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The physiological stress response to amputation in the eleven-armed sea star (*Coscinasterias muricata*)

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ABSTRACT

The eleven-armed sea star, *Coscinasterias muricata*, is subject to human-induced stressors, such as invasive fishing activities, that can cause limb loss (amputation), and heavy metal discharge into their habitat. The well-being and survival of a keystone predator such as *C. muricata* has important ecological implications as their presence influences community structure in the marine environment.

Understanding the stress physiology of an animal can provide insight into their overall health and survival. While the stress physiology of northern asteroid species (such as *Asterias rubens*) has been well documented, this has not been well studied with *C. muricata*. In this thesis, I was able to identify time-dependent changes in two physiological parameters (total coelomocyte count and dopamine levels in the coelomic fluid) in *C. muricata* subjected to amputation. There was a synchronous increase in both of these parameters 24 hours post amputation.

Dopamine in the coelomic fluid was measured by using high performance liquid chromatography (HPLC). I adapted a pre-existing method involving pre-column derivatisation and fluorescent detection, which was initially developed for the detection of dopamine in porcine muscle. However, this method requires further development as it could not detect dopamine to the same sensitivity as previously reported HPLC methods using electrochemical detection.

Lastly, the initial attempts at developing an *in vitro* cytotoxicity bioassay using *C. muricata* coelomocytes is described in detail. The initial aim of this experiment was to understand the effect of heavy metals on cellular parameters. However the experiment was hampered by unusually low cell counts in this species, which has not been previously reported. The knowledge gleaned from this study may provide the groundwork for future studies that use *C. muricata* coelomocytes for cytotoxicity testing or as a biomarker.

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1.1 Stressors

Even small changes from the optimum environmental conditions for an animal can be a challenge for the organism. These changes can be a “stressor”, which challenges homeostasis and induces a counteractive stress response in order to maintain bodily function and survival. Common stressors currently of serious concern in the marine environment in New Zealand and overseas include (but are not restricted to); trampling on the organism or their habitat, anthropogenic pollutant discharge and invasive fishing activities such as trawling (Crowe *et al*. 2000). Marine organisms are particularly vulnerable to chemical stressors as the oceans are often the final sink for water-soluble inorganic and heavy metal compounds. As two-thirds of Earth’s surface is oceanic, the potential impact of such anthropogenic disturbance on marine biota is substantial. This thesis investigates the impact of two stressors, amputation and heavy metal exposure, in a marine invertebrate species, the eleven-armed sea star, *Coscinasterias muricata* (Figure 1.1).

Figure 1.1 An eleven-armed sea star with all eleven arms intact. Photographed by author at the site of sampling (Point Halswell, Wellington Harbour, New Zealand).

1.1.1 Amputation

In their natural habitat, asteroids may lose appendages as a result of predation, human disturbance, or the process can be self-induced (Figure 1.2). Voluntary amputation, termed autotomy, refers to an animal's ability to remove or sever parts of its own body, under the control of the animal itself, in response to biotic and, to a lesser extent, abiotic factors. In the wild, autotomy typically results from biotic factors such as injury from predation and asexual reproduction. Superficial wounds inflicted by a predator on the dorsal surface have been shown to induce autotomy of the entire arm in *Asterias forbsei* and *Acanthaster planci* (Aldrich, 1976; Glynn, 1982; Lawrence, 1991). Abiotic factors are considered 'accidental' causes, in which an animal undergoes autotomy to free itself if an appendage becomes immobilised under rocks or in crevices (Lawrence, 1991).

Figure 1.2 An eleven-armed sea star with several appendages missing, and one smaller, recently regenerated limb. Photographed by author at the site of sampling (Point Halswell, Wellington Harbour, New Zealand).

As autotomy is a self-induced process, the stress response following autotomy may vary from that occurring after forceful mutilation of the body. Predation is the main cause of involuntary amputation in the wild. However, studies have also demonstrated that invasive fishing equipment can cause amputation. Using the common sea star *Asterias rubens*, Rogers *et al*. (2001) found a positive correlation between the number of injured sea stars and the intensity of trawling activity in the area. Trawling nets and apparatus have been reported to damage and/or sever appendages of echinoderms caught as bycatch (Kaiser, 1996; Ramsay *et al*., 2001). Bergmann and Moore (2001) reported an increased mortality rate in sea stars that were injured and discarded from trawling in comparison to those that remained uninjured from the event. The high mortality rate suggests that forceful amputation is a stressful event for the animal that compromises the physiology and survival of the animal.

Like all stressors, physical trauma will activate a physiological stress response. The stress response following injury includes the repair phase, which is defined as the complete healing and re-epithelisation of the injured area (Candia Carnevali *et al*., 1993). In echinoderms, this phase is aimed at closing the wound to prevent further loss of coelomic fluid and parasite entry (Candia Carnevali *et al*., 1993; Hernroth *et al*., 2010).

1.1.2 Heavy metals

In biology, the term ‘heavy metals’ typically includes arsenic, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, nickel, selenium, silver, and zinc. The widespread biological definition is based on the environmental toxicity of these metals rather than on their chemical or physical properties (Depledge and Fossi, 1994). They are further categorized based on how essential they are to biological processes. Iron, magnesium, manganese, cobalt, zinc and copper are essential metals with wide-ranging cellular functions, such as contributing to the structure and function of many essential enzymes (Stern *et al*., 2007). On the other hand, the remaining metals have no apparent biological role, and therefore no molecular mechanism has evolved to facilitate their uptake. However, these heavy metals are of concern as they enter cells by molecular and/or ionic mimicry. Molecular mimicry occurs as a result of non-essential metal ions binding to biomolecules that have a donatable electron pair. The resulting organo-metal complex behaves similarly, structurally and/or functionally to

other biomolecules or the original biomolecule (Bridges and Zalups, 2005). Furthermore, non-essential heavy metals can also enter by ionic mimicry, in which free ions simply exploit transporters originally utilised for the transport of essential metals.

1.1.2.1 Cadmium

Cadmium is emitted from the combustion of fossil fuels and agricultural phosphate fertilisers (Bennet-Chambers *et al.,* 1999). Cadmium is only present in minute concentrations in the ocean and has no biological role. However, up to 1,011 tonnes of cadmium from human activity enters the ocean annually from rivers and the atmosphere (Neff, 2002). In Western Australia, 137 tonnes of water soluble cadmium was emitted by agricultural activity between 1983 – 1999, which leeches out from soil and eventually accumulates in the ocean (Bennet-Chambers *et al.,* 1999).

Several transporters and channels intended for the uptake of calcium, iron and zinc are speculated to be involved in cadmium uptake through ionic mimicry (Chmielowska-Bak *et al*., 2013). The use of channel blockers and gene knockout models to test the effect of blocking calcium channels has been associated with decreased cadmium toxicity in several organisms, ranging from plants to humans (El Azzouzi *et al.,* 1994; Perfus-Barbeoch *et al*., 2002). Cadmium also enters cells by molecular mimicry, forming complexes with sulfhydryl-containing molecules, such as the amino acid cysteine and the antioxidant peptide glutathione (GSH; Bridges and Zalups, 2005).

Cadmium induces apoptosis and interferes with normal cell proliferation and differentiation (Bertin and Averbeck, 2006). It is also known to increase the production of reactive oxygen species (ROS), causing indirect genotoxic effects (Bertin and Averbeck, 2006). Cadmium exposure is known to cause lysosomal damage in a wide range of aquatic animals (the common mussel – Coles *et al.,* 1995; Asari clam – Matozzo *et al.*, 2001; Sea bass – Vazzana *et al*., 2009). In addition, field and laboratory studies have demonstrated dose-dependent cadmium accumulation in the pyloric caeca, gonads and body wall of *A. rubens* (den Besten *et al.*, 1989; Coteur *et al.*, 2003). Cadmium also retards the maturation of oocytes and embryonic development in *A. rubens* (den Besten *et al*., 1989; den Besten *et al.*, 1991). Toughness and size of ossicles in sea stars are known to be adversely affected by increased environmental heavy metal levels, including cadmium (Moureaux, 2011). Furthermore, exposure to cadmium for 48 hours delays the righting time of the starlet cushion star (*Asterina*

gibbosa), which is a behavioural indicator of increased stress experienced by asteroids (Bowett, 2002; Canty, 2009).

1.1.2.2 Zinc

Zinc is used in the production of metal alloys such as brass and bronze, and large amounts are also emitted from fly ash, a product of coal combustion (Callender and Rice, 2000). Up to 60,000 tonnes of zinc enters the ocean from the atmosphere, and an additional 6,000 tonnes enters via smaller water bodies, each year (Neff, 2002). Unlike cadmium, zinc is an essential metal for animals, plants and micro-organisms (Eide, 2006). It is the second most abundant transition metal in an organism, and plays an essential role as a cofactor in many enzymes, as well as contributing to protein and membrane stability (Bettger and O'Dell, 1981).

The uptake of essential metals is a regulated process which allows organisms to maintain a relatively constant internal concentration, unlike non-essential metals (Neff, 2002). However, even essential metals can be toxic in excess amounts (Viarengo and Nott, 1993). The majority of the zinc present in seawater is not bioavailable as it is bound to organic ligands. However, the small fraction of zinc existing in the free ionic form can be toxic, ranging between concentrations of 100 to 50,000 µg/L, depending on the species and developmental stage (Neff, 2002).

High concentrations of zinc have shown to impair the development and survival of the red sea bream (*Pagrus major*) and Pacific oyster (*Crassostrea gigas*) larvae (Brereton *et al*., 1973; Huang *et al*., 2010). Furthermore, necrosis of haemocytes and acute inflammatory reactions have been observed in the gills of the common mussel, *Mytilus edulis* (Hietanen *et al*., 1988). Zinc toxicity is reported to inhibit lysozyme activity in the spiny sea star, *Marthasterias glacialis* (Stabili and Pagliara, 2009).

1.2 Biology of *C. muricata*

The phylum Echinodermata includes five extant classes; Asteroidea (sea stars and sea daisies), Ophiuroidea (brittle stars), Echinoidea (sea urchins and sand dollars), Holothuroidea (sea cucumbers), and Crinoidea (feather stars and sea lilies). Asteroidea is the most diverse class within Echinodermata, with 1,900 species in 370 extant genera (Mah and Blake, 2012).

The genus *Coscinasterias* includes four species; *C. acutispina, C. tenuispina, C. calamaria* and *C. muricata.* The latter two taxa were previously considered synonymous but are now classified as separate species (Waters and Roy, 2003; Barker, 2013). *C. acutispina* and *C. tenuispina* are found in the Northern Hemisphere, while the other two species are prevalent around the Indo-Pacific region. *C. calamaria* occurs around South Africa, while *C. muricata* is restricted to Southern Australia and New Zealand (Waters and Roy, 2003). *C. muricata* is found abundantly in the intertidal zone of sheltered harbours. Field studies have suggested that they are most abundant around 5 m in depth throughout the year, although during summer they also occur at shallower depths (Witman and Grange, 1998).

Sea stars play several important ecological roles in the marine ecosystem as predators of algae, sponges and other marine invertebrates, and also as detritivores that contribute to decomposition and nutrient cycles (Barker, 2013). In particular, keystone predators such as *C. muricata* are important to their environment as they regulate the diversity, abundance, composition, size, as well as the spatial and temporal distribution of many other species through top-down control (Paine, 1966; Menge *et al*., 1994; Day *et al*. 1995).

C. muricata predominantly feed on molluscs including the ribbed mussel (*Aulacomyna maoriana*), nesting mussel (*Musculus impactus*), common mussel (*M. edulis*), Mediterranean mussel (*Mytilus galloprovincialis*) around southern New Zealand, and the green lipped mussel (*Perna canalicus*) around the North Island (Day *et al*., 1995; Temara *et al*., 1998; Lamare *et al*., 2009). A study carried out in Rapid Bay, South Australia indicates that *C. muricata* is also a direct predator of several other mollusc species and crustaceans (Keough and Butler, 1979; Figure 1.3). *C. muricata* is predated upon by a species of giant spider crab (*Leptomithrax australiensis*), the Port Jackson shark (*Heterodontus portusjacksoni*) and conspecifics exhibiting cannibalism (Keough and Butler, 1979).

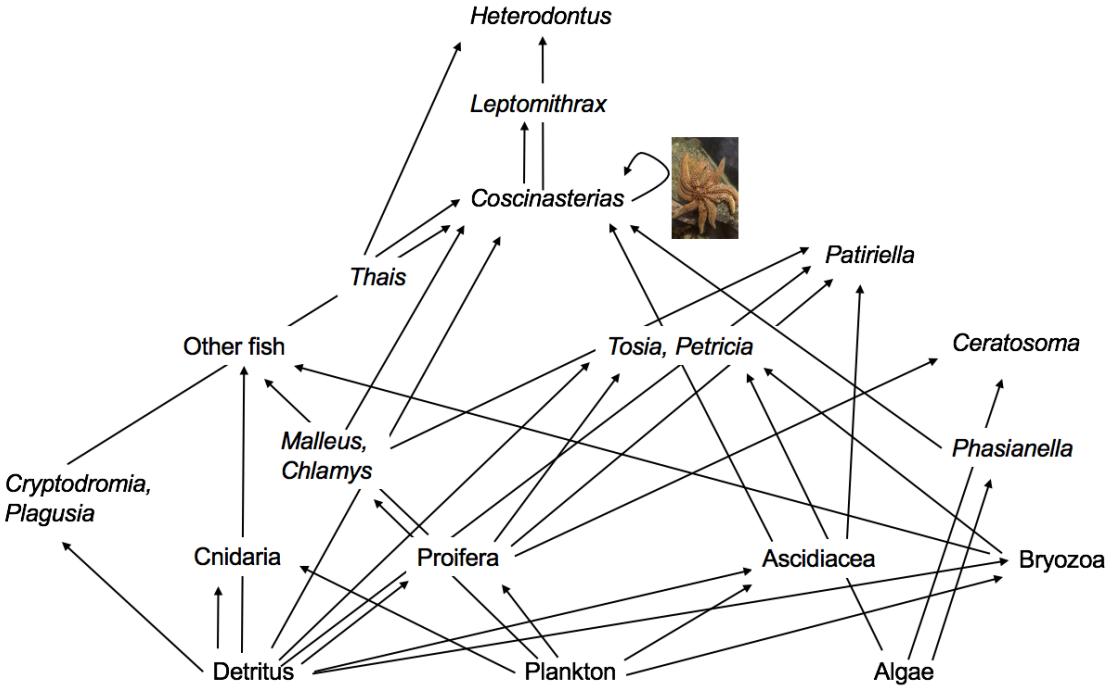

Figure 1.3 Food web involving *C. muricata* adapted from Keough and Butler (1979). For full list of taxa involved in this food web, refer to Keough and Butler (1979).

1.2.1 Coelomic fluid

Coelomic fluid in the perivisceral coelom bathes the internal organs, and serves as an important fluid medium for humoral immunity, endocrine factors and nutrient transport (Ferguson, 1964; da Silva Laires, 2012). The total volume of coelomic fluid has not yet been reported for *C. muricata,* but body weight is supposedly an accurate indicator of coelomic fluid content in echinoderms, and is approximately 20% of body weight in *Pisaster ochraceus* (Ferguson, 1964; Geise, 1996; Yui and Bayne, 1983). However, the volume is known to fluctuate with changing salinity (Ellington and Lawrence, 1974). The ionic composition of the coelomic fluid of sea stars is almost identical to sea water with the exception of higher potassium ion (K^+) levels (Ferguson, 1964). Moreover, coelomic fluid of sea stars contain high levels of asterosaponin, which is a family of secondary metabolites with antibacterial, antiviral, antitumor properties (Cheng *et al*., 2006; da Silva Laires, 2012). Other humoral factors, such as lectins, have been found in the coelomic fluid of *Asterina pectinifera,* and play a role in cell-cell recognition (Kamiya *et al*., 1992). Furthermore, an analogue of the vertebrate inflammatory cytokine, interleukin 6, was characterised in *Asterias forbesi* (Beck and Habicht, 1996).

A number of endocrine factors have also been reported in the coelomic fluid of sea stars. Catecholamines such as dopamine, serotonin and noradrenaline, as well as acetylcholine, octopamine, nitric oxide, and melanocyte stimulating hormone (MSH) have been identified in the coelomic fluid of asteroids (Thorndyke and Candia Carnevali, 2001). Many of these endocrine factors have been studied for their implication in either reproduction or regeneration.

1.2.2 Coelomocytes

Coelomic fluid also contains a heterogeneous population of immunoactive cells, collectively referred to as coelomocytes (Figure 1.4). Echinoderm coelomocytes have been studied for their remarkable ability to interchange between differentiated and undifferentiated states after injury, which may have future implications in medical research (Candia Carnevali *et al*., 2009). Furthermore, echinoderm coelomocytes have been studied in biomonitoring and toxicology studies due to their crucial role in the echinoderm immune system (Coteur *et al*., 2003; Békri and Pelletier, 2004; Ronning, 2005).

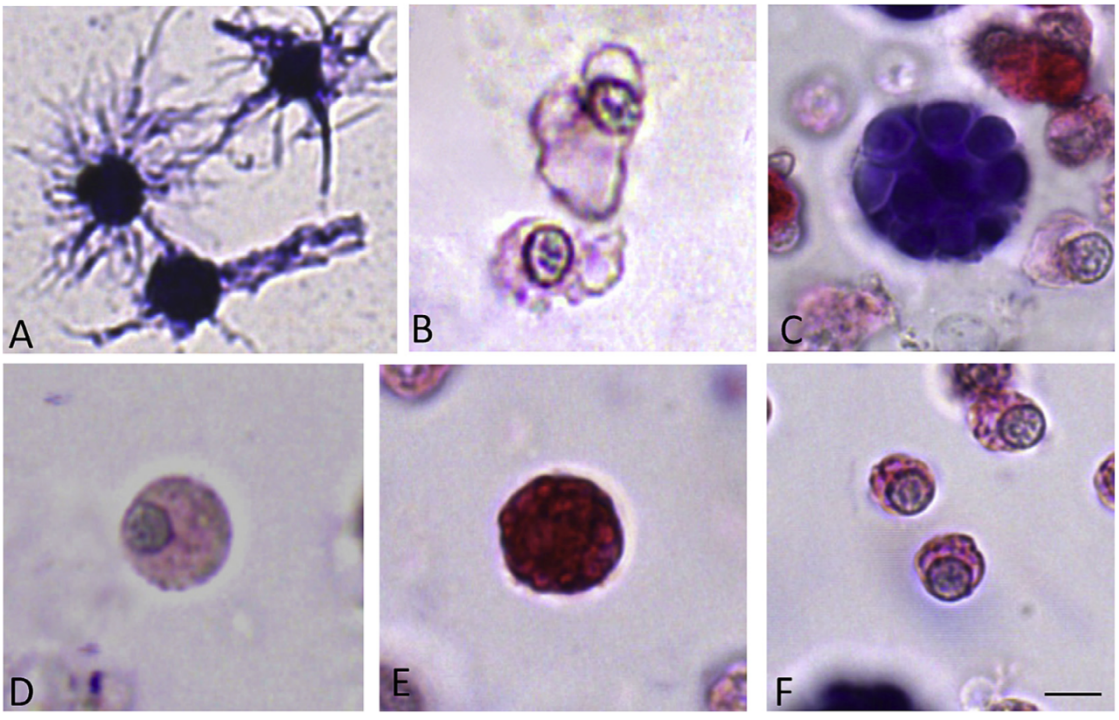

Figure 1.4 Coelomocyte types of *Holothuria tubulosa*. A = filopodial phagocyte; B = petaloid phagocyte; C = morula cell; D = spherulocyte; E = acidophilic spherulocyte; F = progenitor cell. Bar = 5 µm. Note here

that these coelomocytes from a sea cucumber species; asteroids only possess phagocytes and spherulocytes. Figure and caption courtesy of Vazzana *et al.* (2015).

Coelomocytes are further categorized into groups based on their morphology, size, or function. Over the years different authors have used varying terms to refer to essentially the same cell types, which has led to inconsistent usage and confusion of terminology. Smith *et al*. (2010) recognizes three echinoderm coelomocyte groups (phagocytes, spherule cells and vibratile cells), while Coteur *et al*. (2002) lists five groups (spherule cells, crystal cells, progenitor cells, vibratile cells and amoebocytes). Holm *et al*. (2008) divides coelomocytes into four categories: phagocytes (synonymous with amoebocytes in older literature), vibratile cells, morula cells, and a group of irregularly shaped, slow moving cells that are recently being referred to as amoebocytes. Moreover, Smith *et al*. (2010) lists amoebocytes, morula cells, pigment cells, granulocytes and eleocytes as synonymous terms, all referring to spherule cells. On the other hand, Sharlaimova *et al.* (2014) and Vazzana *et al*. (2015) use the term amoebocyte and phagocyte interchangeably. Canicatti *et al*. (1989) attributes this inconsistency to the different methodology that authors have used to observe and characterise the cells. The function of different cell types is discussed in further detail in section 2.5.1 of chapter 2.

1.3 Objectives and outlines

Coleman (2004) reported *C. muricata* as one of the most common bycatch species from scallop fisheries. Furthermore, studies have revealed that echinoderm species with low commercial value (such as sea stars and brittle stars) that are caught, injured and discarded as by-catch from trawling are adversely affected by such traumatic events (Bergmann and Moore, 2001). Moreover, as noted above, the presence of heavy metals in oceans has raised environmental concerns.

Although intensive trawling and heavy metal pollution in the marine environment is known to disturb the benthic community, the synergistic effect of the two disturbances at a physiological level is yet to be investigated. Knowledge gleaned from this study will broaden our understanding of the traumatic effects induced by amputation, and how it may subsequently affect survival in heavy metal polluted areas.

Sea stars have been demonstrated to be effective biomarkers of chemical pollution by assessing their stress response, or as sentinels that are used to assess

bioaccumulation of toxins within the body (Pelletier and Larocque, 1987; Temara *et al*., 1998; Coteur *et al*., 2003; Danis, 2004; Ronning, 2005). However, with no established asteroid cell line for use in the lab, asteroids have rarely been used for *in vitro* cytotoxicity testing (Ronning, 2005).

While it is not practical to investigate how each and every species responds to a pollutant, a keystone species would be a useful model for *in vitro* cytotoxicity testing. *C. muricata* may bioaccumulate heavy metals from both direct exposure through the environment, and ingestion of contaminated prey (Boisson *et al*., 2002). Factors influencing the survival and wellbeing of a keystone marine invertebrate predator, such as *C. muricata*, can influence the overall ecosystem of the rocky shore community.

Sea star coelomocytes will be used to test the cytotoxicity of levels of cadmium and zinc that are recommended by the Australian and New Zealand Environment and Conservation Council/Agriculture and Resource Management Council of Australia and New Zealand (2000; hereafter referred to as ANZECC) for the protection of marine species. In doing so, I also investigate the potential for *C. muricata* coelomocytes to be an effective cytotoxicity testing tool, as well as a biomarker for detecting environmental pollution. The underlying hypothesis of this thesis is that amputation is a stressor that will induce physiological changes in sea stars.

1.3.1 Chapter outline

Chapter 2 – Changes in total coelomocyte count in response to amputation

The overall aim of this chapter is to understand the time-dependent changes in total coelomocyte count during the first 48 hours following amputation. In this chapter, the total coelomocyte count is used as an indicator of stress experienced by the sea stars.

Chapter 3 – Quantification of dopamine in coelomic fluid of sea stars using high-performance liquid chromatography (HPLC)

Dopamine is a catecholamine, which has previously been reported as a stress hormone in marine invertebrates (Huet and Franquinet, 1981; Tan *et al*., 2015). The aim of this chapter is to develop a method for detecting dopamine levels in the coelomic fluid of sea stars using high performance liquid chromatography with fluorescence detection. To my knowledge, this technique has not been used with

coelomic fluid of asteroids. The time-dependent changes in dopamine levels following amputation will be quantified in this chapter.

Chapter 4 – Initial steps made in the development of an in vitro *cytotoxicity bioassay using coelomocytes harvested from sea stars*

This chapter outlines the challenges associated with establishing coelomocyte cell cultures and assays to understand the effect of *in vitro* heavy metal exposure. Here, I also discuss two of the initial objective of this thesis: 1) to examine the effect of ANZECC recommended guideline values on the physiology of coelomocytes; and 2) to investigate the synergistic effect of amputation and heavy metal exposure on the physiology of coelomocytes. Ultimately, this chapter aims to provide groundwork for utilising sea star coelomocytes as a tool for *in vitro* cytotoxicity testing.

Chapter 5 – General discussion

This chapter collates the findings from the above chapters and provides direction for future research aimed at understanding the stress physiology of asteroids in response to amputation. This chapter also discusses the potential to use echinoderms for *in vitro* cytotoxicity testing and as biomarkers of environmental pollution.

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Chapter 2

Changes in total coelomocyte count in response to amputation

Chapter 2 – Changes in total coelomocyte count in response to amputation

2.1 Abstract

The total coelomocyte count is a simple and affordable method of understanding stress in echinoderms. The time dependent changes in coelomocyte numbers after amputation were investigated by counting the number of cells in the coelomic fluid samples collected in the field (baseline), 3 hours before amputation, and 6, 24, and 48 hours after amputation. The coelomocyte numbers were higher in amputated animals 24 hours after amputation. However, the difference in cell count between the amputated and non-amputated animals were insignificant at all other time points. The increase in cell numbers 24 hours after amputation is likely to be associated with the proliferation and recruitment of coelomocytes to the area of the wound, as these cells play a crucial role in preventing pathogen entry and excessive loss of coelomic fluid. The subsequent decrease in cell numbers 48 hours after amputation may mark the end of the repair phase, at which point the coelomocytes are recruited into the body wall tissue for regeneration.

2.2 Introduction

The total coelomocyte count is a widely used indicator of stress in echinoderms, not just in response to amputation, but to other stressors such as human handling, low salinity, exposure to inorganic compounds and heavy metals (Coteur *et al*., 2003; Pinsino *et al*., 2007; Shannon *et al*., 2015; Tan *et al*., 2015). As coelomocytes are immunocytes, their numbers are expected to increase in individuals under stress, in a way similar to how stressors such as injury and infection increase white blood cell counts in vertebrates (Davis *et al*., 2008). In asteroids, the increase in coelomocyte numbers following amputation can be attributed to two main reasons; first, to replenish lost tissue (including coelomic fluid), and second, to increase the number of coelomocytes for participating in the immune response (Pinsino *et al*., 2007).

While this thesis focuses extensively on only the repair phase, the cellular processes that occur during this phase differ between the two types of regeneration in asteroids (morphallaxis or epimorphosis). The change in cell count after amputation may vary, depending on the type of regeneration, as morphallactic regeneration is a slower

process than epimorphic regeneration. The regenerative processes will therefore be outlined briefly in this section (for a detailed review of echinoderm regeneration, refer to Candia Carnevali (2006)). Regeneration occurs over a longer time-span and restores the presence and function of the lost body part. Regeneration is typically divided into three phases: repair, early regeneration, and advanced regeneration. The repair phase is characterised by the wound healing process involving the recruitment of coelomocytes to the area of the wound (Pinsino *et al*., 2007; Khadra *et al*., 2015b). During the early regeneration phases, the first signs of differentiation of stem cells can be observed. Finally, the advanced regeneration phase is defined by tissue restoration and subsequent formation of a new arm that resembles the structures and functions of a fully grown arm (Khadra *et al*., 2015a, Khadra *et al*., 2015b).

Morphallactic regeneration begins with the reorganization and subsequent proliferation of the components already existing in the stump (King and Newmark, 2012). It differs to epimorphic regeneration, which relies on the formation of undifferentiated cells from the dedifferentiation of specialised cells. In this case, the aggregation of dedifferentiated and undifferentiated cells under the epidermis is referred to as the blastema (Agata *et al*., 2007). Blastemal cells acquire a new function (redifferentiate) and begin to form the regenerating limb (Agata *et al*., 2007). The absence of the blastema and reduced degree of cell differentiation characterises morphallactic regeneration.

In *Echinaster sepositus*, regeneration is suggested to be morphallactic, as the newly formed epidermal monolayer derived cells are from the existing epithelium of the stump (Khadra *et al*., 2015b). These cells do not possess typical characteristics of completely dedifferentiated cells, and instead retain features resembling epidermal cells, such as a prominent nucleus, granules and vacuoles. Furthermore, a true localised blastema that is characteristic of epimorphic regeneration has not observed. Similarly, morphallactic regeneration has also been reported in the six-rayed star, *Leptasterias hexactis* (Mladenov *et al*., 1989).

Regeneration in *Asterias rubens* was originally believed to be morphallactic but research by Fan *et al*. (2011) provided contrary evidence, suggesting epimorphic regeneration instead (Moss *et al*., 1998; Candia Carnevali, 2006). Wound healing was achieved after approximately four days in *A. rubens*, with a 2-4 layered primitive epithelium originating from dedifferentiated cells already present in the epidermis around the wound (Fan *et al*., 2011). These dedifferentiated cells proliferate, migrate, and are then recruited as new epidermal cells, which is characteristic of epimorphic

regeneration. Huet (1975) also suggested epimorphic regeneration in the starlet cushion star *Asterias gibbosa*. Similarly, Ducati *et al.* (2004) reported the formation of blastemal-like structure after fission in *Coscinasterias muricata*, which is indicative of epimorphic regeneration.

Coelomocytes play a central role after amputation, especially during the repair phase where they contribute to clotting and wound healing (Khadra *et al*., 2015b). Furthermore, aggregation of non-proliferating cells beneath the epidermis around the wound has an important immune function as the first line of defence against pathogen entry (Holm *et al*., 2008a; Khadra *et al*., 2015b). The time dependent changes in coelomocyte numbers have been used as an indicator of stress in wounded echinoderms (Pinsino *et al.,* 2007; Vazzana *et al*., 2015). Sea cucumbers (*Holothuria tubulosa*) exhibit significantly higher coelomocyte numbers compared to control animals after 1, 2.5, 6, 24 and 48 hours after injury, while only a slight increase in cell number was observed 6 hours after amputation in *A. rubens* (Pinsino *et al.,* 2007; Vazzana *et al*., 2015)

This chapter aimed to determine the time dependent change in total coelomocyte count of *C. muricata* during the first 48 hours following amputation. It should be noted here that this chapter initially had two objectives, the second one aimed at understanding the population dynamics of coelomocytes following amputation. I was unsuccessful in achieving this aim, but the details of this part of the experiment are discussed in section 2.5.1.

2.3 Methods and materials

2.3.1 Collection and field sampling

Sixteen adult sea stars were collected via snorkeling on 28 October, 2015 (water temperature 12°C) from Point Halswell, Wellington Harbour, New Zealand. Animals that were less than 20 cm in diameter and those that had stumps of recently amputated appendages were not collected. Following capture, baseline sampling was carried out in the field before transportation and in the absence of any additional stressors other than handling. A total of 1.8 ml of coelomic fluid was collected from individuals in the field using a sterile 22-gauge needle and 5 ml syringes that contained 2.2 ml of sterile Leibowitz's culture medium. The needle was inserted into the distal third of an arm into

the coelomic cavity, following the technique described by Békri and Pelletier (2004). The coelomic fluid was transported in these syringes on ice to avoid air-borne contamination associated with transferring samples to tubes in non-sterile field conditions. An additional 700 µl of coelomic fluid was collected and immediately frozen in liquid nitrogen for dopamine detection as described in chapter 3.

2.3.2 Transport and housing

Sea stars were transported back to Massey University, Palmerston North, New Zealand (approximately 2 hours' drive) in 24 L chilly bins to avoid hyperthermal stress. No more than two animals were placed in the same chilly bin for transport. Upon arrival, each chilly bin was fitted with and external filter and placed in a temperature and light-controlled room (temperature 12°C; light cycle 12h:12h). Water pH, ammonia, nitrite and nitrate levels were monitored using commercially available colorimetric assays (API™ Saltwater Master Test Kit, Aquarium Pharmaceuticals, PA, USA). Daily 20% water changes were carried out with artificial red salt (Crystal Sea MarineMix, Marine Enterprises International, Baltimore, MD, USA) dissolved in reverse osmosis water to replicate the composition of natural saltwater.

2.3.3 Experimental design

After approximately three days of acclimation to laboratory conditions, 1.8 ml of coelomic fluid was collected from each animal. Samples were then stored as outlined in section 2.3.4. Sea stars were returned to their respective tanks and allowed 6 hours to replenish coelomic fluid before amputation was carried out. Animals were randomly assigned into an untreated control group (n=8) and a treatment group (n=8). Animals in the treated group had the distal third of one of their arms amputated with a scalpel while those in the control group were not amputated. Subsequently, 1.8 ml of coelomic fluid was collected 6, 24 and 48 hours after amputation. The same volume of coelomic fluid was collected from control animals at the same time intervals. Animals were returned to their tanks between sampling.

2.3.4 Sample treatment

For the total coelomocyte count, 100 µl of coelomic fluid was set aside and further processed according to section 2.3.5. Of the remaining coelomic fluid, 700 µl was immediately frozen at -80°C for dopamine detection in chapter 3, and the rest was used for the *in vitro* cytotoxicity testing experiment in chapter 4.

2.3.5 Total coelomocyte count

The trypan blue exclusion test was used for this study as it is one of the most common and affordable methods for distinguishing dead from living cells. The dye only penetrates cells that do not possess a functional membrane, i.e. damaged or dead cells are stained blue while live cells do not take up the dye.

Trypan blue solution (0.2%) was prepared in sterile PBS, and 100 µl was added to an equal volume of coelomic fluid. The percentage of live cells was counted using a haemocytometer under an upright brightfield microscope at 400x magnification. Cell counts were later corrected to account for the dilution factors.

2.3.6 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Repeated measures two-way ANOVA was used to analyze the effect of amputation across five different time points. A post hoc multiple comparison of means was carried out using the Bonferroni test ($p = 0.05$).

2.4 Results

A repeated measures two-way analysis of variance (ANOVA) indicated that the cell count between amputated and non-amputated animals was significantly different ($F_{4,70} = 3.935$; $p = 0.05$; Table 2.1). However, time since amputation had no significant effect on the cell count of amputated animals ($p = 0.13$).

Multiple comparison of means using the Bonferroni test revealed that the mean cell count was only significantly higher in the amputated group 24 hours after injury (X+24, $p = 0.02$; Table 2.2). Mean cell count did not differ between the treatment and non-treatment group at the two time points before amputation (i.e. at baseline and 3 hours

before amputation (X-3); Table 2.2). Coelomic fluid cell count did not differ between the two groups 6 hours after amputation (X+6), despite the difference between the means (Table 2.2). Mean cell counts were the same for the amputation and non-amputation group 48 hours after amputation (X+48).

Table 2.1. Repeated measures two-way ANOVA for total coelomocyte count. DFn: Degrees of freedom in the numerator; DFd: Degrees of freedom in the denominator.

	F (DFn, DFd)	***p* value**
Interaction (Treatment x Time)	F (4, 70) = 1.839	0.13
Time	F (4, 70) = 2.170	0.08
Treatment (Amputation/Control)	F (1, 70) = 3.935	0.05

Table 2.2. Bonferroni's multiple comparisons test for total coelomocyte count. Baseline refers to coelomic fluid sample in the field before transporting sea stars in to captivity; X-3: 3 hours before amputation; X+6, 24, 48: 6, 24, and 48 hours after amputation, respectively. Means are given as x 10^5 cells/ml. ns: not significant; *: p = < 0.05

	Treatment group (x 10^5 cells/ml; mean ± SEM)	**Control group (x 10^5 cells/ml; mean ± SEM)**	**Significance**
Baseline	1.11 x 10^5 ± 0.27	1.36 x 10^5 ± 0.28	ns
X-3	0.75 x 10^5 ± 0.15	0.53 x 10^5 ± 0.12	ns
X+6	2.38 x 10^5 ± 0.79	0.83 x 10^5 ± 0.09	ns
X+24	3.59 x 10^5 ± 1.49	0.74 x 10^5 ± 0.19	*
X+48	2.38 x 10^5 ± 1.03	2.44 x 10^5 ± 0.76	ns

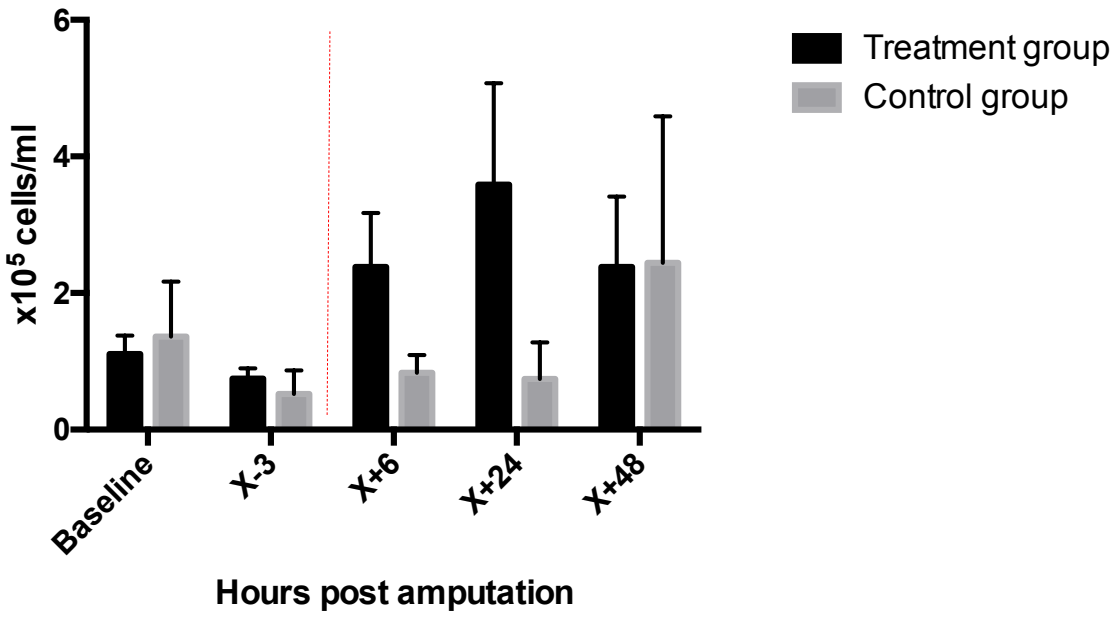

Figure 2.1 Total coelomocyte count (TCC). Baseline refers to coelomic fluid sample in the field before transporting sea stars in to captivity; X-3: 3 hours before amputation; X+6, 24, 48: 6, 24, and 48 hours after amputation, respectively. Red dotted line indicates time of amputation. Data are expressed as the mean values ± SEM.

2.5 Discussion

The cell count of amputated animals was significantly higher than that of control animals 24 hours post amputation, which is considered as the end of the repair phase and where wound closing and initiation of clotting occurs. The increase in cell count 24 hours after amputation may be associated with increased proliferation and recruitment of coelomocytes to the site of injury to prevent pathogen entry and further loss of coelomic fluid (Khadra *et al*., 2015a, 2015b). Interestingly, Pinsino *et al.* (2007) reported a time-dependent change in coelomocyte numbers in *A. rubens*, in which cell count was lowest 24 hours post amputation. Mean cell count was highest around 6 hours after amputation, which is in disagreement with the findings from this study. Vazzana *et al.* (2015) found a progressive increase in cell count between 6 and 48 hours post injury in sea cumbers (*H. tubulosa*), with maximum cell count being observed 48 hours after amputation.

The discrepancy with other studies in terms of timing may be associated to species-specific differences in the duration of the repair phase. The repair phase is defined as

the complete healing and re-epithelisation of the injured area (Candia Carnevali *et al.*, 1993). Subsequently, the occurrence of cell differentiation is often used as the milestone for the beginning of the regeneration phase. The timing of these events may vary between species, and may even be influenced by temperature (Candia Carnevali, 2006). In some echinoderms species, the repair phase may last for more than a week, unlike the conventional claim that the the first 24 – 48 hours after amputation constitutes the repair phase (Candia Carnevali, 2006; da Silva Laires, 2012).

A surprising finding here was that the cell count 6 hours after amputation was not significantly greater than that of the control animals, although the mean cell count was higher in the amputated animals at this time point. This is inconsistent with earlier studies that have consistently reported a significant difference between the injured and control animals at this time (Pinsino *et al.*, 2007; Vazzana *et al.*, 2015). As Coteur *et al.* (2003) state, the total cell count is very variable between individuals, thus leading to statistical insignificance despite the apparent difference in the mean of the two groups at this particular time point. Nonetheless, in another study with *A. rubens*, the coelomocyte number in wounded animals does not appear to differ significantly between 1 hour or 6 hours after amputation (Holm *et al.*, 2008b) The progressive increase in cell count after amputation has been attributed to the rapid division of circulating stem cells.

In this study, coelomic fluid was not collected 3 hours after amputation, in order to minimise sampling frequency. Nonetheless, Vazzana *et al.* (2015) observed a gradual decrease after amputation at 1 and 2.5 hours post injury, while Pinsino *et al.* (2007) reported a rapid increase in cell numbers after 1 hour, and then a sudden decrease at 3 hours after amputation. The decrease could be attributed to the recruitment of coelomocytes around the area of injury, for either clotting and wound repair processes, or associated with the immune response (Canicatti and Farina-Lipari, 1990; Khadra *et al.*, 2015b; Vazzana *et al.*, 2015).

In the Japanese sea cucumber *Apostichopus japonicus*, mechanical stress also induced a similar pattern in coelomocyte numbers (Tan *et al.*, 2015). Cell counts decreased from 16 x 10^6 cells/ml at 1 hour post handling, to around 11 x 10^6 cells/ml after 2 hours (Tan *et al*., 2015). As these sea cucumbers were not wounded but still exhibited a similar decrease as observed by Pinsino *et al.* (2007) and Vazzana *et al.* (2015), this may be associated with a general stress response, rather than wound healing. In other words, the decrease in cell count around this time point after

application of a stressor may not be in fact to do with clotting as suggested previously. Other physiological processes such as an increase in heat shock protein 70 (HSP70; or other molecular chaperones), and/or endocrine factors involved in the stress response may be associated with this decrease in cell count, but this remains to be confirmed.

The findings from this study are inconsistent with Pinsino *et al.* (2007) who showed a significantly different time-dependent pattern to ours. The average cell count at 24 hours post injury reported by Pinsino *et al.* (2007) was approximately 1.8×10^6 cells/ml which is substantially greater than what was observed in this study (3.6×10^5 cells/ml). Pinsino *et al.* (2007) reported that the cell count 24 hours after amputation was even lower than before amputation (2.8×10^6 cells/ml), although the cause of this remains to be determined. In this study, the cell count of amputated animals decreased 48 hours post amputation, and the difference between the control and treatment groups was insignificant at this time. This may mark the end of the repair phase, and the initiation of the subsequent phases in which coelomocytes are no longer circulating, but are instead recruited at the site of injury for regeneration.

The decrease in cell count observed 48 hours following injury is inconsistent with the findings of Vazzana *et al.* (2015) who found that the cell count increased from 24 hours post injury. The discrepancies may be due to species differences, between members of the same echinodermata class, and even between two asteroid species. This may be associated with the two different regenerative processes (epimorphic or morphallactic). However, the more likely explanation is that it is a result of different sampling and analytical methods, as both Holm *et al.* (2008b) and Pinsino *et al.* (2007) studied *A. rubens* but report results that are not comparable. A comparative study using the same laboratory technique and several different echinoderm species would be able to provide more information on inter-species differences. The discrepancy between studies using the same species may also be attributed to the difference in temperature in which the animals were housed in, as temperature is suggested to affect the duration of the repair phase (Thorndyke and Carnevali, 2001; da Silva Laires, 2012). However, in the two aforementioned experiments with *A.rubens*, the ambient water temperatures were similar. Holm *et al.* (2008) housed their animals at 14 °C, while Pinsino *et al*. (2007) maintained the temperature between 12 – 14 °C.

Sea stars that are injured from invasive trawling activities have a greater mortality rate than those that are uninjured (Bergmann and Moore, 2001). Whether the results observed in laboratory amputation studies reflect what occurs in sea stars with

trawling-induced injury is yet to be investigated. *A. rubens* caught as bycatch from lobster fishing have been reported to exhibit white lesions which is followed by disintegration of the entire animal (Bergman and Moore, 2001). This has not been reported in laboratory amputation studies, which demonstrates the need to investigate the stress response of bycatch sea stars for physiological parameters to be applicable to the fishing industry.

Mortality of bycatch sea stars has also been linked to the duration of aerial exposure, temperature, hypoxia and compression by the weight of catch (Bergman and Moore, 2001). Holm *et al.* (2008b) reported a dramatic increase in total coelomocyte count in sea stars subjected to both amputation and hypoxia. Total coelomocyte count is an affordable and easy parameter to measure, which can be used to determine the stress response of bycatch echinoderms and ultimately allow better management strategies to be implemented to minimise the stress experienced by these animals.

2.5.1 Differential coelomocyte count

As the term 'coelomocytes' actually refers to a heterogeneous population of cells in the coelomic fluid, I intended to carry out a differential coelomocyte count as well, in which different cell types would be classified based on characteristics provided by previous studies. Unfortunately, the coelomocytes in this study failed to adhere to the poly-L-lysine coated slides and could not provide informative results. For this reason, only the results from the total coelomocyte count are presented.

As study was unsuccessful in identifying the different coelomocyte types and their proportions in the coelomic fluid, this section collates and discusses the findings of previous studies. Due to the disagreement in naming of cell types and their presence in asteroid coelomic fluid, this section only deals with two asteroid coelomocytes (phagocytes and spherule cells) according to an extensive review by Smith *et al*. (2010).

2.5.1.1 Phagocytes

Cells capable of engulfing foreign material or dead cells are collectively referred to as phagocytes, which have also been called agranulocytes, lymphocytes or amoebocytes (Sharlaimova *et al.*, 2014). Despite the non-standardised naming of coelomocytes, there is a unanimous agreement that phagocytes constitute the majority of the coelomocyte population in several echinoderm species (Pinsino *et al*., 2007; Smith *et*

al., 2010; Sharlaimova *et al*., 2014; Vazzana *et al*., 2015). In addition, authors have consistently reported different morphs of phagocytes which are capable of spontaneous transition from one form to another. The petaloid or bladder form has been reported in the sea urchins and sea cucumbers, as well as in sea stars (Johnson, 1969; Kaneshiro and Karp, 1980; Vazzana *et al*., 2015). Pinsino *et al*. (2007) reported rapid morphological transition of *A. rubens* petaloid phagocytes to filopodial forms (5 – 8 minutes). The transition involves the formation of micro spikes on the petaloid phagocyte, which is followed by cytoplasm retraction and filopodial elongation.

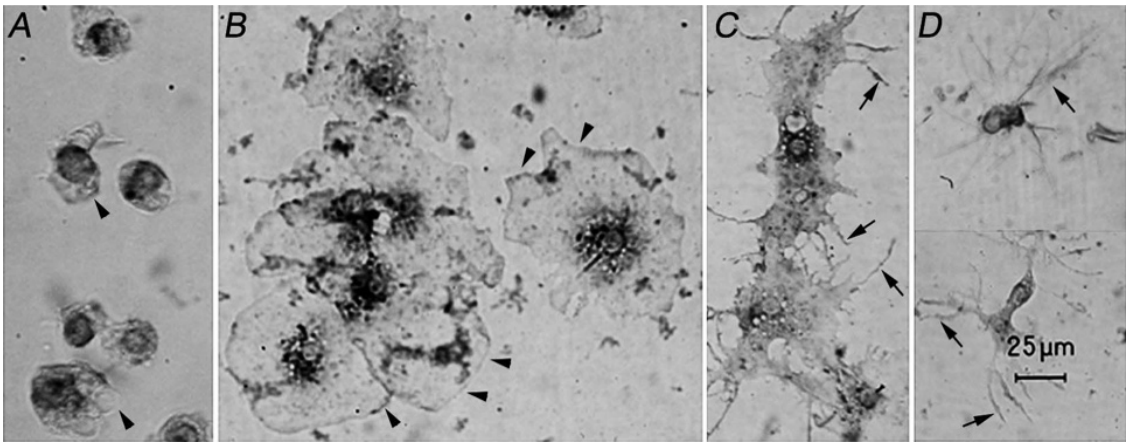

Figure 2.2 Petaloid phagocytes of *A. rubens* transitioning into the filopodial form. Cells were attached to glass slides and observed after (A) 1, (B) 2, (C) 4, and (D) 8 minutes. Arrowheads and arrows point to petaloid and filopodial cytoplasmic protrusions, respectively. Figure and caption courtesy of Pinsino *et al.* (2007).

Following amputation, the filopodial phagocyte has been implicated in forming the cicatrix layer by forming a syncytial network of phagocytes that lack internal cellular membrane divisions (thereby appearing multinucleated), which separates the stump and the newly developing epithelium (Khadra *et al*. 2015b). The syncytial phagocyte layer is likely to act as a physical barrier against further coelomic fluid loss and parasite entry. The latter is further supported by earlier research that has reported the formation of phagocytic syncytia after exposure to xenogenic cells *in vitro* (Dales, 1992).

In *A. rubens*, clot formation reportedly does not occur before 24 hours post amputation (Pinsino *et al*., 2007). Loss of coelomic fluid is reduced by muscle contraction around the amputation site and maintaining the amputated arm in an upright position (Moss *et al*., 1998; Pinsino *et al*., 2007). Similarly, in *E. sepositus*, closure of the perivisceral

coelomic cavity is achieved by densely packed collagen fibres and constriction of the circular muscles persisting until 24 hours after amputation (Khadra *et al*., 2015b). Therefore, if filopodial phagocytyes are involved in the clotting process, an increase in their proportion in the coelomic fluid is expected after this muscle mediated process.

Sharlaimova *et al*. (2014) identified large and small petaloid agranulocytes (referring to the lack of a granular appearance in the cytoplasm) in *A. rubens* as the dominant cell type in the coelomic fluid. An earlier study by Gorshkov *et al*. (2009) identified two coelomocyte types that were referred to as young and mature cells. The mature cells were characterised by the presence of phagocytic vacuoles and several lysosomes, which is characteristic of an active phagocyte. Conversely, the young coelomocytes lacked signs of differentiation, leading the authors to suggest that the young coelomocytes develop into the mature phagocytic cells. The mature coelomocytes constituted 97-98% of the whole cell population, which is consistent with previous reports that phagocytes are the dominant cell type in the coelomic fluid. Nonetheless, Coteur *et al*. (2002) reported three phagocytic amoebocyte groups (named G1, G2 and G3) based on their size. The smallest amoebocyte (G1) possessed fewer granules in comparison to the amoebocytes in the intermediate size group (G2) and the largest size group (G3). G2 and G3 cells are suggested to be involved in wound healing and/or clotting of the coelomic fluid. Moreover, G3 cells are continuously responsive to immune challenges, and this is a probable explanation for the fast reaction time exhibited by these cells. On the other hand, G1 cells are suggested to be precursor cells with minimal phagocytic ability. G1 cells may therefore be comparable to the young coelomocytes described by Gorshkov *et al*. (2009).

2.5.1.2 Spherule cells

Asteroid coelomic fluid also possesses another cell type, spherule cells (also referred to as morula cells or granulocytes). These cells have not been well studied compared to the more common phagocytes; however, Canty (2009) found spherule cells to be the second most common cell type in *A. rubens*. Spherule cells are characterised by the presence of irregularly shaped granules in the cytoplasm, measuring between 2-5 μM in diameter (Chia and Xing, 1996). Two types of spherule cells have been discovered, the echinochrome A containing red spherule cell, and the colourless cell present in all echinoderm classes. Asteroids are known to only possess the colourless form (Smith *et al*., 2010).

The red spherule cell is involved in oxygen transport, while echinochrome A is known to have strong anti-bacterial properties (Service and Wardlaw, 1984; Haug *et al*., 2002; Smith *et al*., 2010). The colourless form is involved in the inflammatory response, wound healing and remodelling of the extracellular matrix (Smith *et al*., 2010). Vazzana *et al*. (2015) observed the breakdown and despherulation of these cells at the site of injury, suggesting a possible role in forming the fibrous matrix of the body wall with collagenous material.

In the extensive review on echinoderm coelomocytes by Chia and Xing (1996), spherule cells are further classified into three types. Granules of type I cells are homogenously electron dense, type II cells exhibit an electron dense centre with the surrounding area being less electron packed. Finally, type III cells which possess irregularly shaped and apparently empty granules. Khadra *et al*. (2015b) identified type III spherule cells in *E. sepositus* and implicated their role in wound healing after amputation. Type III cells contain a greater protein content in comparison to type I and II cells (Chia and Xing, 1996). Furthermore, type III cells exhibit high acid phosphatase content, which is an enzyme involved in protein degradation (D'Ancona Lunetta and Canicatti, 1990; D'Ancona Lunetta, 2009). Vazzana *et al*. (2015) reported spherule cells to breakdown rapidly and despherulate at the site of injury, suggesting involvement in forming the fibrous matrix of the body wall by synthesising collagenous material.

Several authors have suggested the presence of melanin in type III spherule cells (Canicatti *et al.*, 1989; D'Ancona Lunetta and Canicatti, 1990; Chia and Xing, 1996). Melanin is a highly conserved pigment that is capable of defending cells from free radicals (i.e. oxidative stress) by scavenging reactive oxygen species. It is also associated with sequestering parasites and plays a role in the repair phase during regeneration and replenishment of the coelomic fluid (Smith *et al*., 2010; Grimaldi *et al*., 2012; Sharlaimova *et al*., 2014). An earlier study by Chia and Koss (1994) reported the presence of type II spherule cells in the Pacific blood star *Henricia leviuscula*. More recently, Sharlaimova *et al.* (2014) also found spherule cells (referred to as eosinophilic granulocytes by the authors), with visible cytoplasmic granules. However, whether or not these spherule cells are of the same type to those found by Chia and Koss (1994) in the Pacific blood star is uncertain.

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Chapter 3

Quantification of dopamine in coelomic fluid of sea stars using high-performance liquid chromatography (HPLC)

Chapter 3 – Quantification of dopamine in coelomic fluid of sea stars using high-performance liquid chromatography (HPLC)

3.1 Abstract

Dopamine is a hormone secreted in response to stress in marine invertebrates, which diverts bioenergetics to maintain immediate homeostasis. The time dependent changes in dopamine concentration following amputation in *Coscinasterias muricata* were investigated by detecting dopamine using high performance liquid chromatography (HPLC). A pre-existing HPLC method with pre-column derivatisation and fluorescent detection was adapted to detect dopamine in the coelomic fluid of *C. muricata*. This is the first report on detecting dopamine with fluorescent detection in coelomic fluid of an echinoderm species. Dopamine was measured in the coelomic fluid samples collected in the field (baseline), 3 hours before amputation, and 6, 24, and 48 hours after amputation. Dopamine was higher in amputated animals 24 hours after amputation. However, the difference in dopamine concentration between the amputated and non-amputated animals was insignificant at all other time points. Dopamine may play a role in inducing cell migration, and therefore elevated dopamine levels 24 hours after amputation could be linked to the proliferation and recruitment of coelomocytes to the area of the wound.

3.2 Introduction

Stress stimulates the release of several hormones such as glucocorticoids and catecholamines into the circulation system (Axelrod and Reisine; 1984). Secretion of these hormones forms part of the stress response which is defined as a series of coordinated physiological changes that increase the ability of an organism to maintain homeostasis in the presence of stressors (Lacoste *et al*., 2001). The primary stress hormone in mammals is cortisol (a glucocorticoid), while dopamine (a catecholamine) is associated with several psychological disorders in humans (Segman *et al*., 2002; Mehler-Wex *et al*., 2006). In marine invertebrates, dopamine is a stress hormone, which diverts bioenergetics to maintain immediate homeostasis, such as clotting, replenishing lost coelomic fluid, and maintaining the composition of the coelomic fluid (Lacoste *et al*., 2002; Tan *et al*., 2015).

An increase in dopamine has been observed in echinoderms subjected to mechanical stress (human handling) and amputation (Huet and Franquet, 1981; Thorndyke and Candia Carnevali, 2001; Tan *et al*., 2015). In asteroids, regeneration of amputated body parts is facilitated by the nervous system through the secretion of monoamine neuromediators such as serotonin, noradrenaline and dopamine (Huet and Franquinet, 1981). The suggested function of these neuromediators include influences on DNA and RNA synthesis, mitosis, and the metabolism of cytoplasmic and nuclear proteins through the regulation of the regulatory enzyme adenylate cyclate that catalyses the conversion of adenosine triphosphosphate (ATP) to 3',5'-cyclic AMP (cAMP). Despite being a stress hormone in marine invertebrates, studies on the presence of dopamine, and the time-dependent changes that occur after amputation in asteroid species, are sparse (Huet and Franquinet, 1981).

High-performance liquid chromatography (HPLC) is a commonly used technique in analytical chemistry. A typical HPLC setup comprises five basic components: the pump, injector, column, detector, and computer for data processing (Figure 3.1). Briefly, the injector delivers the sample into a stream of mobile phase, which is a mixture of solvents whose composition need to be carefully selected to allow separation of the sample compounds. The pump delivers the mobile phase and sample to the column, which is filled with sorbent particles (such as silica and polymers). The column separate the components of the sample based on their chemical and/or physical interaction with the sorbent particles. Subsequently, this passes through a detector and eventually produces a chromatogram with peaks representing particular compounds.

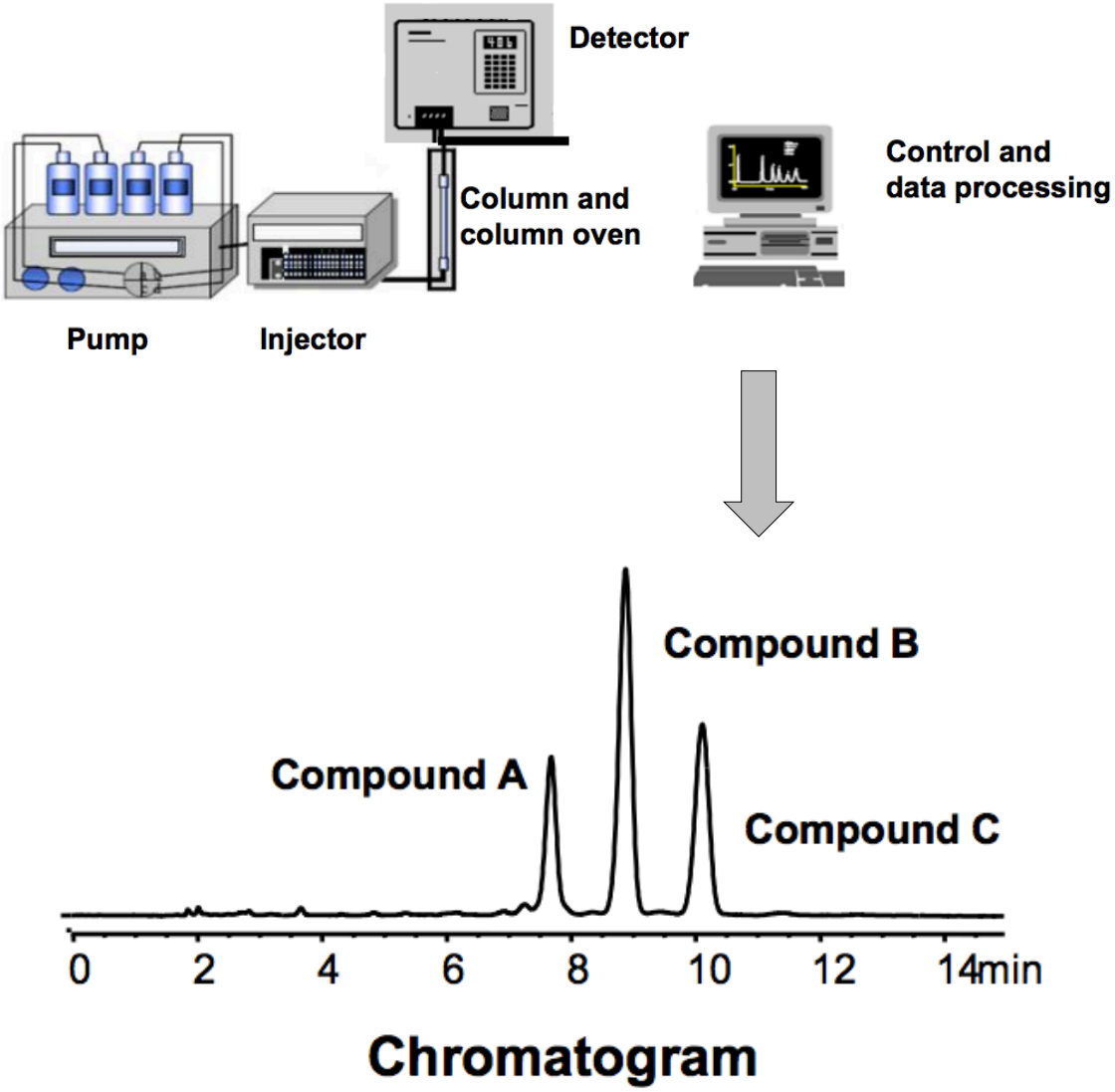

Figure 3.1. Basic components of an HPLC system. Courtesy of Agilent Technologies.

The detection method (Figure 3.1; detector) varies with the compound(s) of interest in the sample. The choice of method depends on the expected concentration of the compound in the sample, sensitivity of the detection method, efficiency and cost of analysis, and the number of samples. For the detection of dopamine in marine invertebrates, the HPLC method using electrochemical detection developed by Li and Vandepeer (2004) has been previously used by Qu *et al*. (2009) and Tan *et al*. (2015). In this study, a method involving pre-column derivatisation and fluorescence detection was used. Pre-column derivatisation involves the chemical reaction between the

analyte of interest (dopamine) and a reagent, and the products of the reaction are separated in the column and detected (*c.f.* post-column derivatisation involves separation of the analyte in the column first, and then derivatising it). Pre-column derivatisation was chosen in this study as it consumes a smaller amount of derivatisation reagent, and because the HPLC system configuration is simpler. Fluorescence detection is acclaimed for its high selectivity where the analyte of interest is well distinguished from endogenous/exogenous compounds which may interfere with the quantification (Lawrence and Frei, 2000).

The present study was aimed at: 1) adapting a pre-existing method developed by Zhao *et al*. (2011) for the detection of dopamine in porcine muscle, to quantify dopamine in coelomic fluid of an asteroid species and; 2) investigating the time-dependent changes in dopamine levels during the first 48 hours following amputation.

3.3 Methods and materials

Test runs of several previously published methods used for a range of marine species were conducted in order to identify a reproducible protocol for the detection of dopamine in sea stars. The UV and fluorescence detection methods by Muzzi *et al.* (2008) were tested but proved unsuccessful in obtaining similar chromatographs as reported by the authors. Detection of dopamine was eventually achieved through pre-column derivatisation by adapting a method described by Zhao *et al.* (2011).

3.3.1 HPLC system configuration

The HPLC system comprised of LC-20AD pumps, an SIl-20AC HT auto-injector, a SPD-M20A diode array detector, a CTO-20A column oven, and a DGU-20A3 online degasser (all Shimadzu Japan). LC Solutions software (Shimadzu Japan) was used to analyse chromatographs, and a Phenomenex C18(2) (150 x 4.6 mm, 5 µm particle size) was used as the analytical column. Fluorescence was monitored at excitation and emission wavelengths of 350 and 450 nm, respectively. Injection volume was 50 µl, and the column temperature was maintained at 32°C. The flow rate was 0.8 ml/min with an end run of 15 minutes.

3.3.2 Reagents

The mobile phase (60% HPLC-grade methanol and 40% deionized water with 0.1% (v/v) acetic acid), was prepared daily. All solvents used for the mobile phase were sourced from Merck Millipore, Auckland, New Zealand. The pre-column derivatisation solution was prepared by dissolving 0.0150 g *o*-phthalaldehyde (OPA) in 2 ml methanol, then adding 100 ml of 2-mercaptoethanol (2-ME) and finally adding 50 mM sodium borate to 25 ml (pH 10). The derivatisation solution was made fresh each day.

OPA was used in the derivatisation reagent as it is a very sensitive fluorescent reagent that reacts with amino acids. Catecholamines, such as dopamine, are derived from tyrosine (an amino acid), and in the presence of compounds known as 'thiols' (such as 2-ME), the reaction produces a highly fluorescent isoindole product (Wagner and McManus, 2003).

3.3.3 Sample treatment

Coelomic fluid samples for analysis were obtained by the methods described in section 2.3 (chapter 2). The samples were frozen at -80°C immediately after collection until required for analysis. Coelomic fluid was thawed at 4°C, and then 350 µl was diluted with equal volume of deionized water to reduce viscosity. The mixture was vortexed vigorously for 30 seconds and then centrifuged for 10 minutes at 14,000 rpm to allow cellular debris to descend to the bottom. 350 µl of the supernatant was collected and mixed with 140 µl of derivatisation reagent for 30 seconds in autosampler vials. A ratio of 5:1 (supernatant:derivatisation reagent) was used by Zhao *et al.* (2011), however preliminary results showed very small dopamine peaks, which was indicative of inadequate dopamine derivatisation. Therefore, the volume of derivatisation reagent was increased from 70 µl to 140 µl (5:2) . All coelomic fluid was analysed within 15 minutes of thawing due to the instability of dopamine.

3.3.4 Method validation

The method validation included the determination of the following parameters: retention time, limit of quantification (LOQ), and the linear range. Retention time refers to the amount of time taken from the injection of sample to the time of detection of the

analyte. The limit of quantification is defined as the lowest concentration of the analyte that can be determined with acceptable precision and accuracy (Shrivastava and Gupta, 2011). Lastly, linear range is determined to verify that the area under the peak Is proportionate to the concentration of the analyte. Calibration curves were constructed by plotting the area under the peak against the concentration. The linearity was determined by linear regression analysis.

The recovery and repeatability of the method was also examined. A stock solution of dopamine standard (2 mg/ml) was prepared daily in methanol and stored at 4°C. Subsequently, the solution was serially diluted with deionized water to achieve three concentrations of standards (100, 25, 1.56 ng/ml). To quantify the recovery rate of dopamine after dilution with water, coelomic fluid samples were spiked with 50 µl of dopamine standards at two different concentrations (100 and 25 ng/ml). After detection, the recovery rate was calculated by the following formula: Recovery rate = $(A_{sp}-A_{nsp})/A_{std}) \times 100$, where A_{sp}: dopamine peak area of spiked coelomic fluid; A_{nsp}: peak area of dopamine of non-spiked coelomic fluid; A_{std}: peak area of standard of the same concentration as the spiked coelomic fluid). The repeatability of the method was assessed by running each of the three concentrations of standards (100, 25, 1.56 ng/ml) three times a day, on multiple days. The peak height, area and retention times were noted, and the relative standard deviations (RSD) for these parameters were calculated by: (standard deviation/mean) x 100.

3.3.4 Statistical analysis

Before statistical analysis, data points with dopamine levels below the limit of detection were substituted with the LOQ (1.56 ng/ml) divided by the square root of 2, using the method published by Croghan and Egeghy (2003) and Ogden (2010). Data were then ln-transformed to improve normality. The time-dependent effect of amputation on coelomic fluid dopamine levels was assessed using repeated measure general linear modelling in SAS (version 9.2, SAS Institute, Inc., Cary, NC, USA), with one factor for comparisons between treatments (amputated or non-amputated) and one factor for comparisons across sampling occasions. The Wilk's Lambda test was used for multivariate analysis of variance (MANOVA) and Tukey's post hoc test was used for pairwise comparisons of means between amputated and control animals at each time point.

3.4 Results

3.4.1 HPLC method development

The retention time for dopamine was around 9.2 min (Figure 3.2). The limit of quantification was 1.56 ng/ml, and the relationship between the concentration of dopamine and the area under the peak was linear between 1.56 – 500 ng/ml (Table 3.1). The linear regression equation was y=35870x +110802, with correlation coefficient (r^2) of 0.998 (Figure 3.3).

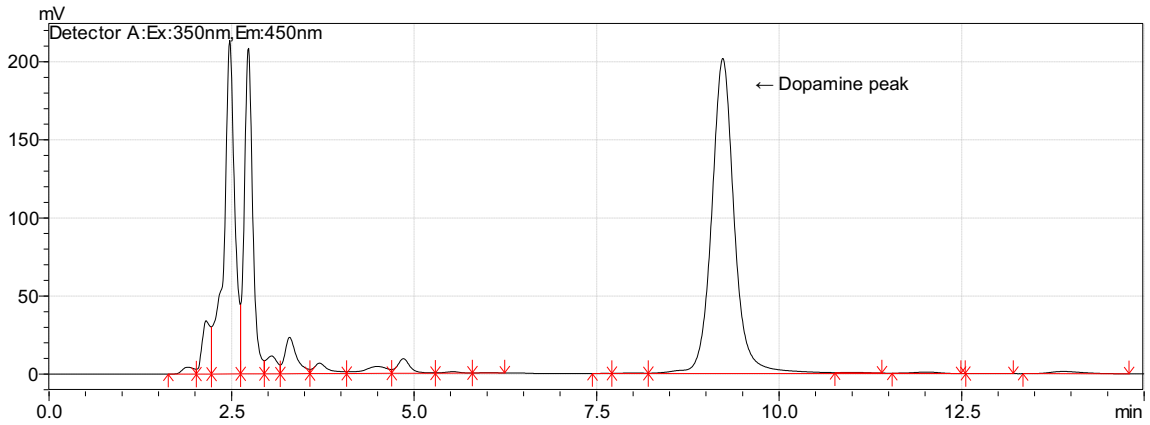

Figure 3.2. Typical chromatograph of dopamine detected by pre-column derivatisation and HPLC with fluorescence detection. Excitation and Emission wavelengths were 350 and 450 nm, respectively. The retention time for dopamine was around 9.2 minutes. This standard contains 100 ng/ml of dopamine.

Table 3.1 Summary of analytical parameters obtained for dopamine.

Limit of quantification (ng/ml)	1.56
Linear range (ng/ml)	1.56 – 500
Linear regression equation	y=35870x +110802
Correlation coefficient (r^2)	0.998
Retention time (minutes)	9.2

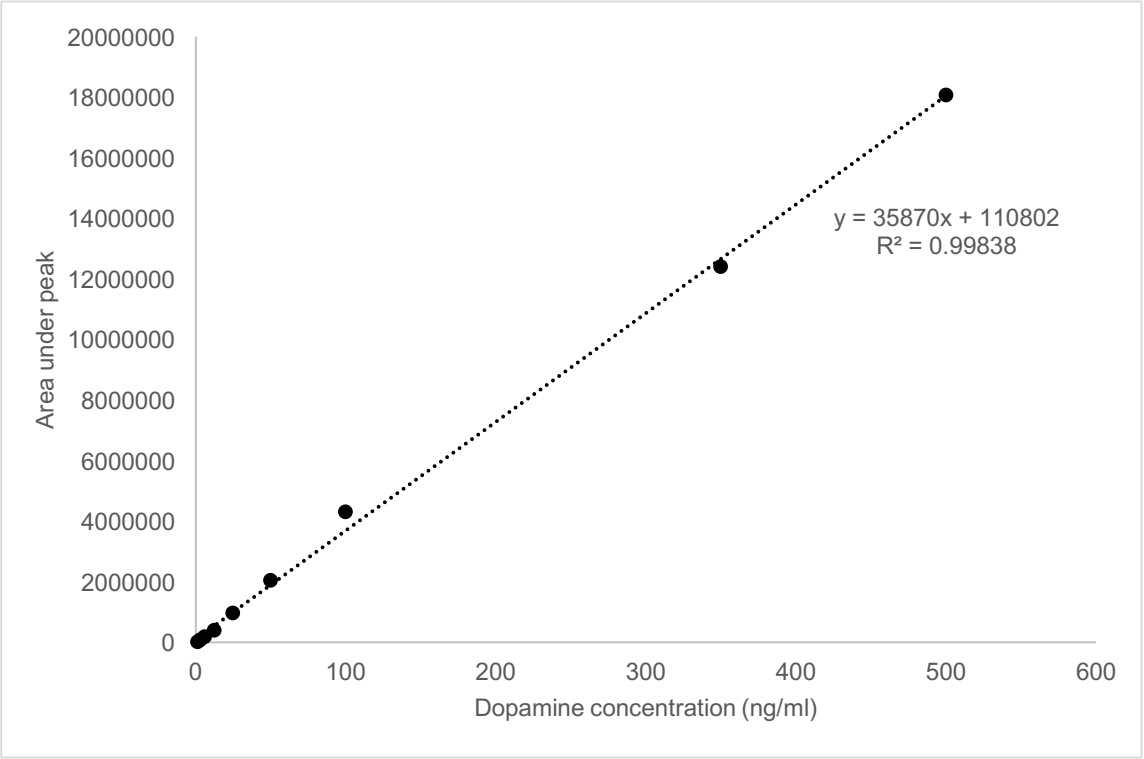

Figure 3.3. Calibration curve for dopamine standards between 1.56 – 500 ng/ml.

3.4.1.1 Intra- and inter- day variation

The intra- (repetitions of each concentration within one day) and the inter- (repetitions of each concentration over multiple days) variation is expressed in relative standard deviation (RSD) for the area under the curve, and the retention time (Table 3.2). The RSDs were lower than 5.5% for intraday, and 5.3% for interday sample runs.

Table 3.2 Intra- and inter- day variation of dopamine detection. The relative standard deviation (RSD) for the area under the peak, and retention time is reported for each concentration of dopamine standard.

	Intraday		Interday	
Concentration of dopamine standard	**Area RSD (%)**	**Ret. Time RSD (%)**	**Area RSD (%)**	**Ret. Time RSD (%)**
100	1.3 (n=3)	0.016	3.4 (n=9)	0.799
25	3.2 (n=3)	0.021	5.3 (n=9)	0.851
1.56	5.5 (n=3)	0.011	4.9 (n=5)	1.283

3.4.1.2 Recovery rate

Coelomic fluid samples spiked with either 25 or 100 ng/ml of dopamine were tested to elucidate the recovery rate of dopamine after dilution and centrifugation of the samples. Recovery rate of dopamine in coelomic fluid spiked with 25 and 100 ng/ml were 104.6 and 101.3 %, respectively. The results indicated that the method of sample treatment did not affect the concentration of dopamine in coelomic fluid.

3.4.2 Dopamine levels following amputation

The group-by-time interaction was significant, indicating that amputation did have a significant effect on the time-dependent changes in dopamine (Table 3.4). The mean was only significantly higher in the amputated group 24 hours after injury (X+24; Table 3.5; Figure 3.3; p = <0.05). The means did not differ between the treatment and non-treatment group at all other time points (p = >0.05).

Table 3.3 Repeated measures general linear modelling for coelomic fluid dopamine. DFn: Degrees of freedom in the numerator; DFd: Degrees of freedom in the denominator.

	Wilks' lambda	**F (DFn, DFd)**	***p* value**
Time	0.747	0.93 (4,11)	0.4819
Treatment * Time	0.387	4.36 (4,11)	0.0236

Table 3.4 Tukey's multiple comparisons test for coelomic fluid dopamine. Baseline refers to coelomic fluid sample in the field before transporting sea stars in to captivity; X-3: 3 hours before amputation; X+6, 24, 48: 6, 24, and 48 hours after amputation, respectively. Means are given as ng/ml of dopamine. ns: not significant; *: p = < 0.05

	Treatment group (ng/ml; mean ± SEM)	**Control group (ng/ml; mean ± SEM)**	**Significance**
Baseline	1.32 ± 0.22	2.18 ± 0.87	ns
X-3	9.00 ± 7.20	13.71 ± 10.56	ns
X+6	4.16 ± 1.63	2.06 ± 0.76	ns
X+24	2.88 ± 0.71	1.10 ± 0.00	*
X+48	1.10 ± 0.00	3.12 ± 1.56	ns

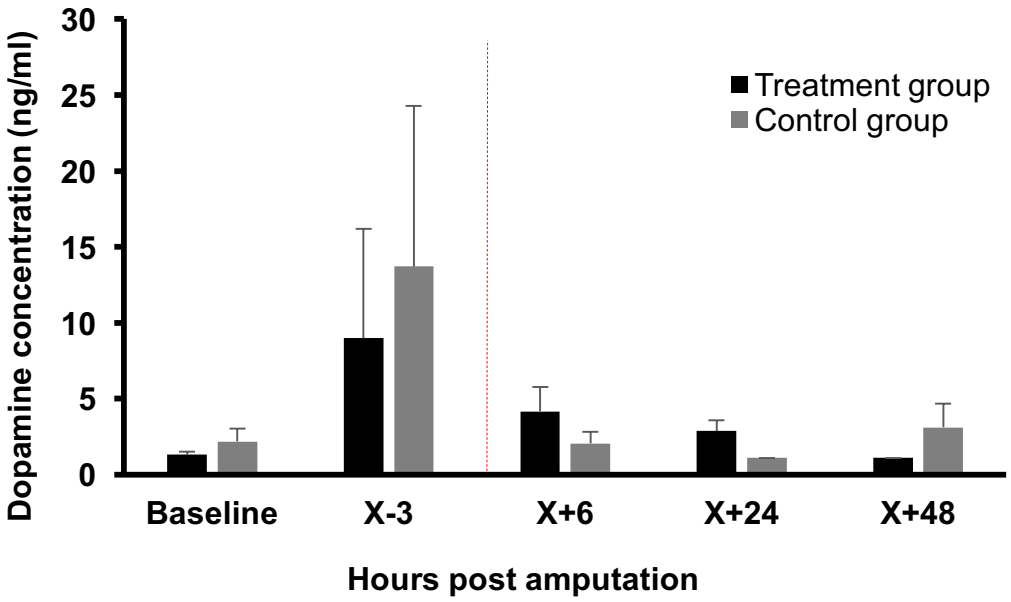

Figure 3.4 Dopamine present in coelomic fluid. Baseline refers to coelomic fluid sample in the field before transporting sea stars in to captivity; X-3: 3 hours before amputation; X+6, 24, 48: 6, 24, and 48 hours after amputation, respectively. Red dotted line indicates time of amputation. Data are expressed as the mean values ± SEM.

3.5 Discussion

Here, an existing method (originally developed by Zhao *et al*., (2011) for the detection of dopamine in porcine muscle), was successfully adapted to quantify dopamine in the coelomic fluid of an asteroid species. In this study, the limit of quantification was lower than the lowest concentration (100 ng/ml) tested by Zhao *et al.* (2011), and I was able to achieve a similar correlation coefficient value (r^2) as the previous study (r^2 = 0.999). Furthermore, the maximum RSDs in this study were lower than those reported by the authors for both intra- and inter- day variation (7.8% and 9.4% respectively), indicating that the adapted method in this study exhibited acceptable reproducibility.

However, this method is not without limitations, especially regarding the limit of quantification which was 1.56 ng/ml. Concentrations below 1.56 ng/ml could not be quantified, ultimately leading to gaps in the data set. Although this is a commonly encountered problem in chemical analyses, there is considerable debate surrounding the correct way of handling such data, and whether such results should be accepted for publication (Croghan and Egeghy, 2003; Ogden, 2010). In this study, these gaps

have been substituted with the limit of quantification (1.56 ng/ml) divided by the square root of 2, which has been shown to have the smallest overall error rate compared to other substitution methods such as replacement with zero, or limit of quantification divided by 2 as reported by Croghan and Egeghy (2003). Furthermore, Croghan and Egeghy (2003) recommended that this substitution approach be used only when a small percentage of values are below the detection limit. The data did not comply with this, but it should be noted here that when dopamine concentrations above the limit of quantification were observed in this study, they were typically more than an order of magnitude greater than 1.56 ng/ml, and so the reported significant result corresponded with a large increase in coelomic fluid dopamine concentrations 24 hours after amputation. I used this substitution method as the aim of this study was to report significant time-dependent changes in dopamine levels following amputation, rather than reporting the absolute concentration of dopamine occurring in the coelomic fluid. Nevertheless, I acknowledge that the substitution method affects the estimates of the mean and standard deviation, and results in a uniform and misleading distribution for the values below the limit. Going forward, improving the sensitivity of the HPLC method might help remedy this issue.

Nonetheless, this study demonstrated that amputated animals exhibit elevated dopamine levels 24 hours after amputation. Interestingly, there was a synchronized increase in both the total coelomocyte count and dopamine levels 24 hours post amputation (chapter 2). Increased dopamine levels during stress have been documented in many marine invertebrate species, and are often accompanied by impaired immunofunction (Lacoste *et al*., 2002, Malham *et al*., 2002). However, there is currently disagreement on the effect of dopamine on the number of circulating coelomocytes, as injection of dopamine supposedly decreases cell numbers in the haemolymph of white shrimp (*Litopenaeus vannamei*) but increased dopamine is also associated with raised coelomocyte counts in the Japanese sea cucumber, *Apostichopus japonicus* (Chang *et al*., 2007; Pan *et al*., 2011; Tan *et al*., 2015). The results from this study are more aligned with Tan *et al.* (2015), who reported an increase in both coelomocyte numbers and dopamine in response to a stressor. This is not surprising since the sea cucumber is an echinoderm that is more closely related to *C. muricata* than the white shrimp (an arthropod). As mentioned in chapter 2, the increase in cell numbers 24 hours post amputation is likely to be associated with the re-epithelisation of the wounded area. As dopamine is suggested to initiate a number of cellular processes such as mitosis (Huet and Franquinet, 1981), an increase in

dopamine is likely to be associated with the re-epithelisation process that requires the migration and proliferation of cells to the wounded area. Unfortunately, there is currently little evidence to support dopamine-induced migration of cells in echinoderms. Dopamine is, however, known to play a role in migration of human immune cells via the dopamine receptor, D3 (Watanabe *et al*. 2006).

Dopamine in invertebrates diverts bioenergetics to maintain immediate homeostasis (such as preventing further loss of coelomic fluid), and it has been associated with reduced immunofunction in sea cucumbers (Tan *et al*., 2015). Decreased phenoloxidase (an enzyme involved in melanogensis in invertebrates) and phagocytosis activity have been reported, and such may be the case with sea stars exhibiting elevated dopamine levels (Gonzalez-Santoyo and Cordoba-Aguilar, 2012; Tan *et al*., 2015). Similarly, Chang *et al*. (2007) suggested that dopamine causes redirection of resources to processes such as glycolysis, lipolysis and respiration, causing a reduction in immunofunction in three decapod species. In this study, dopamine levels were elevated 24 hours post amputation, suggesting that bioenergetics may be prioritised, at least at this time, to the maintenance of coelomic fluid composition and volume. However, it is uncertain whether the immune function is reduced at this time, since there was also an increase in coelomocyte numbers in chapter 2. Coelomocytes have also been suggested to be the first line of defence against after amputation by phagocytosing cell debris and pathogens (Holm *et al*., 2008; Khadra *et al*., 2015). To further investigate this, an examination of the differential coelomocyte count is required to identify whether dopamine causes an increase in coelomocytes participating in the immune response, or whether dopamine increases numbers of coelomocytes that are predominantly involved in re-epithelisation of the wounded area. Reduced immune function may in turn adversely affect the survival of these animals, however further investigation is required to understand the relationship between dopamine, immune function and survival.

Lacoste *et al.* (2001) reported a rapid increase in catecholamines in Pacific oysters (*Crassostrea gigas*) subjected to mechanical shaking, in which values returned to basal levels within 90 minutes. The authors also reported that catecholamine responses to temperature and salinity variations were more prolonged, lasting up to 72 hours, suggesting that the type of stressor (acute or chronic) has an influence on the time-dependent changes in hormone levels. Investigating the dopamine response of sea stars that are uninjured but subject to acute stressors such as human handling would be interesting to investigate. In this study, both the cell count and dopamine levels were

elevated 24 hours after amputation. However acute short lasting stressors may increase the coelomocyte count and dopamine levels more quickly than what was observed in this study. Furthermore, the relationship between the physiological stress findings in this species and survival rates (both short- and long-term) following fishing activities need to be established. Since injured sea stars have a higher mortality rate than those that are unscathed by the fishing equipment, investigating the stress response of sea stars subjected to varying types and intensities of stressors would allow better management of these animals caught as bycatch from commercial fisheries.

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Chapter 4

Initial steps made in the development of an *in vitro* cytotoxicity bioassay using coelomocytes harvested from sea stars

Chapter 4 – Initial steps made in the development of an *in vitro* cytotoxicity bioassay using coelomocytes harvested from sea stars

4.1 Abstract

In vitro cell cultures are an effective method for testing toxicity of xenobiotic compounds. Using cell cultures allows more experimental replicates and treatments, which minimises ethical concerns over using live animals. This chapter aimed to investigate the effects of cadmium and zinc on cellular parameters (cellular metabolic activity, membrane integrity, and phagocytosis activity), and, whether amputation acts synergistically with heavy metal exposure to compromise these cellular parameters. Coelomocytes harvested from *Coscinasterias muricata* in the field (baseline), 3 hours before amputation, and 6, 24, and 48 hours after amputation were set up for this *in vitro* experiment. However, this experiment was impeded by unexpectedly low cell counts present in coelomic fluid of *C. muricata* (in comparison to other echinoderm species), which ultimately led to failure to achieve the initial aims of this study. Nonetheless, this chapter collates the findings gleaned from this experiment in order to provide groundwork for future studies with *C. muricata* cell cultures. This is the first study to attempt *in vitro* experiments with *C. muricata* coelomocytes.

4.2 Introduction

The Australian and New Zealand Environment and Conservation Council (ANZECC) provide numerical guidelines, termed trigger values, for the management of toxicants in aquatic systems. The guideline handbook defines trigger values as:

> "…concentrations that, if exceeded, would indicate a potential environmental problem, and so 'trigger' a management response, e.g. further investigation…they are calculated to protect a predetermined percentage of species with a specified level of confidence"."

For cadmium and zinc in marine waters, the ANZECC (2000) trigger values for the protection of 95, 90, and 80% of species are 5.5, 14, 36 μg L^{-1}, and, 15, 23, and 43 μg

L^{-1}, respectively. For example, to preserve 90% of the species in a given environment or habitat, the cadmium concentration must not exceed 14 µg L^{-1}.

Current biomonitoring practices focus on change at the ecological level such as population, community and ecosystem disturbances, rather than physiological changes occurring in a single organism or species. Nonetheless, all changes observed at these larger scales stem from disturbances occurring at the biochemical and cellular level of each individual. The direct effect of these guideline values on the physiology of biota is notably overlooked. As 'environmental problem' is a broad term encompassing effects on the abiotic and biotic components of the environment, investigating the effect of trigger values on particular species, especially keystone species, has merit. If a toxicant is found to be deleterious on a keystone species, the cascade effect on the food chain may indeed lead to a broader 'environmental problem'.

Exposure to high levels of heavy metals is associated with brain lesions, compromised fertility, kidney and liver failure in a wide range of species, from fish to humans (Patra *et al.*, 2001; Berntssen *et al.*, 1999; Sheweita *et al.*, 2005; Ahmad *et al.*, 2011; Agarwal *et al.*, 2012;). Furthermore, the accumulation of high levels of heavy metals is known to interfere with proper embryonic development, steroid metabolism, immune function and skeletogenesis in asteroids (Voogt *et al*., 1987; den Besten *et al*., 1989; Temara *et al*., 1997a; Coteur *et al.*, 2003; Matranga *et al*., 2012). Heavy metal uptake must be regulated to achieve homeostatic levels (Viarengo and Nott, 1993).

The eleven-armed sea star *C. muricata* presents an ideal species to investigate the biological effects of the ANZECC trigger values, as factors affecting their survival may lead to an uncontrolled overexpansion of prey species and cause a collapse in the community structure. Keystone predators are subject to pollutant exposure through direct exposure from the environment, as well as consumption of contaminated prey. Boisson *et al.* (2002) found that approximately 50% of lead (Pb) accumulated in soft tissue of mussels was transferred trophically to *A. rubens*. In addition, sea stars also accumulate heavy metals directly from the environment, with accumulation within the animals reflecting the concentrations of heavy metals present in the environment (Bjerregaard, 1988; Temara *et al*., 1997b; Temara *et al*., 1998). Furthermore, *C. muricata* reproduces both sexually and asexually, and since heavy metals are known to have adverse effects on embryonic development, fertilization, and steroid metabolism in asteroids, heavy metal exposure may be a threat to the species (Voogt *et al*., 1987; Lee *et al*., 2004).

The synergistic effect of arm amputation and toxicant challenge may also present a threat to the survival of *C. muricata*. Although arm amputation does occur in the wild (due to predation), invasive anthropogenic fishing activities such as trawling combined with heavy metal contamination may exacerbate the adverse effects of limb loss. There is an energetic cost associated with the loss of an appendage. This cost decreases the ability of an organism to obtain nutrients, and energy and nutrients are expended on the repair and regeneration of the lost arm which comes at a cost to other body compartments (Lawrence, 2010). Arm amputation in sea stars has been shown to cause a number of physiological changes, including an increase in heat shock protein 70 (HSP70) expression, increased total cell count and increased concentrations of endocrine factors (Huet and Franquinet, 1981; Pinsino *et al*., 2007).

4.2.1 Cellular heavy metal tolerance and detoxification

One of the most well documented cellular effects of heavy metal toxicity is oxidative stress. Oxidative stress is defined as adverse physiological effects that result from the excess accumulation of reactive oxygen species (ROS) such as superoxide (O_2^-). ROS induce lipid peroxidation which change the structure and biochemistry of phospholipids, cholesterol and triglycerides, resulting in the functional loss of biomembranes (Lesser, 2006). In addition, disruption of redox homeostasis is associated with DNA damage and impaired DNA repair responses (Barzilai and Yamamoto, 2004).

Cellular mechanisms to reduce heavy metal induced ROS production include binding the heavy metals to specific ligands that reduce the bioavailable concentration within the cell, compartmentalisation within organelles (mainly lysosomes), and formation of insoluble precipitates (Viarengo and Nott, 1993). Lysosomes are capable of accumulating significant amounts of heavy metals and other xenobiotic compounds. However, there is a limit to how much heavy metal can be stored in lysosomes, and when exceeded, lysosomal membrane damage and a reduction in lysosomal enzyme activity may occur due to increased ROS production (Viarengo and Nott, 1993). Heavy metals in lysosomes are mainly bound to a lipid peroxidation byproduct called lipofuscin (Viarengo and Nott, 1993; Rainbow, 1997). Oxidised and non-functional portions of membranes are incorporated into lysosomes where they form lipofuscin granules that sequester heavy metals.

In cases where ROS production is inevitable (such as when the preventative measures listed above are overwhelmed), the cell relies on both non-enzymatic and enzymatic defence to avoid oxidative stress (Viarengo and Nott, 1993). Non-enzymatic defence refers to antioxidant molecules that are essential for the maintenance of redox homeostasis. Some antioxidant molecules act early in the oxidative process by scavenging free radicals, while some antioxidant molecules are capable of removing undesirable products of oxidative damage such as lipid peroxidation and DNA oxidation (El-Beltagi and Mohamed, 2013; Fourquet *et al.*, 2008). Important biosynthesised antioxidant molecules such as glutathione (GSH) and melatonin are highly conserved and present in invertebrates as well as mammals (Tan *et al*., 2007).

Enzymatic defence involves the conversion of O_2^- radicals to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 produced by SOD is subsequently processed via two main detoxification mechanisms. Glutathione peroxidase (GPx) catalyzes the conversion of H_2O_2 using two glutathione (GSH) molecules as substrates. This process ultimately produces one glutathione disulfide (GSSG) and two H_2O molecules (Equation 1). In addition, the enzyme catalase (CAT) catabolises two H_2O_2 molecules into two H_2O and a O_2 molecule (El-Beltagi and Mohamed, 2013; Fourquet *et al.*, 2008; Equation 2).

$$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O \qquad \text{(Equation 1)}$$

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2 \qquad \text{(Equation 2)}$$

4.2.2 *In vitro* cytotoxicity testing

With increasing efforts to reduce the use of live animals in scientific research, *in vitro* cytotoxicity testing provides an alternative by using primary cell cultures or established immortalised cell lines in the place of live animals allowing more experimental treatments and replicates (Tan *et al.*, 2008). In the case of primary cell culture, live cells are isolated from a living sea star under aseptic conditions and the cells are placed in a solution referred to as culture media, which contains essential components for maintaining cellular function such as amino acids, ionic compounds, carbohydrates and vitamins. Cell culture media are available in many formulations and a form most compatible with the study species must be selected for normal cell function to be

maintained. To test the effect of toxicants on cell function, the cytotoxic compounds are introduced to the cells to elicit a cellular response. Cytotoxic compounds may cause apoptosis (programmed cell death) or cell necrosis (premature cell death caused by external factors). However, depending on the cytotoxic compound and the concentration, cells may not have sufficient time and energy to activate the apoptotic pathway resulting in cell necrosis (Promega Corporation, 2015). Assays which have been previously used with success with isolated echinoderm coelomocytes include: cell viability, cellular metabolism, cell growth and proliferation, lysosome integrity and DNA integrity (Ronning, 2005; Canty, 2009). The assays that were employed in this study are described in further detail in following sections.

The obvious disadvantage associated with using cell cultures is that the results obtained from *in vitro* experiments may not directly depict what occurs within a complex organism where the cells are *in situ* with other tissues. Nonetheless, this can be overcome with careful extrapolation using physiologically based pharmacokinetic (PBPK) modelling and an appropriate understanding of the limitations of the *in vitro* model. *In vitro* cytotoxicity testing has been proven to be an effective tool for quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) by estimating the absorption, distribution, metabolism and excretion of substances using human cells (Yoon *et al.*, 2011).

The biggest advantage of *in vitro* cytotoxicity testing is that it is a prognostic tool of toxins before pathological change is observed in the whole organism or at ecological levels. Secondly, *in vitro* cytotoxicity testing can be an effective assessment tool for guideline values that were generated based on non-biological data, such as those derived from statistical modelling or chemical monitoring. Cell cultures are also useful as they can elucidate the cellular and biochemical mechanism of xenobiotic toxicity.

In vitro cell cultures may also be developed into an effective diagnostic tool. Recently, biomarkers have become increasingly popular as they allow for non-fatal sampling, as well as repeated sampling to monitor change over time. The term ‘biomarker’ is used when an aspect of the organism, such as the biochemistry or physiology, reveals information about the overall condition of the animal, as well as the degree of environmental pollution (Bartell, 2006). Employing biomarkers can be beneficial as they can act as early warning signals of a disturbance, which allows for intervention before irreversible changes occur in the community structure. In addition, biomarkers are effective as they reflect the effect of only the biologically active proportion of the

toxicant accumulated in the body. Such is not the case with sentinel organisms that merely represent the amount of accumulation, rather than the effect of accumulation. Preliminary experiments with cell cultures provide the foundations of developing effective biomarkers by identifying appropriate culturing conditions and informative assays that reveal physiological disturbance.

In vitro cytotoxicity testing often uses mammalian or microbial cell lines that are already established, making them easy to acquire and maintain. However, the effect of toxicants on the aquatic macrobiota cannot be easily translated from most mammalian or microbial cell lines (Babich *et al.*, 1986; Kamer and Rinkevich, 2002). As a result, fish cell lines have become the preferred choice for aquatic cytotoxicity testing. While several fish cell lines have been developed over the years, numerous past attempts to develop permanent and proliferative cell lines of aquatic invertebrates (excluding insects) have been unsuccessful (Bayne, 1998; Rinkevich, 1999; Mothersill and Austin, 2000; Kamer and Rinkevich, 2002; Rinkevich, 2005). Primary cell culture using common starfish *Asterias rubens* coelomocytes has been reported to be an effective biomonitoring and toxicity testing tool for several xenobiotic substances such as brominated flame retardants, tributyltin (TBT) and perfluorinated substances (Ronning, 2005). *A. rubens* is by far the most commonly used asteroid species ecotoxicological studies. However, it is only a prominent species in the northern hemisphere and is not found in New Zealand waters. There are no previous studies on cell cultures or the establishment of cell lines from *C. muricata*.

4.2.2.1 Cellular metabolic activity

Cellular metabolic activity is easily measured using an indicator dye called Alamar Blue produced by ThermoFisher Scientific. The Alamar Blue reagent contains resazurin as its active ingredient. Resazurin is a non-fluorescent membrane permeable molecule that changes colour from blue to bright red when converted to resorufin within active proliferating cells. The fluorescence emitted by resorufin is quantitatively measured as an indicator of cellular metabolism as viable cells are able to continuously convert resazurin to resorufin through reduction reactions.

The precise location and enzymes responsible for the reduction of resazurin remain unclear, as both cytosolic and mitochondrial reductases have been implicated in the process (Rampersad, 2012). Therefore, it is likely that the reduction of resazurin occurs from multiple metabolic reactions in more than one intracellular location. This allows Alamar Blue to be used as an indicator of both cellular metabolic activity and/or

mitochondrial impairment (O'Brien *et al*., 2000; Ronning, 2005; Rampersad, 2012; Bonnier *et al.*, 2015).

4.2.2.2 Cellular membrane integrity

Neutral red (3-Amino-7-dimethylamino-2-methylphenazine hydrochloride or toluylene acid) is a dye that was developed to enter cells via non-ionic diffusion and stain viable lysosomes red. The degree of lysosomal membrane damage is determined microscopically by measuring the amount of time it takes for the dye to penetrate into the organelle (Monteiro *et al*., 2011). Since its development, the dye has been developed further to quantify the degree of membrane damage. The ability of a cell to uptake the dye is lost with membrane damage and this can be quantified spectrophotometrically. The amount of dye taken up by the cell is represented as optical density for red wavelength in the visible spectrum. This technique is widely used for interrogating the cellular membrane integrity of marine invertebrate cells as it can be easily performed on multiple samples and can be analysed for less time and effort (Canty, 2009).

4.2.2.3 Phagocytosis activity

Phagocytes are an integral part of the asteroid immune system. Quantifying phagocytosis activity is a common assay used as an indicator of immune function. The process of phagocytosis is a highly complex series of events involving chaemotaxis of the phagocyte toward the foreign particle, recognition, ingestion and degradation in phagolysosomes. As phagocytes constitute the vast majority of the coelomocyte population in echinoderms, assessing their activity is an ideal assay to understand and assess immunocompetence.

Phagocyte activity can be determined by challenging isolated phagocytes with particles which can be phagocytosed. Several methods have been developed, and these methods differ in that there are variations in the size of the foreign particles used to challenge the phagocytes, the use of colorimetric or fluorescent particles, and compatibility of these particles with certain cell types (Tabata and Ikada, 1988; Hampton and Winterbourn, 1999). For invertebrates, the method developed by Pipe *et al.* (1995) for the blue mussel (*Mytilus edulis*) has been the most widely adopted due to its relative simplicity and affordability, compared to some of the techniques commonly used with human and other mammalian phagocytes. The assay involves using zymosan, a glucan ligand from yeast (*Saccharomyces cerevisiae*) that has been

stained with neutral red. Phagocytes are incubated with the neutral red-stained zymosan, and the phagocytes that have actively phagocytosed the zymosan are quantified colorimetrically by spectrophotometry.

4.2.3 Chapter aims

Originally this chapter aimed to identify: 1) the effect of ANZECC trigger values of cadmium and zinc on coelomocytes; and 2) the effect of amputation on heavy metal tolerance of coelomocytes. However, initial attempts at establishing coelomocyte cultures were unsuccessful, and ultimately resource and time constraints precluded the experiment being further revised and refined. However, the experience gained through this experiment was of value and this chapter now addresses the difficulties and future directions for *in vitro* cytotoxicity experiments using asteroid coelomocytes.

4.3 Method development

Bringing wild animals into captivity involves challenges such as the transportation of the animals to the facility, and providing suitable housing facilities. With marine invertebrates, the most crucial aspect of husbandry is to maintain suitable water quality, which includes oxygenation state and various chemical parameters such as ionic composition and waste product (e.g. ammonia and nitrite) build up. A constant supply of oxygenated clean saltwater can be achieved in laboratories with an in- and out- flow piping system to and from the sea (i.e. an open-circuit sea water reticulation system). This facility was not available for this experiment as the laboratory was not near the sea. In such cases, echinoderms have been successfully housed in aquaria fitted with filters and daily changes of water (Canty, 2009). However, this is a strenuous and time consuming method, therefore I was restricted to keeping sea stars alive in captivity for a maximum of two weeks.

4.3.1 First trial

As a pilot trial, 3 ml of coelomic fluid was collected from live *C. muricata* specimens in the field using a sterile 22-gauge needle and syringe. Coelomic fluid samples were placed into sterile tubes containing 9 ml of Leibovitz's L-15 medium (1:3; Gibco™ Cat.

No. 11415064, Thermofisher Scientific, Auckland, New Zealand). Leibovitz's L-15 medium was selected as the cell culture medium as its compatibility with asteroid coelomocytes has been tested previously (Ronning, 2005). Leibovitz's culture medium was used as cell clumping was previously observed with anticoagulant buffer composed of: 0.435 M NaCl; 10.7 mM MgCl; 27 mM Na_2SO_4; 16.6 mM glucose; 12 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 5 mM ethylene glycol tetraacetic acid (EGTA); pH 7.4 (Holm *et al*., 2008). All reagents were sourced from Sigma-Aldrich (Sydney, Australia).

Animals were transported from Point Halswell, Wellington, to Palmerston North in 24 L chilly bins containing sea water. Animals were housed in chilly bins in pairs and the tubes containing cell suspensions were kept on ice during this period. Upon arrival at the lab, 50 µl of the diluted cell suspension from the field was seeded into each well of a sterile 96-well flat-bottom microtitre plate, with 8 replicates per individual (column-wise). This was performed in a biological safety cabinet (SAFE 2020, ThermoFisher Scientific). The plate was incubated in the dark for 24 hours at 15°C in a chilling incubator (EchoTherm™ IN40, Torrey Pines Scientific LLC, San Marcos, CA, USA) to allow the cells to settle in a single layer at the bottom of the well. Unattached cells were removed after this period. Subsequently, samples were collected 3 hours prior to amputation, and then 3, 6, 8, 12, and 24 hours post amputation.

The baseline cell cultures showed signs of fungal contamination. In hindsight, this most likely occurred while transferring the coelomic fluid to tubes containing culture medium in open air during field sampling. Samples were collected out in the open and the sterile coelomic fluid samples were aliquotted into 50 ml tube containing Leibovitz's culture media in open air conditions. The 50 ml tubes, syringes, needles and Leibovitz's culture medium were all sterile, and the tube would have only been open for less than 10 seconds, but contamination undoubtedly occurred during this period. It was consequently not possible to carry out any further analyses of the baseline samples. Samples collected thereafter were handled in a biological safety cabinet to avoid microbial contamination as previously experienced. Coelomic fluid was seeded into 96-well microtitre plates and followed the same protocol as described above for the baseline samples.

During this pilot trial, cadmium chloride ($CdCl_2$; Sigma-Aldrich, Sydney, Australia) was tested in three different concentrations. $CdCl_2$ was serially diluted using Leibovitz's culture medium to achieve three test solution concentrations: 5.5, 14, and 36 µg/L

which were the cadmium concentrations suggested by ANZECC (2000) for the protection of 95, 90, and 80% of species in the environment, respectively. Two replicates of each individual were exposed to either one of the three concentrations of $CdCl_2$ or culture medium (to serve as controls) for 72 hours at 15°C (Figure 4.1). At the end of the exposure period the cell culture was uncontaminated and a significant portion of coelomocytes remained adhered to the bottom of the well, indicating live cells.

4.3.1.1 Protein determination

The protein content of each well was determined to account for any differences in cell seeding densities and differences in rates of cell growth. Plates were frozen and thawed to facilitate cell membrane disruption. Fifty µl of NaOH (0.1 M) was added to each well to solubilize the protein and plates were incubated at 27°C for one hour.

Two different protein assay kits with varying measurement ranges were tested. Firstly, a protein determination was carried out using the *DC* Protein Assay Kit (Bio-Rad Laboratories, Auckland, New Zealand), with a working range of 200 – 1,500 µg/ml protein. Protein standards were prepared with bovine serum albumin (stock solution 2.0 mg ml^{-1}; Sigma-Aldrich, Sydney, Australia) in 0.1 M NaOH. Preparation of all reagents used was in accordance with the manufacturer's guidelines. Briefly, 5 µl of each concentration of standard was placed in duplicate on a new 96-well microtitre plate. The same quantity of Leibovitz's culture medium was also added to wells in duplicates as blanks. Modified reagent A was prepared by adding 20 µl of reagent S to every ml of reagent A (an alkaline copper tartate solution). Twenty-five µl of modified reagent A was added into each well. Two hundred µl of reagent B (a dilute Folin Reagent) was added into each well and mixed gently for 5 seconds. Fifteen minutes later, the absorbance was read at 650 nm using a colorimetric plate reader (BioTek Instruments Inc., Winooski, VT, USA). Absorbance was read within one hour of mixing the reagents in the wells as they become unstable after this time.

Absorbance readings using this assay kit indicated that several wells contained lower protein concentrations than the working range of the Bio-Rad DC protein assay (<200 µg/ml). Therefore, a second more sensitive protein assay (BCA Protein Assay Reagent Kit, Pierce Biotechnology) with a working range of 20 – 2000 µg/ml was used and this reduced the number of wells with protein concentration outside the measurement range. However, there were still several wells that had protein concentrations less than the minimum working range concentration (<20 µg/ml). As a result, for the second

study the Coomassie Plus (Bradford) Assay Kit (Pierce Biotechnology; working range: 1 – 25 µg/ml) was used.

4.3.2 Second trial

Two different heavy metals were used in this study ($CdCl_2$ and $ZnCl_2$; Sigma-Aldrich, Sydney, Australia), and the three assays described in sections 4.2.2.1 – 4.2.2.3) were carried out in the second trial. In order avoid transferring coelomic fluid into tubes containing culture medium in open air, field sampling was carried out using sterile syringes that were pre-filled with sterile Leibovitz's culture medium in a biological safety cabinet in the lab. Coelomic fluid collected in the field (baseline samples), 6 hours before amputation and, 6, 24 and 48 hours after amputation were exposed to heavy metals. Different time points were selected for this trial because, although no fatalities resulted from withdrawing 3 ml of coelomic fluid at all seven time points, it did become sequentially more difficult to draw out the coelomic fluid. Decreased tube feet activity, turgidity, and movement of sea stars were also observed. This was probably due to the lack of time for the sea stars to replenish the lost coelomic fluid between sampling points. Consequently, only 1.1 ml of coelomic fluid was withdrawn at each sampling point in this trial, and placed in 2.2 ml of Leibovitz culture medium (1:2; GIBCO). The volume of coelomic fluid collected at each time-point was also decreased to 1.1 ml as losing 3 ml of coelomic fluid per sample may have been a substantial stressor that contributed to decreased tube feet activity, turgidity and movement of the sea stars. The dilution ratio of coelomic fluid in Leibovitz's cell culture medium was decreased from 1:3 to 1:2 in order to achieve a higher a cell concentration than in the first trial, because determination of protein content in the previous trial was unsuccessful as there were not enough cells in each well.

Fifty µl of the cell suspension was seeded in to each well of a 96-well flat-bottom microtitre plate, with 48 replicates per individual. The plate was incubated in the dark for 24 hours at 15°C to allow the cells to settle in a single layer and adhere to the bottom of the well. Then, for each assay, two replicates of each individual were exposed to one concentration of heavy metal or culture medium (to serve as controls) for 72 hours at 15°C.

4.3.2.1 Heavy metal exposure

Stock solutions of $ZnCl_2$ (Cat. No. 793523; Sigma-Aldrich), and $CdCl_2$ (Cat. No. 20899; Sigma-Aldrich) were diluted with Leibovitz's culture medium. The three concentrations of each heavy metal are as follows: 15, 23 and 43 µg L^{-1} of zinc; 5.5, 14, and 36 µg L^{-1} of cadmium. Samples were also filter sterilized through 0.20 micron syringe filters prior to use in cell cultures. Fifty µl of each exposure media was added to designated wells (Figure 4.1). After 72 hours, the exposure media was taken out to remove any dead cells which would inhibit survival of any living cells.

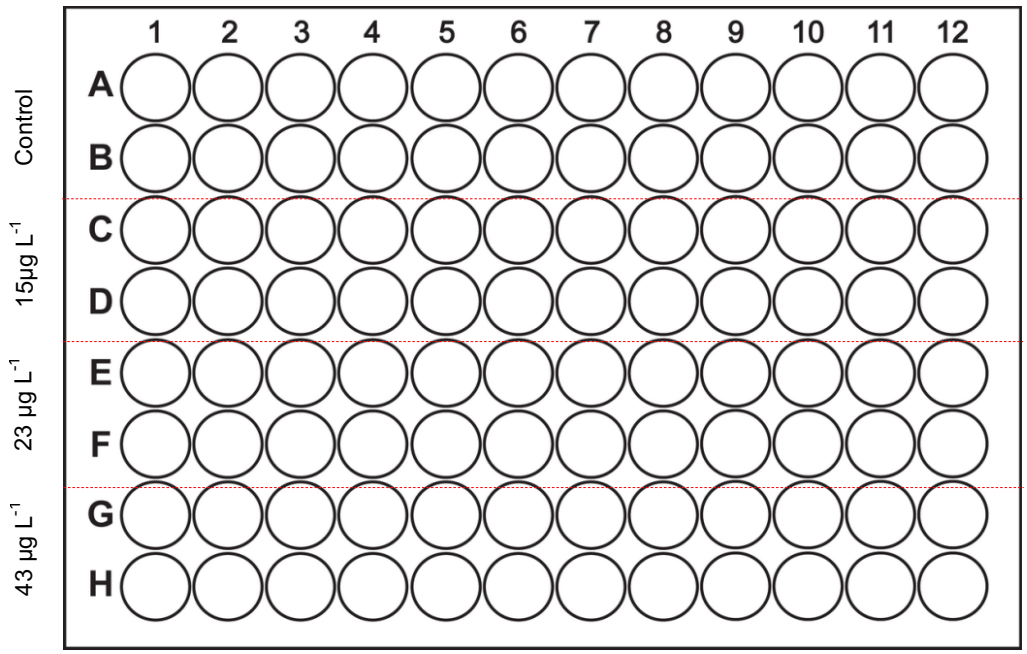

Figure 4.1 Experimental layout of 96-well microtitre plates. Exposure concentrations for zinc are depicted on the left hand side. The numbers along the top indicate individual animals. This layout was repeated for three assays at each time point for each of the two heavy metal (zinc and cadmium).

4.3.2.2 Cell metabolic activity

To each well, 100 µl of PBS containing 5% (v/v) Alamar Blue was added, and the plates were incubated in the dark for 30 minutes at room temperature. Fluorometric readings were performed on a Fluoroskan Ascent® FL fluorescence plate reader

(ThermoFisher Scientific, Auckland, New Zealand) using excitation and emission wavelengths of 544 nm and 590 nm, respectively.

4.3.2.3 Cellular membrane integrity

Two hundred µl of 0.004% of neutral red dye (diluted in milliQ water) was added to each well and the plates were incubated 10°C for 3 hours. Each well was washed twice with PBS. The dye was resolubilised by adding 100 µl of acidified ethanol (1% acetic acid and 50% ethanol in milliQ water). The plates were agitated briefly before absorbance was read at 540 nm using a colorimetric plate reader (BioTek Instruments Inc., Winooski, VT, USA).

4.3.2.4 Phagocytosis activity

4.3.2.4.1 Staining of zymosan particles

Zymosan particles were stained with neutral red according to the method described in Pipe *et al.* (1995b). Firstly, 10 mg of zymosan particles (Zymosan A, Cat. No. Z4250; Sigma-Aldrich, Sydney, Australia) were suspended in 100 ml of milliQ water and then centrifuged at 300 *g* for 5 min. Following centrifugation the supernatant was carefully discarded and the pellet washed with milliQ water and centrifuged again at 300 *g* for 5 min. The supernatant was carefully aspirated and discarded while the pellet was resuspended in Tris/HCl buffer with (w/v) 2% NaCl (0.05M; pH 7.6; Sigma-Aldrich, Sydney, Australia). The mixture was centrifuged again at 300 *g* for 5 minutes, and the supernatant discarded. The pellet was mixed with 1% neutral red solution (Cat. No. N4638; Sigma-Aldrich, Sydney, Australia). The suspension was boiled at 100°C for 1 hour and cooled. The mixture was then centrifuged at 300 *g* for 5 min. The supernatant was discarded, and 1.8% of phosphomolybdic acid (Cat. No. 221856; Sigma-Aldrich, Sydney, Australia) added. The mixture was left cooled and kept at 4°C for 30 min. The suspension was then centrifuged at 300 *g* for 5 min, the supernatant removed, and the pellet washed in milliQ water and centrifuged again at 300 *g* for 5 min. The pellet was resuspended in 6% ammonium molybdate (Cat. No. 277908; Sigma-Aldrich, Sydney, Australia) and left for 1 hour at 4°C. The mixture was centrifuged again at 300 *g* for 5 min, the supernatant was discarded and the pellet was washed with milliQ water. The mixture was centrifuged a final time at 300 *g* for 5 min. Finally, the stained zymosan particle concentration was quantified using a haemocytometer on an upright microscope (Olympus BX-2) and the pellet resuspended with the appropriate amount of Tris/HCl buffer to produce a zymosan concentration of 1 x 10^7 particles ml^{-1}.

4.3.2.4.2 Quantifying zymosan uptake

Quantification of zymosan uptake was done by following the procedure described in Canty (2009). Fifty µl of the dyed zymosan suspension was added to each well containing cells and the plates were incubated for 30 minutes at 10°C. The cells were fixed by adding 100 µl of Baker's Formol Calcium (2 g NaCl, 1 g $C_4H_6CaO_4$ (calcium acetate), 4 ml formaldehyde, made up to 1L with milliQ). The plate was centrifuged at 79 *g* for 5 min. The supernatant was removed and the cells were washed twice with 100 µl of PBS. The dye was resolubilised by adding 100 µl of acidified ethanol (1% acetic acid and 50% ethanol in milliQ water). The absorbance at 540 nm was determined using a colorimetric plate reader, and the amount of particles phagocytosed was calibrated against a standard curve of stained zymosan suspensions (5×10^6 – 0.313×10^6 particles ml^{-1}).

4.3.2.5 Protein determination

Protein determination was carried out using the Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, U.S.A). A standard curve of absorbance vs. protein concentration was constructed by preparing seven concentrations (1, 2.5, 5, 10, 15, 20, 25 µg ml^{-1}) of bovine serum albumin (stock solution 2.0 mg ml^{-1}; Sigma, U.K.) in milliQ water.

Plates were frozen and thawed to facilitate protein solubilisation. Having experienced very low protein concentrations in the wells from the previous trial, the protein was concentrated in this trial before detection. One hundred µl of acetone was added to each well to facilitated protein precipitation. The plates were then centrifuged at 800 rpm for 10 min to separate the supernatant and protein precipitates. The supernatant was carefully removed and 150 µl Coomassie Plus Reagent to each well and mixed for 30 seconds. The plates were incubated for 10 minutes at room temperature and absorbance was measured at 595 nm on a plate reader.

4.4 Discussion

The biggest challenge encountered during the experiments outlined in this chapter was achieving the optimum seeding density (2×10^5 cells/ml) in every well. Cell counts were highly variable between individuals, ranging between 1×10^4 - 2.8×10^5 cells/ml at baseline. Pinsino *et al*. (2007) reported between 2 – 5×10^6 cells/ml in the coelomic fluid of *A. rubens*, and Coteur *et al*. (2004) found between 3 – 9×10^6 cells/ml. As well

as the highly variable cell density, the cell counts were significantly lower than what was reported in *A. rubens* (which were also collected in the Winter/Spring, albeit in the Northern Hemisphere). To verify the method used for counting cells, coelomic fluid of another echinoderm species, the New Zealand endemic sea urchin (*Evechinus chloroticus*), was collected and assessed. The cell count of five individuals ranged from 7.35 – 8.98 x 10^6 cells/ml, which is slightly higher than the average (5.4 x 10^6 cells/ml) reported by Johnstone (2013), who studied the same species. Nonetheless, this verifies that the haemocytometer method used for cell counting was not responsible for the apparent lack of cells that was observed in this study.

Békri and Pelletier (2004) also reported high variability between individuals of the polar six-rayed star (*Leptasterias polaris)*, where cell counts ranged from 8.4 x 10^4 – 7.2 x 10^5 cells/ml, with an average of 2.4 x 10^5 cells/ml. The authors reported that the wet weight of animals was not correlated with the number of cells. In addition, Coteur *et al.* (2003) also reported high variability of cell counts between individuals. In this study, there was a much greater range of cell numbers, with almost at 30-fold difference between the minimum and maximum, which is greater than the 10-fold difference reported by Békri and Pelletier (2004). *In vitro* cytotoxicity testing studies on asteroids are extremely sparse, making it difficult to establish the reasons for the observed variability by using the published literature.

Canty (2009) did not account for cell numbers in each well but instead determined protein concentrations to normalize for the cell number in each well. Nonetheless, in this study, protein concentration was also extremely difficult to quantify, despite using three commercially available protein assay kits. As the optimum seeding density could not be achieved, the cells' ability to survive and proliferate were hindered, resulting in low protein concentrations that were undetectable by commercially available kits. Ronning (2005) was successful in establishing primary cell cultures of *A. rubens* coelomocytes when they seeded 40,000 cells/well (equates to 2.0 x 10^5 cells/ml). Similarly, Matranga *et al.* (2006) were able to establish short-term sea urchin coelomocyte cultures at a density of 2.0 x 10^5 cells/ml. This was not achievable in this study, due to the highly variable cell count between individuals which in general were well below 2 x 10^5 cells/ml. In other words, a larger volume of coelomic fluid would have had to be removed from individuals with very low cell counts, and a lower dilution factor in Leibovitz's medium used in order to seed the cells at the optimum density of 2 x 10^5 cells/ml. This could become an additional cofounding variable when comparing

results between individuals. Matranga *et al.* (2006) removed a larger amount of coelomic fluid from individuals (between 5 – 10 ml), however due to differences in anatomy between these two families of Echinodermata, sea urchins may have more coelomic fluid than sea stars. Consequently, removal of such an amount may not have been significant. Furthermore, Canty (2009) used incubation times for *in vitro* experiments that were much less (approximately 4 hours), while the incubation in the current studies was much more prolonged (several days). The nature of this experiment required a longer incubation time, which may have hindered the longevity of the coelomocytes in cell culture especially when seeded at sub-optimal density.

One of the major advantages listed for *in vitro* cytotoxicity testing is that it allows multiple assays to be carried out simultaneously. Therefore, this experiment was designed to maximize this advantage by carrying out three cell assays. Coelomic fluid was diluted with culture medium in a 1:2 ratio, and 50 µl of the cell suspension was placed in each well in order to yield sufficient volume of cell suspension for two heavy metals at three concentrations for each, and three bioassays (total of 48 replicates per individual). In hindsight, it was extremely challenging to seed cells at the optimum density in this many replicates, as well as minimising the volume of coelomic fluid withdrawn to minimize stress in the animals.

4.4.1 Heavy metal exposure on coelomocyte physiology

Adverse effects of cadmium have been demonstrated in several marine species. Coles *et al.* (1995) reported significantly decreased phagocytosis activity of *M. edulis* that were exposed to 40 µg L^{-1} of cadmium chloride *in vivo* for seven days. Furthermore, Canty (2009) found reduced phagocytosis activity in *A. rubens* exposed to 100 µg L^{-1} and 180 µg L^{-1} of cadmium for 5 days. A small number of *in vitro* studies have investigated the effects on cadmium and zinc in other marine invertebrate species. Brousseau *et al.* (2000) reported that the phagocytosis activity of *Mya arenaria* (clam) haemocytes increased at low concentrations of both cadmium (0.18 µg L^{-1} and 1.83 µg L^{-1}) and zinc (0.14 µg L^{-1} and 1.36 µg L^{-1}). However, on the contrary, haemocytes exhibited a dose dependent increase in phagocytosis activity at higher concentrations of both metals (cadmium: 18.33 µg L^{-1} and 183.32 µg L^{-1}; zinc: 13.63 µg L^{-1} and 136.32 µg L^{-1}). Furthermore, inhibition of 50% of phagocytic activity (IC50) was observed at even higher concentrations (12649 µg L^{-1} and 10360 µg L^{-1} for cadmium and zinc chloride, respectively). The hormesis-like effect of low dose mercury has to be

attributed to the increase in intracellular calcium levels which acts as secondary cellular messenger for the initiation of phagocytosis (Duchemin *et al.*, 2008). Cadmium and zinc may increase phagocytosis activity via a similar mechanism, however no reports of increased intracellular calcium levels during low-level cadmium and zinc exposure has been reported for sea stars. In sea cucumbers (*Holothuria polii*), the haemolytic ability is slightly increased during low-concentration zinc exposure (0.1 – 0.5 mM), possibly by acting as a divalent cation stabilizing agent on haemolysins (Canicatti and Grasso, 1988; Chia and Xing, 1996).

Canty (2009) reported no significant difference in cellular membrane integrity in sea stars that were exposed to cadmium concentrations that were much higher than those in this study (100 – 320 μg L^{-1}). This suggests that at these concentrations the lysosomes and other heavy metal detoxifying mechanisms are not compromised. Interestingly, Bowett (2002) reported lysosomal damage in the starlet cushion star (*Asterina gibbosa*) exposed to 0.5 μg L^{-1} of cadmium for two days (Canty, 2009). Both these studies were *in vivo* studies, which highlights the need for *in vitro* exposure experiments to elucidate the cellular level changes that occur during heavy metal exposure with greater clarity.

4.4.2 Amputation and heavy metal challenge

Most of the literature on environmental toxicology of echinoderms is focused on the adverse effects of pollutants alone. As amputation is a naturally occurring phenomenon, the idea that it may be acting synergistically with other environmental disturbances has largely been unexamined. Research on how environmental disturbances, such as pollution, retards regeneration after amputation has gained some attention, while the reverse of cause and effect (i.e. how amputation decreases tolerance to environmental disturbance) has not been explored.

A marked increase in heat shock protein 70 (HSP70) is a commonly used indicator of stress in echinoderms after injury (Pinisino *et al.*, 2007; Holm, 2008). HSP70 overexpression has been demonstrated to have deleterious effects on growth, development and survival of *Drosophila melanogaster* (Krebs and Feder, 1997). Similarly, overexpression of HSP70 has adverse effects on sea urchins as it decreases the reactivity of immune system cells, as indicated by the inhibition of cell spreading (Browne *et al.*, 2007). Cell spreading involves deformation of the cellular membrane,

with the cell acquiring a flattened shape as it attaches to the substrate. This process is essential as the adhesion of the cell to the extracellular matrix may progresses into either cell proliferation, quiescence or senescence (McGrath, 2007).

Excessive HSP70 expression can have adverse effects such as increasing cell mortality as the cell cycle and apoptosis require strict regulation of HSP70 levels (Garrido *et al.*, 2001). Furthermore, overexpression of HSP70 is also reported to impair activity of alcohol dehydrogenase and lactate dehydrogenase, two highly conserved enzymes involved in cellular metabolism (Krebs and Holbrook, 2001). Therefore, overexpression of HSP70 may act synergistically with excess heavy metal exposure to reduce coelomocyte functionality.

Overexpression of HSP70 is extremely energy costly; however, during stress, energy is allocated to increase HSP70 expression to maintain protein integrity (Hernroth *et al.*, 2011). Therefore, the energy allocation may be prioritized to maintain cellular proteins and in turn compromise heavy metal tolerance. The aim of this experiment was to determine whether the stress of amputation has an effect on the physiology of coelomocytes. If setting up cell cultures had been successful, identifying the correlation between HSP70 and cellular parameters during heavy metal exposure would have been desirable. However due to time and resource constraints, further amendments to this experiment could not be made.

4.5 Conclusion

Although the experiment in this study unfortunately did not succeed, in the future a successful short-term culture may reveal important information for the management of injured echinoderm bycatch and their subsequent survival in heavy metal contaminated areas. Sea star coelomocyte cultures do have promise to be an effective cytotoxicity testing tool or biomarker. However, without an established cell line, maintaining live animals and cell cultures was extremely problematic, especially as the laboratory was not in close proximity to the sea star habitat. Further research into species specific culturing conditions is required, especially regarding the optimum cell density in cultures, as it was difficult to achieve the optimum seeding density suggested for other echinoderm species. Furthermore, it would be of merit to gain knowledge on the maximum volume of coelomic fluid that can be withdrawn with minimal effect on the animal.

4.6 References

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Chapter 5

General discussion

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5.1 Synthesis and future directions for assessing the stress response of amputated sea stars

The aim of this thesis was to determine the physiological changes that occur in response to arm amputation in the eleven-armed sea star (*Coscinasterias muricata*). Together, the results from chapter 2 and 3 supported each other in providing insight into when and how physiological changes occur in response to amputation in this species.

In chapter 2, the coelomocyte count of amputated individuals was greater than that of non-amputated individuals 24 hours post amputation. However, the coelomocyte numbers of amputated animals were not greater than those of the non-amputated controls at any other times before or after amputation. These results suggested that active cell proliferation for wound healing began 24 hours following amputation. Cell recruitment into bodywall tissue by 48 hours after amputation would have meant that circulating coelomocyte numbers in the coelomic cavity would have decreased by this time. Generally, the numbers of coelomocytes were an order of a magnitude (10-times) less in *C. muricata* in comparison to other asteroid species for unknown reasons. Coelomocyte numbers between amputated and control animals 6 hours after amputation did not differ and this was in agreement with previous reports that clot formation does not occur until at least 24 hours has elapsed since amputation (Pinsino *et al*., 2007). In general, the time-dependent changes in coelomocyte count did not align with findings from previous studies (Pinsino *et al*., 2007; Vazzana *et al*., 2015). This could be due to the different sampling techniques employed where the volume and frequency of coelomic fluid sampling may have had an effect on the magnitude of the stress response in the animals.

In chapter 3, the dopamine concentration of the coelomic fluid of amputated animals was higher than in non-amputated animals 24 hours following amputation. Interestingly, a significant increase in total coelomocyte numbers in amputated animals coincided at the same time point (chapter 2). Dopamine is indirectly involved in mitosis (by regulating cAMP synthesis by increasing adenylate cyclase activity), which suggests that dopamine induced cell proliferation at 24 hours post amputation may be the reason behind the increased total coelomocyte numbers at the same time point (Huet and Franquinet, 1981).

Dopamine in coelomic fluid was measured by adapting an existing HPLC method by Zhao *et al.* (2011) with pre-column derivatisation and fluorescent detection. The method in this study was able to detect much lower concentrations of dopamine than Zhao *et al.* (2011) reported. The adapted method did have limitations, particularly regarding the lowest quantifiable concentration; however, this study demonstrated that dopamine can be detected by an HPLC method that has not been previously used with echinoderm coelomic fluid. As the sensitivity of the adapted HPLC assay could not be improved further, I was unable to quantify the absolute concentrations of dopamine present in all the coelomic fluid samples. Instead, the time-dependent changes in dopamine concentration that occur after amputation are reported. Previous publications used HPLC with electrochemical detection but fluorescent detection was used in this study since I did not have access to an electrochemical detector which is able to quantify concentrations lower than 1.56 ng/ml (Tan *et al.*, 2015). It would be beneficial to develop several detection methods that can quantify dopamine to the same sensitivity as the electrochemical detection method. In order to do this, further investigation is required into appropriate extraction methods, as the extraction method used by Tan *et al.* (2015) was incompatible with the detection method in this study.

Understanding the stress physiology of *C. muricata* has important implications for the management of this species. Michael (2007) listed *C. muricata* as one of the five most common motile bycatch species, with a 63% occurrence in dredge oyster (*Ostrea chilensis*) fisheries in New Zealand. Continued harvest of commercial species may incur an unintended increase in mortality rates of *C. muricata*, especially if the sea stars are injured from the fishing equipment. I was unable to collaborate with commercial fisheries to provide a more comprehensive study that would have used both animals collected in the field, and sea stars that were caught as bycatch. However, the findings of this study provide groundwork for further investigations into this subject. In addition, the experience of being caught and injured by trawling equipment may be more traumatic for sea stars than the amputation conducted in this study, therefore the stress response also needs to be assessed with injured bycatch sea stars. Future investigations should also involve the short-term and long-term survival rates of amputated animals to establish the relationship between stress and survival.

The stress physiology of a keystone predator, such as *C. muricata*, has wider consequences for the community structure. To accomplish this, the relationship between physiological stress in response to involuntary amputation, and ecological

changes, such as reproduction and feeding rates, needs to be established. Channon (2010) monitored the feeding ecology of *C. muricata* in their natural environment with small archival electronic tags, and the tags had no adverse effect on survival, feeding or movement of the sea stars. In future studies, it would be worth implementing tagging techniques to monitor the ecology of sea stars injured from trawling equipment, to compliment studies reporting on the stress physiology following involuntary amputation.

5.2 Synthesis and future directions for *in vitro* experiments with sea star coelomocytes

Chapter 4 had two aims, and one of them was to identify the cytotoxic effects of ANZECC (2000) trigger values on coelomocytes of *C. muricata*. Secondly, I hoped to understand how some cellular processes may be affected by amputation, as dopamine is known to divert resources to prioritise homeostatic maintenance, and in turn may potentially compromise immunofunction.

To test this, I set up *in vitro* cytotoxicity experiments with cadmium and zinc at ANZECC (2000) trigger value concentrations, and intended to measure cellular metabolic activity, cellular membrane integrity, and phagocytic activity of coelomocytes collected from both amputated and non-amputated animals. Unfortunately, due to the study species exhibiting unusually low cell counts compared to other asteroid species, the optimum seeding density used for other echinoderm species in cell cultures was not achievable in this experiment. As a result, further investigation was hampered by the low numbers of cells harvested and these cells could not be used in cellular assays. This experiment was unable to yield informative results apart from the data on coelomocyte numbers. In the future, this problem may be overcome by determining the optimum cell density per well before setting up the cell cultures. This can be done by determining a range of cell densities in which the fluorescence yield of a dye (such Alamar Blue) would be linear to cell density. The maximum cell density before fluorescence emitted per cell begins to decline has been used as the seeding density by Ronning (2005), who was successful in establishing primary cell cultures of *A. rubens* coelomocytes, and using these in bioassays. The low cell counts in *C. muricata* were unexpected, and ultimately this investigation could not be carried out due to time and resource restrictions. However, this study has established this basic information about *C. muricata* physiology, and future studies involving *in vitro* cell cultures with *C. muricata* coelomocytes would benefit from establishing the optimum cell seeding

density before cell cultures are set up, as values suggested for other echinoderm cell cultures do not comply with this species.

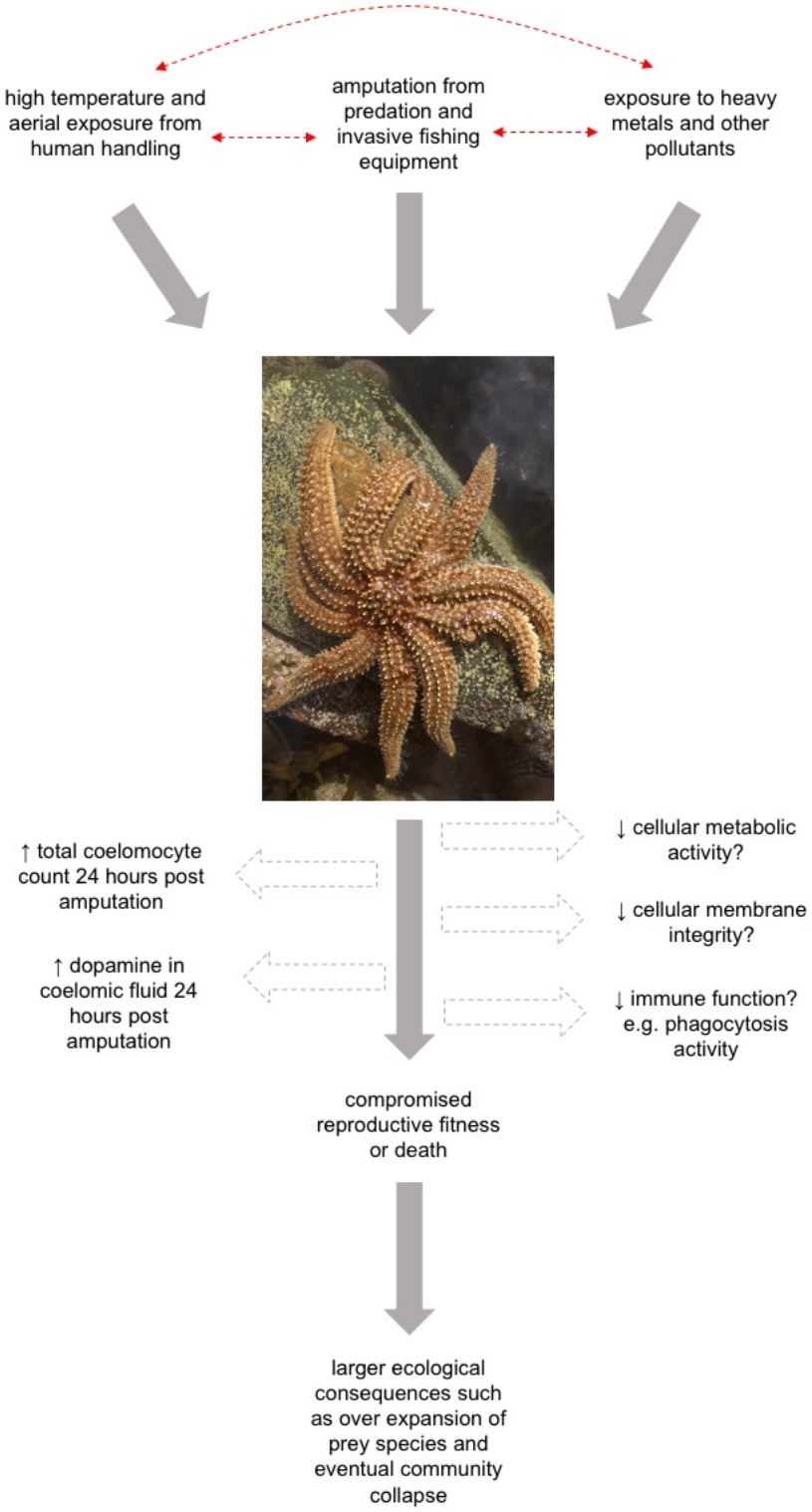

Figure 5.1 Flow chart illustrating the integrated relationship between stressors, physiology and ecology. Red dotted lines represent potential synergetic interaction between the stressors acting on *C. muricata*.

This study is the first to report on the cell counts of *C. muricata*, and I also report the high variability of cell counts between individuals. This observation could be extremely useful for future research as there is increasing interest in using echinoderms as biomarkers of environmental pollution (Temara *et al*., 1997; Reinecke and Reinecke, 2014). As total coelomocyte count is often used as an indicator of stress in echinoderms, it would be of benefit to note this observation if this parameter in this species is to be used as an effective biomarker tool.

I aimed to understand the relationship between the stress response to amputation, how this affects some cellular parameters and, finally, how changes in these cellular parameters influence heavy metal tolerance. Although I was unsuccessful in achieving these aims, Georgiades *et al.* (2006) reported lowered cytochrome P450 in *C. muricata* that were exposed to crude oil contaminated sediment, and Temara *et al.* (1999) reported abnormal foraging behaviour of *C. muricata* subjected to crude oil exposure. Thus, seemingly small changes in cellular parameters may have ecological significance. Similarly, changes in cellular parameters in response to heavy metal challenge may affect the overall physiology, behaviour, and ecology of the sea stars. Amputation stress may act synergistically with heavy metal exposure to further compromise the behaviour and survival of sea stars. Further detailed work is required here, and in order to achieve minimal ecological disturbance, such findings could potentially be relevant to fisheries that carry out invasive fishing activities in heavy metal present waters.

5.3 References

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