic growth and gamete production) and oxygen/nitrogen ratios (Corner and Cowry, 1968; Bayne and Scullard, 1977; Sindermann, 1984).

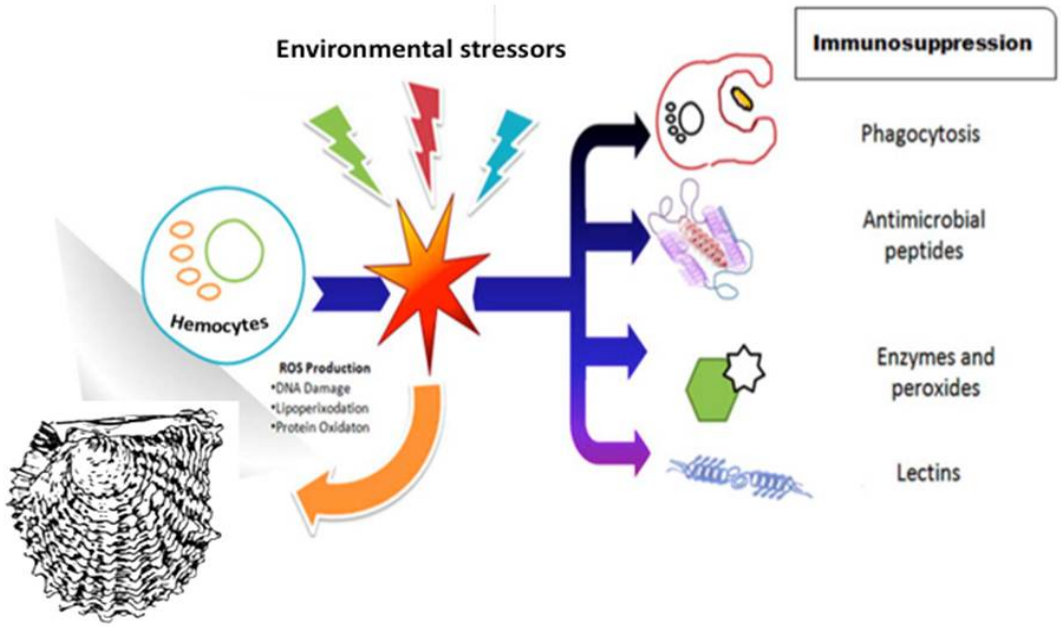

Figure 7. Schematic representation of the effects of environmental stressors on the immune system in oysters. Figure adapted from Perez 2010.

There is also a strong interrelationship between host immunological responses, pathogen virulence and the environment in outbreaks of QX disease among Sydney rock oysters. Initially, environmental pH was tentatively linked to QX disease outbreaks. Early studies of QX disease in southern Queensland suggested that outbreaks occurred after heavy summer rainfall (Haysom 1978; Lester 1986). This provided the basis for studies to determine whether outbreaks of *M. sydneyi* were triggered by a drop in environmental pH, associated with runoff from acid sulphate soils. An initial field study by Anderson et al. (1995) found that *M. sydneyi* infection still occurred during periods when no major pH fluctuations were observed. Even though subsequent work by Wesche (1995) found that infection outbreaks did occur soon after a major drop in environmental pH, no causal relationship between pH and

QX disease outbreaks could be established. It was also demonstrated that a subsequent drop in pH could occur without resulting in an infection outbreak.

Despite this, Peters and Raftos (2003) found that inhibition of immunological function in Sydney rock oysters, specifically decreased PO activity, is associated with outbreaks of QX disease. The fact that PO activity was suppressed in oysters that were not actively infected with *M. sydneyi* suggested that external influences, and not the presence of *M. sydneyi*, were responsible for this immunological inhibition. Subsequent work found a strong link between decreased salinity after heavy summer rainfall, inhibition of PO activity and the onset of QX disease in *S. glomerata* (Butt et al., 2007b). Similarly, Haskin and Ford (1982) found density gradients of the pathogen, *Haplosporidium nelsoni* (the agent of MSX disease) to be directly related to salinity concentrations. At salinities ranging from 9 - 18 p.p.t. the parasite was found to enter *C. virginica*, but its development was severely retarded (Haskin and Ford, 1982).

10. Conclusions and Perspectives

The continuing emergence of infectious diseases is the biggest limiting factor in world aquaculture production. A growing body of evidence suggests that environmental stress plays a major role in the initiation and severity of disease epizootics in aquaculture. In many cases stress seems to inhibit the immunological function of aquaculture species, leaving them susceptible to infectious disease. We are now coming to a mechanistic understanding of that relationship between stress, the immune system and disease. Many marine invertebrates are known to have neuroendocrine systems that are activated by stress, and the activation of these systems often results in suppressed immune function. It seems that there are two

options for aquaculture industries to mitigate the effects of environmental stress on disease susceptibility. The first is to systematically assess their farming practices to eliminate unwarranted stress. The second is to breed disease resistant stock, the result of which is often an enhancement of the immune system.

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