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Stress and immune responses in abalone: limitations in current knowledge and investigative methods based on other models.

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Abstract
Increasing mariculture of abalone focuses attention on their immune and stress responses. For abalone, as well as many invertebrates, the function and relationship of these systems and how *in vitro* tests relate to them are not fully understood. This review focuses on research into the immune system and stress response conducted on abalone and on aspects that can be monitored *in vitro*. To fill the considerable knowledge gaps, we discuss work on other invertebrate taxa, concentrating on those closest to abalone, and making explicit the phylogenetic relations involved. The stress response appears to be very similar to that in vertebrates, but interpreting most immune responses remains problematic. Phylogeny must be considered: immune function tests derived from research into vertebrates or distantly related invertebrates should not be used in abalone until they have been validated in abalone by studies of susceptibility to pathogens. We suggest phagocytic activity of haemocytes and their efficiency in clearing bacteria are reliable parameters to measure, because they have been directly related to immune competency and are consistently depressed by stress. Carefully designed assays of antimicrobial activity may also be useful. Important aims of future research will be to investigate the relationship between growth, stress and robust immunity, and to develop tests that can be run on production animals, which accurately depict immune status.

Key Words: abalone, stress, immunology, invertebrate, *in vitro* assays.

1. Introduction

Invertebrates are increasingly being brought into mariculture, where it is important to monitor immune function and to minimise stresses that could suppress immunity. Our understanding of immune systems and stress responses in abalone is based on limited work on abalone, variable amounts of work on other molluscs (mostly focused on a few gastropods and bivalves), work on other invertebrates and a huge body of work on vertebrates. We do not yet know whether the various *in vitro* tests applicable in vertebrates and/or in other species of invertebrates are good indicators of the immune response in abalone. There is a need to develop relevant *in vitro* tests specific to abalone,

for both laboratory based research and on-farm testing. On-farm health monitoring requires commercial development of test kits based on one or a few parameters that are tightly controlled in the animal, repeatable, cheap and relevant. The background research is far from complete in identifying which stress or immune parameters accurately predict what is happening in the animal itself. The fact that an *in vitro* test in abalone (increased superoxide production after application of a mild stressor) can suggest immune stimulation, but susceptibility to disease increases concurrently, illustrates the importance of this.

The term "stress" has diverse meanings in current usage. It has been used to describe both the stressor and the stress response [1]. The term "stressor" refers to the cause; and "stress" or "stress response" are the terms used for the animal's response to the stressor. Different stressors may produce a different specific response, e.g. bacterial invasion may produce increased phagocytic activity while the presence of a predator may produce neuromuscular reactions involved in flight. In addition to the specific response caused by the stressor however, there is also the generalized stress response. The generalized stress response is a nonspecific pattern of neuroendocrine reactions to a situation that threatens homeostasis [2,3].

The stress response has a complex relationship with disease and has been implicated in disease outbreaks in many animals, including abalone. Table 1 summarises the limited research that has been undertaken to date on stress and/or immune responses in abalone. Recently, a link has been established in abalone between increased stress and decreased immune functional capacity [4-10], leading to increased rates of bacterial infections and increased mortality [6-10]. This link is based on immune function tests carried out after applying stressors such as altered salinity, shaking, decreased dissolved oxygen, increased concentrations of ammonia and nitrate and increased temperature. In abalone and other molluscs, both the stress response and the immune response appear to be centred on the haemocyte, as these blood cells produce mediators of stress and the main immune responses [3,11,12].

2. The Importance of Taxonomy in Assessing the Value of *in-vitro* tests.

Due to the limited work on invertebrates to date, there has been a tendency to fill the gaps in our knowledge about immunity or stress responses of one invertebrate species by using what is known about another invertebrate species. Recent work however, has uncovered major divergences between groups of invertebrate phyla [13]. For this reason, the taxonomic relationships of abalone to other commonly studied invertebrates are shown in Fig. 1. The immune and stress response systems of molluscs and invertebrates from other phyla (e.g. lobsters) do have some similarities, which may have developed prior to evolutionary divergence. Molluscs and lobsters however, are now known to be more distantly related than for example, mammals and sea urchins, so that generalisations about "invertebrate immunity" should be avoided. Work on immunity in other classes of molluscs should also be considered in the light of taxonomic differences, as these all diverged more than 500 million years ago [14], while reptiles and mammals diverged perhaps 350 million years ago. There may be differences even within the Gastropoda.

Extensive recent revisions of the relations between gastropod taxa are described in Beesley *et al*. [15] and shown in Fig 1.

Examples that illustrate the need to pay attention to taxonomy in understanding immune systems have begun to appear. Sea urchins are of the deuterostome lineage and contain a complement cascade not seen in molluscs but comparable to the vertebrate alternative complement pathway [16-20]. Phenoloxidase testing is useful in investigating immune responses in several invertebrate species including arthropods [21,22] and oysters [23] but has yielded mixed results in immune studies of bacterial infection in abalone [6-10,24] suggesting it is of limited use in this species, at least when dealing with bacterial infection. The immune mechanisms used to deal with bacterial, viral and parasitic infections could be different, and we do not know if phenoloxidase activity is important in parasitic or viral infections in abalone. Significant differences in immunity can also be seen among gastropods. Ottaviani *et al.* [25] demonstrated that IL-1α and TNFα increased the motility of haemocytes in *Planorbarius corneus*, a pulmonate in the Heterobranchia, but not in *Viviparus ater*, a caenogastropod. Significant variation has been reported in the immune system even within species; for example granulocytes of genotypically different strains of oysters from different sites may show different lectin binding patterns and react differently to pathogens [26]. If a field project tested only lectins then the results could be misleading.

3. Stress and Immunosuppression in Abalone

Several recent studies have greatly advanced our knowledge of the effect of stress on the immune response of abalone [5-10]. Malham *et al*. [5] demonstrated that after mechanical shaking of *Haliotis tuberculata*, hemolymph concentrations of the stress hormones Noradrenaline and Dopamine rise, then decrease back to basal levels. They reported transient immune depression followed by stimulation. There was an initial depression in haemocyte counts, migratory activity, phagocytosis and superoxide production during the stress, followed by an increase in some immune responses (haemocyte counts, migratory activity and phagocytic ability) after the stress, peaking after 2- 4 hours, followed by a return towards baseline values [5]. Each parameter varied in a different way over time, and intracellular superoxide remained below initial values. The results show that tests at a single time point do not indicate accurately the state of an abalone's immune functional capacity, because the results would depend on whether they were measured at 15 minutes, 2 hours or 4 hours. The series of experiments on *Haliotis diversicolor supertexta* by Cheng *et al*. [6-10] showed a similar period of apparent immune stimulation with milder stressors. During exposure to low levels of the stressor nitrite, haemocyte counts increased for 72 hours followed by decreased counts [7] and there was a transient rise in haemocyte counts for 24 hours during exposure to low ammonia levels, followed by significant decreases [6]. Higher concentrations of ammonia and nitrite both caused decreased haemocyte counts. This indicates mild stressors have a different effect to severe stressors on the immune response.

In each case a stressor was applied, including mild stressors, the experiments by Cheng *et al*. [6-10] showed increased susceptibility to infection by *Vibrio parahaemolyticus* and increased mortality. This is evidence that stress leads to immunosuppression, which may

be exploited by opportunistic pathogens. It also indicates that the apparent immune stimulation seen transiently does not correspond to increased immune functional capacity, because at the same time there was increased susceptibility to infection and increased mortality. For example, abalone exposed to mildly increased nitrite concentrations showed elevated phenoloxidase activity and superoxide production at the same time as increasing mortality in infected abalone. The mortality of the abalone after infection with *V. parahaemolyticus* increased in direct parallel to the nitrite concentration [7]. This indicates that a single parameter of immunity such as phenoloxidase activity or superoxide production taken in a single time-point does not summarise the state of an abalone's immunity.

Cheng *et al*.'s research [6-10] concerned the stressors of elevated temperature, nitrite, ammonia, reduced dissolved oxygen and increased/decreased salinity outside the normal ranges of *H. diversicolor*. As haemocyte counts, phenoloxidase measurements and superoxide production varied as described above, these assays should not be regarded as reliable indicators of immunosuppression in abalone, until the cause and timing of this variation is better understood. By comparison, assays of phagocytosis and efficiency in clearing bacteria were all depressed while abalone were subjected to these stressors. Malham also reported a decreased percentage of phagocytosing cells and a reduced phagocytic rate during application of a shaking stressor [5]. As the phagocytic ability of abalone haemocytes does appear to decrease consistently under stress, and correlates to increased susceptibility and mortality in Cheng's research [6-10], phagocytosis assays may be a reliable single immune parameter, but also require further investigation.

4. Biochemical Links between the Stress Response and Immune System in Molluscs

Vertebrate species rely on nervous, endocrine and immune systems for cell-to-cell communication, with these systems being considerably integrated. Ottaviani & Franceschi [11] point out that a relationship exists between these three systems in various invertebrates, including gastropods and bivalves, but it is still poorly characterised. The stress response originates in the endocrine system, with corticotropin releasing hormone (CRH) stimulating the release of adrenocorticotrophic hormone (ACTH), leading to the release of biogenic amines, which then mediate secondary effects in the molluscs, annelids and insects that have been studied [3,11,12]. The secondary stress response involves metabolic changes in other organs, as the animal responds to the stressor and attempts to maintain homeostasis. There is considerable overlap between the extracellular signals used by the immune and nervous systems in both gastropods and bivalves, and the molecules involved in both systems are present in the haemocytes, as opposed to being spread through multiple organs as seen in vertebrates [11].

The molecules of the stress response in molluscs are comparable to those in mammals: CRH, ACTH, cytokine-like molecules, biogenic amines (noradrenaline, adrenaline and dopamine) and cortisol-like molecules have been found in the haemocytes of gastropods and bivalves [11,27]. The nervous system, haemocytes and cell-free haemolymph contain the neuroactive factors dopamine and noradrenaline. Haemocytes of the Caenogastropod *Viviparus ater* also contain other neuroactive peptides, including substance P, somatostatin and neurotensin [3].

The CRH - ACTH - biogenic amine cascade has not been studied in abalone, but has been studied in other gastropods. The haemolymph of gastropods in two superorders; the pulmonate gastropod *Planorbarius corneus* and the Caenogastropod *Viviparus ater*, when incubated with CRH and ACTH, showed a rapid and marked release of biogenic amines, especially noradrenaline and dopamine [3]. CRH added to the haemolymph provoked a release of ACTH from the haemocytes. CRH and anti-ACTH antibodies did not induce an elevation in biogenic amines, indicating that the ACTH, not the CRH, was responsible for the release of the biogenic amines. This confirms the CRH-ACTH-biogenic amine cascade in these molluscs [3]. Platelet derived growth factor (PDGF) inhibited, whereas transforming growth factor (TGF) stimulated the release of adrenaline and noradrenaline. Neither growth factor had a significant effect on dopamine release. The growth factors influenced each other's effects. When the hemolymph was preincubated with PDGF, adding TGF did not overcome the inhibition on release of biogenic amines. Similarly, preincubation with TGF caused elevation in noradrenaline that was not countered by the subsequent addition of PDGF, though the adrenaline levels were depressed. While added CRH and ACTH both caused increased release of noradrenaline and adrenaline, if added after the growth factors, adrenaline and noradrenaline levels were decreased [12]. These results show an integration of the effects of the molecules involved in the stress response.

Noradrenaline will induce the expression of heat shock protein (hsp) 70 in *Haliotis tuberculata* [28]. Heat shock proteins such as hsp 70 have several functions in mammals, including as chaperones and in stimulating macrophages via toll-like receptors to produce inflammatory cytokines, which are involved in the effector phase of immune responses [29]. ACTH, β-endorphin, the interleukins IL-1α, IL-1β, IL-2, IL-6 and tumor necrosis factor (TNF)α have been demonstrated in haemocytes in both the pulmonate gastropod *P. corneus* and the Caenogastropod *V. ater* [11]. Interleukin-1, IL-6, INF-γ and TNF have been identified in the pulmonate gastropod *Lymnaea stagnalis* [30]. In these gastropods, Interleukin-2 elicited the release of adrenaline and noradrenaline, but this affect was inhibited in the presence of CRH [3]. Similar inhibition was noted with IL-1a, IL-1b, TNFα and TNFβ [3]. This competition may be a feedback mechanism to prevent over-expression, and it shows a relationship between immune and endocrine functions.

Peptide fragments of ACTH 1-24, β-endorphin 1-31 and some cytokines can influence the migration, chemotaxis and phagocytosis *in vitro* of haemocytes in gastropod and bivalve molluscs and in insects [11,31,32]. Ottaviani *et al.* [25] demonstrated that IL-1α and TNFα increased the motility of haemocytes in *P. corneus*, but not *V. ater*. IL-1α, IL-2 and TNFα increased the phagocytic activity and provoked the induction of nitric oxide synthase (NOS) in the haemocytes of both *P. corneus* and *V. ater* [25]. The similar responses of increased phagocytosis and NOS induction but different effect on motility suggest that some cytokine effects are species specific [25]. The ACTH fragments ACTH 1-24, ACTH 1-4, ACTH 4-9, ACTH 1-13, ACTH 1-17 and ACTH 11-24 increase the migratory activity of the haemocytes of *P. corneus* and *V. ater*, with some differences between these species [33]. The whole sequence ACTH 1-39 and the fragment ACTH 4-11 were inhibitory for haemocyte migration [33]. ACTH can induce modifications of the cytoskeleton of the haemocyte in these molluscs, such as rearrangement of microfilament

bundles under the plasma membrane, the concentration of actin in the protruded lamellipods and an increase in microtubules. ACTH also alters cell-adhesive properties and localisation patterns of extracellular fibronectin, which is likely to be involved in the activation and mobilisation of haemocytes during the immune response [11].

Affect of Stress on Growth

Growth rate has been used as a measure of chronic stress. Growth decreases with increased stocking density of farmed abalone [34-38] and is also inhibited by increased metabolic wastes, especially ammonia, which causes stress in abalone [6,37]. Two growth hormone-like substances have been purified from *Haliotis discus hannai* [39]. A growth hormone isolated from the neurosecretory cells in the cerebral ganglia of *Lymnaea stagnalis* stimulated growth of its shell [40] and influenced metabolic pathways, including decreased haemolymph potassium concentration, occasionally enhanced haemolymph glucose concentration, increased storage of polysaccharides in many tissues and a slight decrease in water concentration in some tissues [41]. The effect of stress on the function of these growth hormones has not been examined. Since there is a neuroendocrine link between stress and immune function, there may also be a link between stress and the neuroendocrine system directly affecting growth hormones, as seen in mammals [42].

***In vitro* biochemical tests of stress in Abalone**

The primary stress response is most typically monitored using *in vitro* assays for the neuroendocrine mediators of stress, such as noradrenaline. As noted above, Malham *et al*. [5] demonstrated that after a stressor, noradrenaline and dopamine in *Haliotis tuberculata* haemolymph rose rapidly, then decreased back to basal levels 15-100 minutes after the stressor ceased. Consistent with these findings, Ottaviani and Franceschi [3] have shown that the release of biogenic amines reached their peak after 15 minutes and lasted approximately 45 minutes in the caenogastropod *V. ater* and the pulmonate gastropod *P. corneus*. Thus the timing of *in vitro* assays for these stress hormones is essential and must be standardized for quantitative comparison of the stress response. In vertebrates, elevated levels of glucocorticoids, such as cortisol, are typically used to indicate a primary stress response, due to the extended period over which these stress hormones can be reliably detected in the blood. However, though cortisol-like molecules have been identified in molluscs they have yet to be characterised and an *in vitro* test for chronic stress developed [27].

Due to the apparent brevity of the response of primary stress hormones [3,5], it may be more reliable to monitor the secondary stress response. Several biochemical parameters indicate physiological stress in abalone. Glucose levels in the haemolymph of *H. laevigata* rise after exercise stress and in response to increasing temperature [43]. This is consistent with findings on the heterobranch molluscs *Aplysia dactylomela* [44,45] and *A. californica* [46]. Haldane [43] found no concurrent drop in the glycogen levels during exercise stress, suggesting that this activity is supported by aerobic metabolism, in contrast to Baldwin *et al*. [47], who reported reduced glycogen levels after exercise in *H. iris*, and that anaerobic glycolysis in *H. iris* also results in the production of lactate and a concomitant fall in the pH. pH also dropped after air exposure in *H. iris* [48], and after

both exercise and high temperature stress in *H. laevigata* [43]. Baldwin *et al*. [47] also reported a concomitant increase in D-lactate and the production of tauropine, another end product of anaerobic glycolysis. Other biochemical parameters, such as adenylate energy charge, inosine monophosphate load, or haemolymph ions were not found to be useful indicators of stress in *H. laevigata* [43].

5. The Immune System of Abalone

In vitro Assessment of Haemocytes

The published research on abalone cell counts has used *in vitro* methods developed in other species [5-10], including haemocytometer counts [49] expedited by the use of image analysis software and flow cytometry [50]. Haemocytes start to aggregate in seconds once removed from the animal and various anticoagulants have been trialed to prevent this, including EDTA and modified Alsever's solution [51]. These anticoagulants chelate calcium ions, which are involved in haemocyte immune function [52]. This alters the rate of phagocytosis and production of reactive oxygen species in the bivalves *Mytilus galloprovincialis* [51] and *Crassostrea gigas* [53] and the level of prophenoloxidase activity in the cockroach *Blaberus craniifer* [54]. Though the anticoagulant affects the haemocyte function, stress studies in bivalve molluscs using anticoagulants have shown statistically significant differences between treated and controlled groups, suggesting that anticoagulants do not invalidate the studies using them [53].

Haemacytopenia

Haemocytes are the main defense cell of molluscs and are capable of chemotaxis, antigen recognition, attachment followed by agglutination, phagocytosis, and elimination of invaders by respiratory burst or exocytosis of antimicrobial factors [55]. Physiological functions of molluscan haemocytes include nutrient transport and digestion, shell repair and excretion [56]. Stressed abalone can show a transient drop in haemocyte counts (haemacytopenia) with mild stressors [5,6-10]. The cause of the decreased count is not clear, as none of the possible mechanisms have been investigated in abalone. Shields *et al.* [57] found a decrease in haemocytes in haemolymph of *Haliotis cracherodii* afflicted with ‘withering syndrome’. They reported apoptosis and haemocyte degradation within the haemolymph of afflicted abalone as the cause of the decreased haemocyte numbers. Senescent haemocytes, like other degenerate tissues, are possibly removed by phagocytosis by other haemocytes.

In cases where there is prolonged haemocytopenia, there may also be suppression in the rate of cell proliferation, but the site of haematopoiesis has not yet been established in abalone. Shield's abstract [57] reported increased numbers of "stem cells" in those animals afflicted with ‘withering syndrome’, but these were identified on morphology alone (personal communication) and may not be precursors of fully differentiated haemocytes. An amoebocyte producing organ (APO) has been identified in the pulmonate gastropod *Biomphalaria glabrata*, located between the pericardium and the posterior epithelium of the mantle cavity and in the blood sinuses of the ventricle [55,58-60]. In *L. truncatula* and *L. stagnalis*, haemocytes divide in the haemolymph, in connective tissue and at the site of wound repair, with no well-defined amoebocyte

producing organ being identified [30,61]. Knowledge of the site of haematopoiesis in abalone is required for further investigation into the effects of stress on haemocyte proliferation.

An alternative explanation for the observed haemacytopenia in stressed abalone is that the haemocytes migrate from the haemolymph towards areas prone to injury or pathogen attack [5], but this alone cannot explain the transient haemacytopenia seen during a mild stressor. Haemocytes that migrate towards an inflammatory response to injury would not return to circulation within 10 minutes after initial decline as occurred in Malham's experiments. Transient haemacytopenia would be seen if the cells become more closely associated with the endothelial cells of the vessel wall, thus avoiding being removed during haemolymph extraction, as has been identified in vertebrates [52]; this would probably be mediated through intercellular adhesion molecules as in vertebrates, but these are virtually undefined in molluscs. Unlike vertebrates, both gastropods and bivalves have an open circulation system [14], which is likely to have an impact on the need and type of cell adhesion molecules and traffic of cells in haemolymph. Haemocytes have been reported to be redistributed from connective tissue into haemolymph after injection of particulate material or bacterial infection in *L. stagnalis* [61] and presumably can equally well leave the vasculature, resulting in decreased circulating haemocyte counts. Haemacytopenia will also occur if the haemocytes leave the abalone via migration across epithelial tissues, as seen in oysters and clams [62,63]. Yeast cells, bacterial spores and carbon particles injected into *Crassostrea virginica* were taken up by haemocytes and removed from the tissues as the cells migrated through epithelial tissues to the exterior [62]. Carbon particles injected into the clam *Tridacna maxima* were phagocytosed by circulating haemocytes, transported across the epithelial surface of the digestive organs into the lumen and then eliminated from the organism [63].

Haemocyte Involvement in the Immune Response

Haemocytes migrate towards and aggregate around pathogens in a process called chemotaxis, which is well-characterised in vertebrates [52] and this model may be useful for studies in abalone. It involves directional migration in response to a chemoattractant gradient. There is an initial signal transduction pathway, in which a chemoattractant binds the cell surface receptor, leading to a release of Ca^{++} from intracytoplasmic stores, causing an increase in cytosolic Ca^{++}. This triggers depolarisation of the membrane, followed by repolarisation [52]. Evidence for the existence in abalone of similar signal transduction pathways comes from work on the induction of heat shock protein 70 (hsp 70) in *Haliotis tuberculata* after incubation with noradrenaline [28]. The production of hsp 70 was prevented by a phospholipase C inhibitor, a protein kinase C inhibitor, a Ca^{++} - dependent protein kinase inhibitor and a phosphatidylinositol 3-kinase inhibitor [28].

Phagocytes crawl through tissue, requiring adhesion molecules for reversible adhesiveness to extracellular matrix (ECM) [52]. In vertebrates, numerous adhesion molecules have been described for leukocytes and endothelial cells [52]. In pulmonate gastropods, haemocyte adhesins including integrins and selectins have been identified but not characterized [64,65]. Davids and Yoshino [65] reported differences in the integrin expression between strains of *B. glabrata* that were resistant or sensitive to parasitism by

the human trematode parasite, *Schistosoma mansoni*. In the resistant strains of *B. glabrata*, cell spreading and adhesion were less affected by integrin inhibitor molecules. An actin-myosin network is responsible for movement of the haemocytes and actin microfilaments; and the ECM protein fibronectin has been identified in the mussel *Mytilus galloprovincialis*, with the haemocytes being identified as the producers of fibronectin [66].

Chemotaxins are chemical mediators of chemotaxis [52]. Chemotaxins that have been identified in molluscs include Interleukin-8 (IL-8), Lipopolysaccharide (LPS) and Formyl-methionyl-leucyl-phenylalanine (fMLP) [67-69]. IL-8 identified in the haemocytes of the mussel *Mytilus galloprovincialis* was found to affect conformational change in haemocytes via reorganization of the actin microfilaments, inducing chemotaxis and increasing bacterial phagocytic activity [69].

There is very little published literature on abalone characterising the action of the haemocyte on invading pathogens (Table 1). Numerous studies have been done on other gastropods however, such as the pulmonate *Biomphalaria glabrata*, which is the intermediate host of *S. mansoni*. Electron microscopy has indicated that granular haemocytes of *B. glabrata* are the dominant effector cells in killing *Schistosoma* sporocysts [70,71]. Killing requires only sufficient cells to adhere to less than half the sporocyst's surface. This indicates that killing is an active process, because complete envelopment of the parasite (encapsulation), leading to suffocation or starvation, is not needed [71]. The parasites are killed by *B. glabrata* haemocytes in the absence of haemolymph plasma, indicating that haemocytes can kill in the absence of humoral factors [71]. Nevertheless, injection of cell free plasma from resistant strains decreased infection frequencies in normally susceptible *B. glabrata*, which implies the presence of facilitating humoral immune factors [71].

Some uncertainty exists as to the types of immunocytes in abalone [72]. Sahaphong *et al*. [56] report two main cell types in *Haliotis asinina* haemolymph: the granulocyte (11.43%) and hyalinocyte (88.57%). It is not clear if these arise from separate stem cell populations or represent variations of one haemocyte due to age and differentiation. The granulocyte cytoplasm has a peripheral zone filled with dense granules of various types, which Sahaphong *et al*. [56] consider morphologically similar to vertebrate eosinophil granules. The hyalinocytes had similar organelles to granulocytes and prominent clear zones in the cytoplasm under light microscopy that in electronmicrographs of the cells were consistent with aggregates of glycogen [56]. Stem cells, hyalinocytes and fibrocytes were identified in abalone afflicted with 'withering syndrome' based on morphology, but the cells were not further classified in terms of function [57]. The 'fibrocytes' may simply have been hyalinocytes starting to spread their pseudopodia. Pore cells (rhogocytes) have also been identified in many mollusc taxa, including Polyplacophora, Bivalvia, Cephalopoda and Gastropoda [73,74]. These cells have cytoplasmic invaginations forming sieve-like structures involved in phagocytosis [73]. They phagocytose materials selectively, based on the size that fits into the cytoplasmic sieve and the nature of the material. They will phagocytose foreign material, but the

digestion rate is very low. Their function is in phagocytosis of haemolymph proteins [30,73,74] and in the metabolism of metal ions and respiratory pigments [74].

Abalone also have abundant fixed granulocytes in connective tissues, which may be related to the circulating haemocytes [72], but the relationship between these fixed and circulating phagocytes is not clear. In the Heterobranch gastropod *Lymnaea stagnalis* there is no essential structural or morphologic difference between the two [61]. The fixed phagocytes studied in *L. stagnalis* have intimate connections with collagenous connective tissue fibrils and are actively phagocytic [73]. In molluscs that have been parasitised, the fixed phagocytes are filled with pigment and have been called brown cells [55]. In bivalves, brown cells aggregate in lesions and around parasites and they may be involved in parasite phagocytosis [75]. Brown cells in bivalves appear to have limited ability to degrade phagocytosed material, but may remove such matter by migrating across epithelial surfaces to the exterior of the animal [75].

Investigation into the functional differences of abalone haemocytes has been minimal and it is tempting to try to directly surmise abalone haemocyte organelle function from what has been established in the better studied pulmonate gastropods [30,71,73,76], bivalves [75,77,78] and crustaceans [79]. As might be expected however, there is substantial variation among these invertebrate taxa, both in the names given to different morphologic groups of haemocytes and in the functioning of the haemocyte subtypes.

Recognition of non-self, lectins and agglutination

Discrimination of self from non-self is central to immune function in all organisms. In vertebrates, pattern recognition proteins (PRPs) that recognize conserved motifs in pathogens, called pathogen associated molecular patterns (PAMPS), achieve this [80]. Molecules recognised in pathogens include lipopolysaccarides (LPS) in Gram negative bacteria, peptidoglycans of Gram positive bacterial cell walls, Beta-1, 3-glucans of fungal cell walls and the double stranded RNA of viruses [52]. In molluscs, attachment of foreign particles to the haemocyte membrane may occur directly or be mediated via opsonins or agglutinins, which are commonly lectins [81]. Lectins are multimeric glycoprotein complexes that carry several sites for binding targets and are able to recognise and bind to "non-self". After the lectin binds the target cell or particle, a conformational change occurs, which makes available binding sites on the lectin. The haemocyte membrane receptors then can interact with the revealed binding site on the lectin [81].

The haemolymph from an abalone species, *Haliotis asinina*, is reported to agglutinate chicken and mouse erythrocytes but not guinea pig, rat or rabbit erythrocytes [81], but the pattern recognition proteins, agglutinins and opsonins involved in the abalone immune system are yet to be characterized. The humoral factors involved in opsonising and agglutinating foreign material in the pulmonate *Lymnaea stagnalis* are produced within the haemocyte and incorporated into the plasma membrane [73]. A member of the Immunoglobulin superfamily identified in *L. stagnalis* and called molluscan defense molecule (MDM) is also produced by granulocytes and is possibly an opsonin involved as a mediator in non-self recognition [82]. Specific agglutinin receptors have been

identified on haemocytes of the pulmonate land snail *Helix pomatia* [83]. An agglutinin is essential to the chemotactic response of the caenogastropod *Viviparus malleatus* haemocytes towards killed *Staphylococcus aureus* cells [84]. The fresh water pulmonate *Biomphalaria glabrata* contains lectins that bind to yeasts, thus targeting them for phagocytosis [85]. *B. glabrata* haemocytes recognised galactose, mannose and fucose; and responded by producing reactive oxygen species. Fibrinogen related peptides (FREPs) in *B. glabrata* are another family of haemolymph proteins that have lectin-like properties, allowing them to precipitate soluble antigens derived from trematode parasites [86]. FREPs contain immunoglobulin domains and are produced in increased abundance after infection with the trematode *Schistosoma mansoni*. The immunoglobulin domain of the FREP3 subfamily is very diverse, implying diversification of non-self recognition molecules functioning in immune defense [86].

Plasma of some bivalve species contains at least two lectins [81,88], composed of subunits differing in mass or isoelectric point. Differing subunits are capable of binding several monosaccarides with different affinity, resulting in a wider spectrum of targets. Unlike bivalve lectins, Achatinin, a lectin of the giant African pulmonate snail *Achatina fulica*, consists of subunits of the same type and is strongly specific to a certain monosaccharide. Achatinin binds specifically to 9-O-acetylsialic acid [81]. Yakovleva *et al.* [81] state that bivalve species have higher agglutination titres than gastropod species, with some gastropods such as *Littorina littorea*, a marine Caenogastropod, having no detectable agglutination ability. The more potent agglutination capacity of Bivalvia is possibly due to a higher concentration of plasma agglutinins and polyreactivity. Since the different classes of molluscs all efficiently remove foreign matter, plasma agglutinating activity must not be the only measure of the efficiency of an immune system.

Agglutinating activity in the haemolymph of invertebrates is most typically studied using *in vitro* assays that assess the clumping of vertebrate red blood cells, e.g. [49]. Given the high specificity of reactivity towards different mammalian erythrocytes [81], the relevance of these assays for assessing agglutination ability towards pathogenic diseases is questionable. A more valid approach would be to directly assess the agglutination of relevant pathogens. *In vitro* agglutination assay kits currently exist for a number of human and agricultural pathogens, but these are yet to be developed for any marine pathogens. Nevertheless, a simple assay could be developed to establish the number of cells remaining in suspension after treatment with the haemolymph plasma.

Phagocytosis

Phagocytosis has not been specifically characterized in abalone, but has been well studied in pulmonate gastropods, the Caenogastropod *Viviparus*, and oysters. It proceeds through well-defined stages, including recognition, chemotaxis, attachment, ingestion and destruction of foreign particles [30,55,71,81,88-90]. After internalisation, foreign particles are enclosed in a primary phagosome, which then fuses with lysosomes to form a phagolysosome. The activation of lysosomes is Ca^{++}/phospholipase A dependent [91]. Foreign agents are enclosed within the phagolysosome to destroy, degrade and eliminate them. Two main systems will do this in gastropods: oxygen dependent systems (respiratory burst) and oxygen independent systems (discussed below).

Undigestible particles, after phagocytosis, may be removed to the external environment by diapedesis across epithelial surfaces as demonstrated in oysters and clams [62,63], but this has not been demonstrated in abalone. Alternately, sequestration is known in gastropods, where the phagocyte becomes metabolically quiescent [88]. Objects too big to be phagocytosed are encapsulated by multiple layers of haemocytes, then phagocytosed bit by bit [92]. An ultrastructural study on *Perkinsus* infection of clams showed encapsulation leading to nodule formation [93]. Encapsulation of *Perkinsus* trophozoites was followed by haemocyte lysis and degeneration, with some of the released granules coalescing to form megagranules. Trophozoites were present within these nodules [93]. This process appears to be analogous to the processing of foreign material in vertebrates by macrophages, leading to granuloma formation in response to agents that cannot be properly eliminated.

***In vitro* assays of phagocytosis**

Phagocytic activity can be recorded by measuring the proportion of ingested particles or the proportion of cells that have ingested labeled particles [5,94]. Either adhered cells or suspended cells may be examined. Haemocytes are easily manipulated when adhered to a glass or plastic surface. As the cell adheres, it commonly spreads and its morphology changes from roughly spherical with short or unapparent pseudopodia to a flattened cell with long pseudopodia extended over the surface [88]. Integral membrane proteins are likely to redistribute during the spreading process, as this is the scenario in chemotaxis and adherence in mammalian phagocytes [52,88]. This redistribution is likely to also include receptors for foreign agents, decreasing their availability on the cell surface away from the solid surface [88]. Therefore, adherent cells may either be more active through upregulation of adhesion molecules, or less active through removal of receptors from available cell surfaces, and consequently comparison of studies on phagocytosis can be problematic, unless methods are identical.

Other assays involving the use of flow cytometry have been developed to examine phagocytic activity of haemocytes in suspension [5,49,95], but there are different problems with these. In particular, there is a need to incorporate an anticoagulant, as discussed previously.

Respiratory Burst

During phagocytosis in all animals, there is an increase in oxygen consumption and an increase in production of reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2): the so-called respiratory burst that occurs within the phagolysosome. Slauson & Cooper [52] reviewed the pathway of production of reactive oxygen species, which commences with activation of the NADPH oxidase complex in the phagocyte lysosomal plasma membrane. The relative cytotoxic roles of specific oxidants vary depending on the nature of infectious agents and their defense mechanisms [52,71].

These oxidants react in processes associated with photon-generating oxidation-reduction reactions that can be measured using *in vitro* assays. The light emitted can be detected by chemiluminescence [96]. This method has been used in abalone to test for reactive

oxygen species. Malham *et al*. [5], used chemiluminescence to test for respiratory burst, as well as directly measuring superoxide anion via the reduction of nitroblue tetrazolium. Cheng *et al.* [6-10] also used the nitroblue tetrazolium assay. Lambert *et al*. [53] developed an alternative procedure for measuring respiratory burst in oyster haemocytes using flow cytometry. This paper highlights the importance of initiating the oxidative burst process prior to analysis using suitable activating reagents. The commonly used chemical reagent phorbol myristate acetate was found to be unsuccessful in activating oxidation, whereas zymosan particles triggered a good response. Lambert *et al*. [53] also reported inhibition of the oxidative process by their anti-coagulant solution, so that samples had to be analyzed immediately with minimal handling using this procedure. The need to filter the samples to remove clumped cells also meant that it is not clear if different subgroups of phagocytes were removed and accurate cell counts could not be obtained simultaneously with the assessment of oxidative metabolism using flow cytometry. Thus there appears to be little advantage of using this technique over the more straightforward *in vitro* assay using nitroblue tetrazolium reduction.

Using the chemiluminescence assay, *H. cracherodii* were found to produce more ROS than *H. rufescens* after applying a salinity stressor combined with the environmental pollutant pentachlorophenol [4]. Varying concentrations of scavengers responsible for neutralising or packaging reactive oxygen species may account for the varying rate of ROS production between these two abalone species after exposure to similar stressors [4]. There has been no further characterization of respiratory burst in abalone, but other gastropods have been investigated. H_2O_2 and cytotoxic NO are mainly responsible for killing the sporocysts of *S. mansoni* by resistant strains of *B. glabrata* [71]. Scavengers for O_2^-, HOCl, $^{\cdot}OH$ and $^1O_2$ did not protect sporocysts from being killed [71], suggesting that these ROS did not play a significant role in killing phagocytosed and encapsulated parasites. Haemocytes in mussels have been shown to produce the potent oxidant, peroxynitrite [97]. Clams and mussels have also been found to contain antioxidants (superoxide dismutase, catalase and glutathione peroxidase) that will protect against oxidative stress [98]. Thus, when the stressor is a toxin, as in the study on abalone by Martello & Tjeerdema [4], it is not known whether the increase in ROS is due to augmented production or suppression of inhibitory antioxidant enzymes that may reflect some level of cellular damage. Further research on the regulatory role of these enzymes is required in abalone.

Non-oxygen dependent cytotoxicity and antimicrobial activity

Non-oxygen dependent cytotoxic or antimicrobial activity can be found in haemocytes and in hemolymph of abalone [24,99-104]. The granules of abalone haemocytes have not been thoroughly investigated as to their content, but they are probably lysosomes. Lysosomes contain a variety of cytotoxic and microbicidal enzymes, but there is evidence that their contents vary between molluscan species. Lysozyme, acid phosphatase, arylsulphate, peroxidase, non-specific esterase, elastase, cathepsin B and G and B-glucuronidase have been identified in haemocytes of various species of molluscs [30,76,105-109]. Four types of antibacterial and antifungal peptides have been isolated from haemocyte granules in the bivalve *Mytilus edulis* [110,111]. Bioassay guided

fractionation of abalone haemolymph, for example using the MTS assay, is required to determine if similar antimicrobial compounds are present.

Studies on non cellular immune responses in abalone are rare (Table 1). The most recent studies [24] have investigated the levels of acid phosphatase (ACP), alkaline phosphatase (ALP), superoxide dismutase (SOD), lysozyme and phenoloxidase in cell-free haemolymph of abalone infected with pathogenic and non-pathogenic bacteria. In the research of Shuhong *et al* [24], abalone injected with the pathogen *Vibrio parahaemolyticus* had increased haemolymph levels of ACP and ALP and decreased SOD. No significant differences were seen in the abalone injected with a non-pathogenic *E. coli*, suggesting that abalone immune responses can distinguish between pathogenic and non-pathogenic bacteria. Lysozyme and phenoloxidase activities were low in both treated and control groups, so perhaps these antimicrobial factors are not important in abalone immunity to bacteria [24].

Other studies on abalone haemolymph have demonstrated the presence of both antibacterial [99-103] and antiviral activities [104]. Cushing *et al*. [112] demonstrated that the antibacterial activity in the haemolymph of three species of abalone is inducible. Preimmunizing the animals with killed bacteria led to a rapid rise in haemolymph bactericidal activity that peaked in a few days before subsiding after five to eight days. Preimmunization may be useful for examining immune response to infection, as opposed to simply screening for activity. It also facilitates the isolation and identification of antimicrobial factors, by increasing the concentrations that can be extracted from the haemolymph. The bactericidal factors have not been identified, although Li [99] suggested the factor (or factors) are most likely proteins. Conversely, Vakalia [102] found activity in lipophilic extracts of the haemolymph and reported that the haemolymph activity is only slightly diminished by treatment with peptidases.

A wide range of different assays are currently available for assessing antimicrobial activity, but some are much more reliable and suited to screening haemolymph samples than others. The CellTitre 96® AQ_{ueous} One Solution Cell Proliferation was originally developed for cytoxicity screening against human cell lines [113], but more recently this assay has been used to examine the effects of molluscan haemolymph against marine bacterial pathogens [100,102,103]. This cell proliferation assay is based on the reduction of the MTS tetrazolium compound to a red formazan product by dehydrogenase enzymes in metabolically active cells. This was found to be a reliable indicator, whereas fluorescein diacetate was hydrolysed by residual esterase activity in the haemolymph and thus unreliable [100]. Turbidometric or liquid growth inhibition assays that do not use colour indicators [e.g. 114,115] are also unreliable due to the variable background absorbance of haemolymph. The zone of inhibition assay [e.g. 116] cannot be used to detect activity in haemolymph samples, unless the active factors are solvent extracted and concentrated prior to testing, and even then it can be unreliable for lipophilic active compounds [117].

Prophenoloxidase system

The prophenoloxidase (proPO) system is directly involved in non-self recognition and defense. It is well characterised in arthropods [21,22], but has also been investigated in other invertebrate groups, including the bivalves *Venus antiqua* [118], *Mytilus edulis* [119] and *Perna viridis*, where *in vitro* activation by exogenous proteases has been described, including trypsin, α-chymotrypsin and the detergent sodium dodecyl sulphate [120]. Prophenoloxidase and the activating enzymes are stored in granules in the haemocytes and their release is stimulated when the haemocyte is activated.

Evidence for the importance of phenoloxidase in molluscs stems from a study by Peters and Raftos [23], who found that phenoloxidase was suppressed in QX disease outbreaks among Sydney rock oysters (*Saccostrea glomerata*). Rock oysters selected for resistance to QX disease had significantly higher levels of phenoloxidase activity than unselected wild type oysters and the resistant oysters were shown to possess a novel isoform of the enzyme phenoloxidase [121]. This implies a genetic basis for the enhanced level of phenoloxidase activity in oysters. Studies on the Pacific oyster (*Crassostrea gigas*) have also shown significant differences in the levels of phenoloxidase activity between family lines [122]. Thus selective breeding in molluscan aquaculture could provide improved resistance to disease via enhanced immune functions, such as phenoloxidase activity.

Colorimetric enzyme assays can be used for the determination of phenoloxidase activity *in vitro*. In previous research on bivalve molluscs, L-3,4-dihydroxyphenylalanine (L-DOPA) has been used as the primary substrate. Phenoloxidase however, catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). Consequently, Espin *et al*. [123] have developed a method for the determination of monophenolase activity, which should distinguish phenoloxidase from other phenol-oxidizing enzymes, such as laccase (oxidizes only diphenols) and peroxidase (oxidizes catechols to quinones) [124]. A recent study on Pacific oysters [122] suggests that both assays are measuring the auto-oxidation of phenoloxidase-like substances, stock substrate solutions and various other phenol or quinone products that are present in the haemolymph. This highlights the importance of including positive and negative controls, as well as the need for further optimization of the assays.

The prophenoloxidase system has not been characterized in abalone, but Cheng *et al*. [6-10] have included phenoloxidase measurement in their investigations into the effects of environmental and pathogen stressors on the abalone immune response. They used L-DOPA to measure phenoloxidase activity. Importantly, sodium alginate was used as an activator to facilitate the release of prophenoloxidase and the associated activating enzymes from the granules in the haemocytes. They used haemolymph with no sodium alginate as a control to detect any background activity or auto-oxidation in the samples. They did detect some background activity in these controls, but the absorbance level in the activated haemolymph samples was more than double [10], suggesting that phenoloxidase activity can be reliably measured in abalone hemolymph using this approach.

6. Conclusions

The stress response in both gastropods and bivalves, as well as insects and annelids, has the same key molecules and cascade of responses as seen in vertebrates; i.e. CRH stimulating release of ACTH stimulating release of biogenic amines. Thus we can assume stress responses are similar across many phyla. In molluscs, these stress response molecules are all produced by the haemocyte. The haemocyte is also the central cell of the immune response. Work to date has shown it is involved in migration, phagocytosis, respiratory burst, phenoloxidase production and exocytosis of other antimicrobial factors, but these processes are not yet well understood. We do not yet know whether granulocyte and hyalinocyte haemocytes are related to each other or have separate stem cell origins. The source of the stem cells has not been located. It is not clear which haemocytes are involved in which immune responses and whether the response varies with bacterial, viral and parasitic infection. Stress sometimes produces an early transient drop in haemocyte counts, but it is not clear whether they lyse or leave the haemolymph in an orchestrated response.

Which *in vitro* tests are good indicators of the immune response in normal or stressed abalone is not known because the relationship between the tests and immune defence is only partially understood. Stress had consistent depressant effects on phagocytosis and efficiency in clearing bacteria, but other *in vitro* tests had variable responses. Increased phenoloxidase activity, for example, after stressing abalone with decreased dissolved oxygen [10] does not reflect immunostimulation, because this alteration is concomitant with increased rates of infection and mortality. On-farm kits to assess immune function are normally developed commercially from *in vitro* tests, which measure a narrow range of immune parameters which accurately reflect what is happening in the animal. Phenoloxidase may not be such a parameter in abalone, though it is of importance in other invertebrate species. Shuhong *et al* [24] queried whether it was of any importance in abalone immunology. It may not be important in bacterial infection but perhaps abalone use it in dealing with protozoal or viral infection. *In vitro* measures based on immune mechanisms known in vertebrates or other animals should be interpreted with caution until it is shown that they reflect an aspect of immune function in the animal considered, or a closely related taxon. Because invertebrates, unlike vertebrates, may be very distantly related, phylogeny must be considered in making assumptions about immune systems based on work in other invertebrates. Increased susceptibility to infection is the most valid indicator of immunosuppression; and the fact that phagocytosis and clearance efficiency are directly related to immune competency and are consistently depressed by stress suggests these are reliable parameters to measure, although we need to understand how adherence of haemocytes to a surface and anticoagulants affect phagocytosis. Some assays of the antimicrobial activity of haemolymph are proving to be useful. Further research to characterise these and other *in vitro* tests of immune responses is needed.

Further research needs to be done to clarify the interrelationship between the endocrine and immune systems in molluscs. There is competition among the hormones and cytokines influencing the immune system in gastropods; and the benefits and costs to the animals ability to survive cannot be ascertained until more is known about how these molecules inter-relate. The report of glucocorticoid-like molecules [27] needs further

investigation, since glucocorticoids in vertebrates mediate important secondary effects including suppression of growth and immunosuppression. Genetic improvement programs need to know the metabolic costs of robust immunity on growth.

How growth hormones interact with the other hormones and the immune system is another area to be investigated in molluscs. After a disease outbreak, dead animals can be removed and the cost of these mortalities measured, but we do not understand the mechanisms involved in stunted growth of the survivors. This is probably of greater economic importance to the farmer. How much stress causes a transient stunting of growth and how much will lead to permanent stunting is not known. The challenge is to develop reliable, robust tests that accurately depict immune status and that can be run on production animals. Something better and earlier than poor growth rates of individuals is needed. It would be interesting to know if the oysters with increased resistance to QX disease due to higher levels of phenoloxidase had similar growth rates to other oysters [23]. A more robust immune system with an increased production of phenoloxidase possibly has higher energy demands.

An important aim of future research will be to characterise the relationship between stress, immune defense and responses in other *in vitro* tests, and to develop tests that are easily conducted outside an experimental research laboratory.

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Table 1. Published Literature in Abalone Immunology

Parameters Studied	**Reference**	**Species**
Cell Mediated Response		
Bacterial killing assays	[6-10]	*H. diversicolor*
Haemocyte counts	[6-10]	*H. diversicolor*
	[5]	*H. tuberculata*
Haemocyte morphology	[56]	*H. asinina*
	[57]	*H. cracherodii*
	[72]	not stated
Phagocytosis assays	[81]	*H. asinina*
	[6-10]	*H. diversicolor*
	[5]	*H. tuberculata*
Respiratory burst assays	[4]	*H. cracherodii, H. rufesens*
	[6-10]	*H. diversicolor*
	[5]	*H. tuberculata*
Humoral Response		
Phenoloxidase assays	[6-10]	*H. diversicolor*
Antimicrobial assays	[112]	*H. cracherodii, H. corrugata, H. rufesens*
	[24]	*H. diversicolor*
	[100,102,103]	*H. rubra*
	[99,104]	not stated
Stress Response		
Catecholamines in abalone	[5,12,27]	*H. tuberculata*
Affect of stress on immunity	[4]	*H. cracherodii, H. rufesens*
	[6-10]	*H. diversicolor*
	[124]	*H. rubra*
	[5]	*H. tuberculata*
Affect of stress on physiology	[35]	*H. asinina*
	[47,48]	*H. iris*
	[43]	*H. laevigata*
	[33-35]	*H. rubra*

Figure Captions

Figure 1: Modern classification of common animal taxa based on molecular and morphological evidence, emphasizing the relations of molluscan taxa. Modified from Lecointre and Guyader [13] and Beesley *et al.* [15]. Genera mentioned in the text are shown in parentheses.

FIGURE 1 – Hooper et al. Stress and immunity review.