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Environmental Sciences

Immunostimulatory effects of different aspects of aquaculture on the host response in the edible sea urchin, *Paracentrotus lividus*

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Submitted for the qualification of *Doctor of Philosophy*

National University of Ireland, Cork

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January 2016

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Declaration

I, Ashley N. Cipriano-Maack, declare that:

Apart from my supervisors' guidance, the content and design of this thesis is all my own work;

Specific aspects of my thesis were supported by colleagues; their contributions are specified in detail in the "Contribution of authors" section at the beginning of the thesis;

At the time of submission, the thesis has been submitted neither partially nor wholly as part of a doctoral degree to another examining body;

Candidate: Ashley N. Cipriano-Maack

Signature:

Date:

Contribution of authors

This thesis consists of seven chapters; six are represented by unpublished manuscripts and one as a published manuscript. A.N. Cipriano-Maack developed original ideas and wrote the manuscripts with major contribution to all manuscripts. Sarah Culloty and Gavin Burnell developed original ideas and supervised research in all manuscripts.

Chapter 1: A.N. Cipriano-Maack, Sarah C. Culloty, and Gavin Burnell. **"General introduction,"** *unpublished review paper.*
A.N. Cipriano-Maack conceived the idea, reviewed the relevant literature, and wrote the manuscript. Sarah Culloty and Gavin Burnell advised on writing, read and improved the manuscript.

Chapter 2: A.N. Cipriano-Maack, Sarah A. Long, Sarah C. Culloty, and Gavin Burnell. **"Evaluation of three tagging methods in the sea urchin, *Paracentrotus lividus*, under both laboratory and field conditions,"** *published in Aquaculture Research and Development.*
A.N. Cipriano-Maack conceived the idea, reviewed the relevant literature, and wrote the manuscript. Sarah A. Long provided urchin care and technical support. Sarah Culloty and Gavin Burnell advised on writing, read and improved the manuscript.

Chapter 3: A.N. Cipriano-Maack, Camila T. Wood, and Sarah C. Culloty. **"Immunostimulatory effect of diet (*Laminaria digitata* and *Mytilus edulis*) in the edible sea urchin, *Paracentrotus lividus*,"** *manuscript submitted to Aquaculture International.*
A.N. Cipriano-Maack conceived the idea, reviewed the relevant literature, and wrote the manuscript. Camila T. Wood provided statistical support using SPSS. Sarah Culloty advised on writing, read and improved the manuscript.

Chapter 4: A.N. Cipriano-Maack, Paul Ryan, Camila, T. Wood, Maria Prado-Alvarez, Catherine Stanton, Gavin Burnell, and Sarah C. Culloty. **"The effects of**

three potential immunostimulants on the host response of the edible sea urchin, *Paracentrotus lividus*," *unpublished review paper.*

A.N. Cipriano-Maack conceived the idea, reviewed the relevant literature, and wrote the manuscript. Paul Ryan and Catherine Stanton provided the strains of PNZ and GTF and accompanying protocols. Camila T. Wood provided statistical support using SPSS. Maria Prado-Alvarez provided technical and data analysis support. Sarah Culloty and Gavin Burnell advised on writing, read and improved the manuscript.

Chapter 5: A.N. Cipriano-Maack, Gavin Burnell, and Sarah C. Culloty. **"The effects of Zymosan A on the host response of the edible sea urchin, *Paracentrotus lividus* during handling and storage,"** *unpublished manuscript.*
A.N. Cipriano-Maack conceived the idea, reviewed the relevant literature, and wrote the manuscript. Sarah Culloty and Gavin Burnell advised on writing, read and improved the manuscript.

Chapter 6: A.N. Cipriano-Maack, Paul Ryan, Camila T. Wood, Gavin Burnell, and Sarah C. Culloty. **"The effects of *Vibrio anguillarum* on the edible sea urchin, *Paracentrotus lividus'* immune function,"** *unpublished manuscript.*
A.N. Cipriano-Maack conceived the idea, reviewed the relevant literature, and wrote the manuscript. Paul Ryan provided assistance in the culturing of the *V. anguillarum.* Camila T. Wood provided statistical support using SPSS. Sarah Culloty and Gavin Burnell advised on writing, read and improved the manuscript.

Chapter 7: A.N. Cipriano-Maack, Maria Prado-Alvarez, Sarah C. Culloty, and Gavin Burnell. **"Seasonal observations of immune parameters in the edible sea urchin, *Paracentrotus lividus*,"** *unpublished manuscript.*
A.N. Cipriano-Maack conceived the idea, reviewed the relevant literature, and wrote the manuscript. Maria Prado-Alvarez helped developed the idea of chapter. Sarah Culloty and Gavin Burnell advised on writing, read and improved the manuscript.

Abstract

Aquaculture is a fast-growing industry contributing to global food security and sustainable aquaculture, which may reduce pressures on capture fisheries. The overall objective of this thesis was to look at the immunostimulatory effects of different aspects of aquaculture on the host response of the edible sea urchin, *Paracentrotus lividus,* which are a prized delicacy (roe) in many Asian and Mediterranean countries. In *Chapter 1,* the importance of understanding the biology, ecology, and physiology of *P. lividus,* as well as the current status in the culture of this organism for mass production and introducing the thesis objectives for following chapters is discussed. As the research commenced, the difficulties of identifying individuals for repeat sampling became clear; therefore, *Chapter 2* was a tagging experiment that indicated PIT tagging was a successful way of identifying individual sea urchins over time with a high tag retention rate. However, it was also found that repeat sampling via syringe to measure host response of an individual caused stress which masked results and thus animals would be sampled and sacrificed going forward. Additionally, from personal observations and discussion with peers, it was suggested to look at the effect that diet has on sea urchin immune function and the parameters I measured which led to *Chapter 3.* In this chapter, both *Laminaria digitata* and *Mytilus edulis* were shown to influence measured immune parameters of differential cell counts, nitric oxide production, and lysozyme activity. Therefore, trials commencing after *Trial 5* in *Chapter 4,* were modified to include starvation in order to remove any effect of diet. Another important aspect of culturing any organism is the study of their immune function and its response to several immunostimulatory agents (*Chapter 4).* Zymosan A was shown to be an effective

immunostimulatory agent in *P. lividus*. Further work on handled/stored animals (*Chapter 5*) showed Zymosan A reduced the measured levels of some immune parameters measured relative to the control, which may reduce the amount of stress in the animals. In *Chapter 6*, animals were infected with *Vibrio anguillarum* and, although *V. anguillarum*, impacted immune parameters of *P. lividus*, it did not cause mortality as predicted. Lastly, throughout this thesis work, it was noted that the immune parameters measured produced different values at different times of the year (*Chapter 7*); therefore, using collated baseline (control) data, results were compiled to observe seasonal effects. It was determined that both seasonality and sourcing sites influenced immune parameter measurements taken at different times throughout the year. In conclusion, this thesis work fits into the framework of development of aquaculture practices that affect immune function of the host and future research focusing on the edible sea urchin, *P. lividus*.

Chapter 1: General introduction

Abstract: Aquaculture is a fast-growing industry contributing to global food security and sustainable aquaculture, which may reduce pressures on capture fisheries. The objective of this thesis was to look at the culture of the edible sea urchin, *Paracentrotus lividus.* Gonads of sea urchins are a prized delicacy in Asian and Mediterranean countries, and in some Western Hemisphere countries. Although a great deal is known about the biology and ecology of sea urchins, the current status of sea urchin fisheries is not well documented and published. What is known is frequent outbreaks of infectious diseases in cultured animals have caused significant economic losses and limit the development of aquaculture. In addition, losses in the industry, due to transport stress and culture techniques, are significant. Furthermore, due to increased focus on aquaculture to support demand for seafood that exceeds fishery stocks, the enhancement of immunocompetence with immunostimulants has become a major focus to mitigate losses. A full understanding of the mechanism of action of these compounds is required to enhance the impact to its maximum potential. The objective of this chapter was to provide background on the current state of echinoculture, with particular reference to *P. lividus*, including biology, ecology, and physiology, and an overview of innate immunology and the terms associated. This chapter concludes with the main objectives of the study and a brief outline of the focus of each chapter.

Research Importance of species in aquaculture and hatcheries

Aquaculture is a fast-growing industry, increasing at a mean rate of 8.6 percent per year world-wide (between 1980 and 2012), which contributes to global food security and sustainable aquaculture (FAO, 2010; 2014). It is estimated that the mean rate will increase to 15% for world fisheries and aquaculture production and 33% for world aquaculture growth (Lott, 2015). This in turn is helping to reduce pressures on capture fisheries (Burnell & Allan, 2009; Bostock *et al.,* 2010) which are under increasing pressures with global food demands.

Aquaculture can contribute to improved food security and nutrition through various channels: increased availability of low-cost fish for locals, employment opportunities and increased wages, and global food demands especially for increased fish consumption (Ftse, 2004). Additionally, an important and often overlooked benefit of aquaculture, is its contribution to increased farm efficiency and sustainability (i.e. agricultural by-products, such as manure and crop residues, can serve as fertilizer and feed inputs for small-scale and commercial aquaculture (Ftse, 2004)). With such a large proportion of the global population reliant on fisheries and aquaculture for food and employment (10-12%; FAO, 2014), investment in mechanisms to enhance productivity in the industry can have a vital impact (Ftse, 2004). Through technological advances, aquaculture has achieved important productivity gains and cost reductions. Over time, this has led to a decrease in seafood prices, especially in the EU and the USA (Ftse, 2004).

As of 2012, 567 species are being cultured within the aquaculture industry (FAO, 2014). One major component of aquaculture, hatcheries, primarily acts to support the aquaculture industry by producing larval and juvenile fish, shellfish, and crustaceans where they are then transferred to on-growing systems (FAO, 2010). Some species

that are commonly raised in hatcheries include Pacific oysters, shrimp, Indian prawns, salmon, tilapia and scallops (Ftse, 2004) and more recently sea urchins. Due to the increase in demand for sea urchin roe in American, Asian, and European markets, sea urchin culture has become a focal point for many researchers and industries. In 2010, marine aquaculture produced an estimated 384,300 tons of echinoderms for consumption world-wide (FAO, 2012); with an estimated 88,000 tons of that being sea urchins alone (Carboni *et al.,* 2012). Challenges with culturing urchins include, but not limited to, disease, variable growth rates, handling and transport stress (including storage), and individual monitoring.

What is harvested?

Gonads of sea urchins are a prized delicacy in Asian and Mediterranean countries, and also in Western Hemisphere countries such as Barbados and Chile (Ding *et al.*, 2007). Sea urchin gonads, or roe, are often harvested when in there immature state. During maturation, gonad ooze is produced during gametogenesis and the tissues become more fragile, giving the gonad a melting appearance upon removal from the skeletal-like test, which considerably reduces its commercial value. In Japan, alum, an aluminum compound is used to suppress the gonad ooze. However, this alters the quality by giving it a bitter taste. Therefore, sea urchin culture has become significantly more valuable through the ability to control favorable conditions for immature gonads (Unuma *et al.*, 2002).

Sea urchin production worldwide

Although we know a great deal about the biology and ecology of sea urchins, the knowledge on the current status of sea urchin fisheries is not well documented. Most

reports are not published and any published material is hard to access. However, it is known that sea urchin fisheries have expanded in the past several decades to the point that the natural populations of sea urchins in the United States, Japan, France, and Chile have become overfished (Lawrence, 2007). In 1998, it was estimated that 117,000 tons of sea urchins were harvested per year, which included 16 different sea urchin species (Keesing & Hall, 1998). With the fisheries in decline, the future outlook is not optimistic; therefore, aquaculture, with the assistance of hatcheries, has become a popular supply method to match increasing demand (Keesing & Hall, 1998).

Current sea urchin production in Ireland

Increasing European demand, especially from the French market (Watson & Stokes, 2004), presents an opportunity for developing the *P. lividus* production industry in Ireland (Hannon, 2015). From the 1970s to the mid-1990s, Ireland's *P. lividus* fishery nearly collapsed due to overfishing with a maximum export of 350 mt per year (Andrew *et al.*, 1995; Hur *et al.*, 2002). Currently, in Ireland, there are two operating hatcheries located in the southwest, who supply to the European market.

The edible sea urchin, *Paracentrotus lividus*

Classification

The edible sea urchin, *Paracentrotus lividus*, discovered by Lamarch in 1816 (Figure 1), is in the phylum Echinodermata, class Echinoidea, and family Parechinidae.

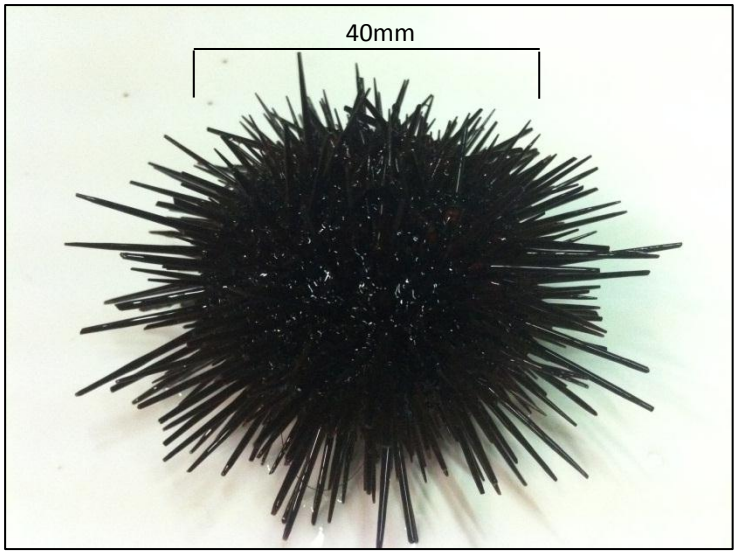


Figure 1: *Paracentrotus lividus*, the focus species of this research (*original photograph by Cipriano-Maack*).

Biology, Ecology, and Physiology

This species is found throughout the Mediterranean Sea and eastern Atlantic Ocean (Figure 2) and is typically restricted to shallow waters where it can reach high densities. This makes it a key species in controlling the dynamics of seagrasses and seaweeds (Sala & Zabala, 1996). *P. lividus* is often thought to be algivorous, however, it has been documented as feeding naturally on sponges, hydrozoa, copepods, dead fish and mussels (Fernandez & Pergent, 1998; Boudouresque & Verlaque, 2007); therefore, defining them as omnivorous scavengers (Lodeiros & Garcí, 2004). Animals grow to a mean diameter of 35–50 mm in about four years, after which growth rates slow down, and it is probable that most of the urchins die of senescence, when normal diploid cells cease to divide (old age), after 6–9 years (Crapp & Willis, 1975).



Figure 2: Distribution range of *P. lividus* throughout the eastern Atlantic Ocean outlined in orange (Map of Eastern Atlantic Ocean, 2015).

(a) Life Cycle and Growth

Most species of sea urchin are indirect developers: the embryo develops first into a swimming larva (Figure 3) that feeds on plankton with a distinct appearance when compared to the adult. The adult structure emerges after metamorphosis, the animal settles onto the substrate of its environment, and grows to sexual maturity (Figure 4). Superficially, the adult displays penta-radial (five-sided) symmetry, while the larva typically has a bilateral organization with left-right and dorsal-ventral (aboral-oral) axes (Warner *et al.*, 2012).

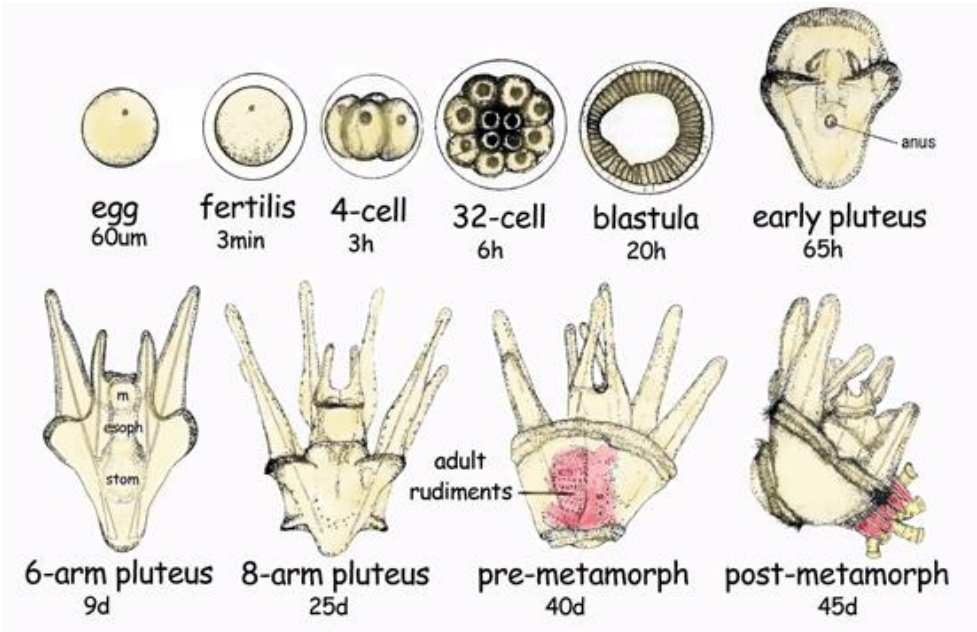


Figure 3: General schematic of planktonic stage development in sea urchins (Planktonic stage development in sea urchins, 2015).

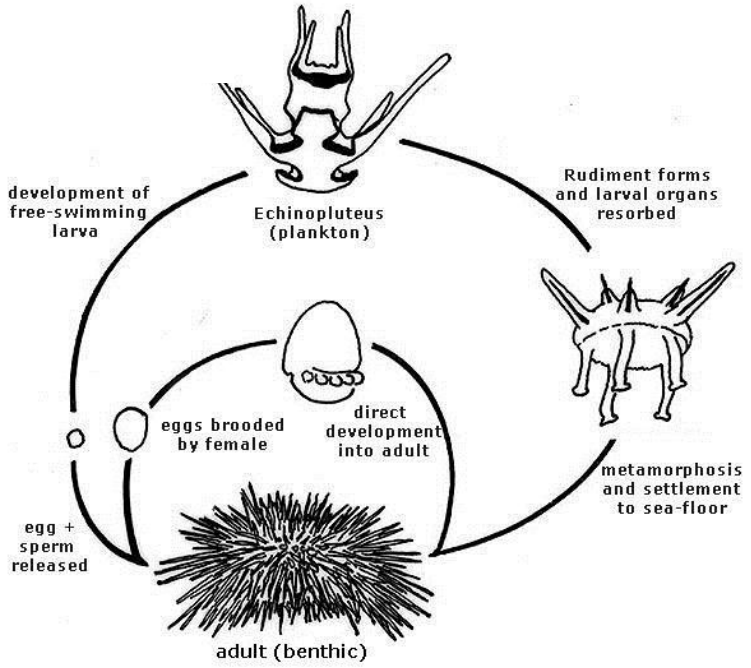


Figure 4: General schematic of sea urchin life cycle (Sea urchin life cycle, 2015).

(b) Adult External Anatomy

P. lividus is a sea urchin with a skeletal-like structure, known as a test, which can reach up to seven centimeters in diameter. The test contains oral and aboral surfaces (Figure 5). The oral surface, or mouth, is made up of five calcium carbonate plates, known as the Aristotle's Lantern (Figure 6), which are responsible for "chewing" the food into bite-sized bits. The Aristotle's Lantern is surrounded by the peristomal membrane, which connects it to the test. The aboral surface, or anus, contains the madreporite, the ocular plate, the genital plate, and the periproct. The periproct is a set of enclosing plates which acts as the anus opening. The test is made up of two sets of plates: the larger interambulacrum (one or more column of plates) and the smaller compact ambulacrum (two or more columns of plates) (Figure 7). The interambulacrum plates have small notches, called tubercles which hold the spines. The ambulacrum plates contain the tube feet, or podia (Agassiz, 1863). On the test, there are rings, not unlike tree rings, that were originally thought to be an accurate determination of age. However, with a wide variation in the season of ring deposition in different individuals, the accuracy of ring count ageing was limited to about ± one year (Crapp & Willis, 1975).

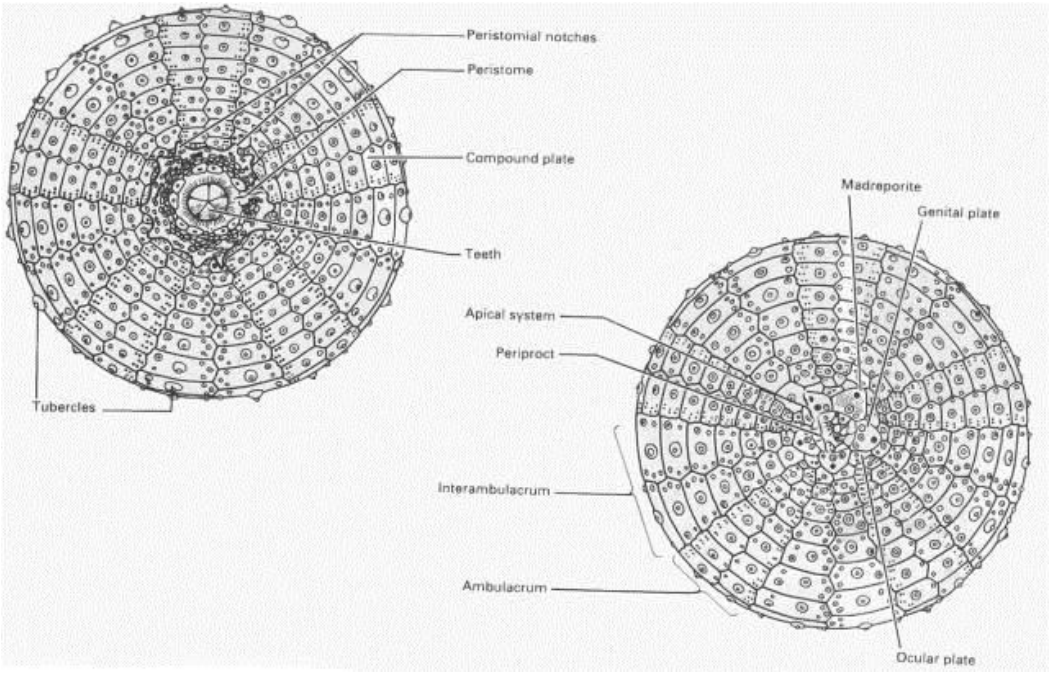


Figure 5: External anatomy schematic of a sea urchin. The oral side pictured on the left and the aboral side pictured on the right (External anatomy schematic of a sea urchin, 2015).

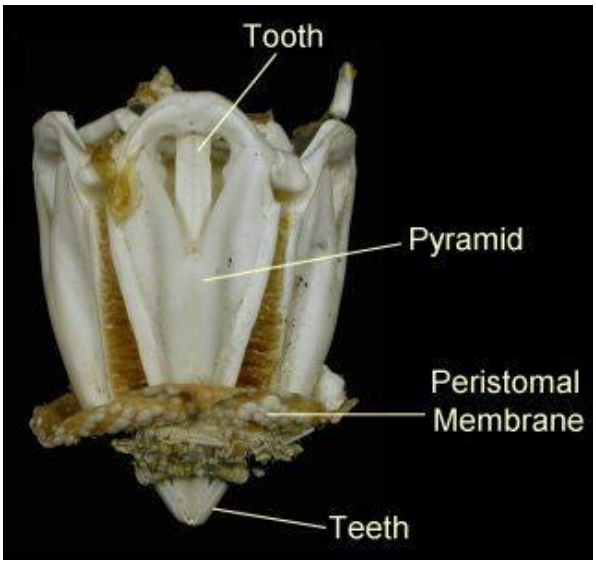


Figure 6: Sea urchin's Aristotle Lantern (Sea urchin's Aristotle Lantern, 2015)

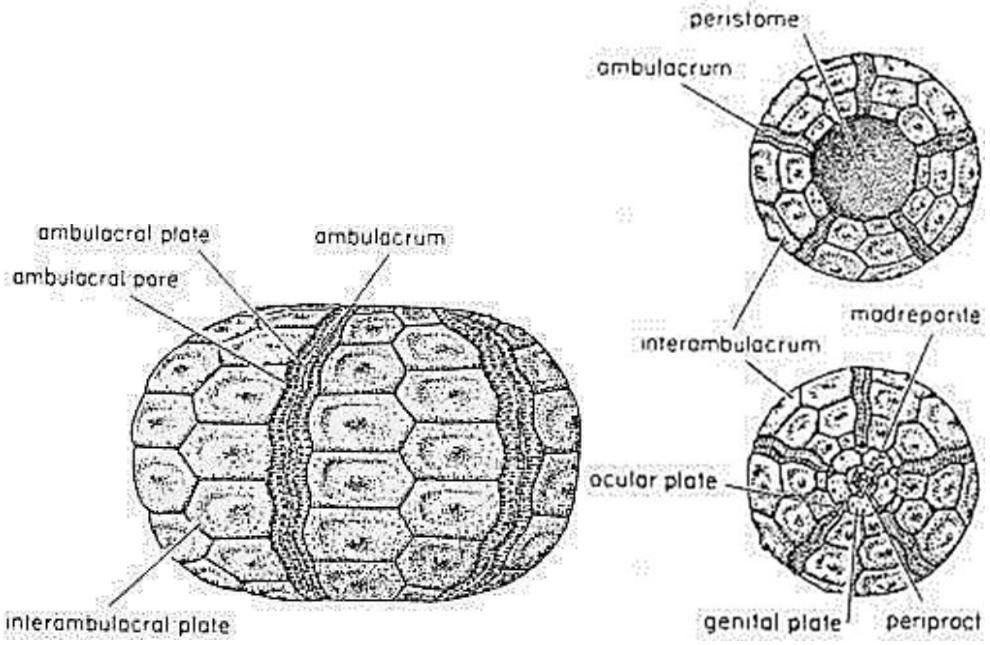


Figure 7: External anatomy schematic of a sea urchin (External anatomy schematic, 2015).

(c) Adult Internal Anatomy

Respiration

Certain sea urchin species possess five pairs of external gills located around the mouth. Fluid is pumped through the gills' interior by muscles associated with the Aristotle's lantern. However, this is not a continuous action, but is conserved for when the animal needs oxygen. Tube feet can also act as respiratory organs through a water vascular system, which is a hydraulic system that aids in locomotion, food and waste transportation, and respiration. The water vascular system is composed of five radial canals that run along the test, arching upwards towards the anus. The ampullae branching off from either side of the radial canals give rise to ten rows of tube feet, which are specifically designed to carry out gas exchange (Barnes, 1982). Many echinoderms, including sea urchins, have low respiratory rates (Lawrence & Lane 1982; Shick 1983). Their oxygen uptake is mostly dependent on the nutritional state,

size, ambient temperature, oxygen tension, seasonality, salinity, and pH (Hiestand 1940; Farmanfarmaian 1966; McPherson 1968; Sabourin & Stickle 1981; Lawrence & Lane 1982; Brockington & Clarke 2001; Talbot & Lawrence 2002; Siikavuopio *et al.*, 2008; Wood *et al.* 2008, 2010, 2011; Christensen *et al.* 2011).

Digestion

The sea urchin has three different fluid systems: the perivisceral fluid, the water vascular system (responsible for locomotion), and the sinuses of the haemal system (responsible for the transportation of elements and dissolved gases). The exact function of these fluids in digestion remains unclear; however, the perivisceral fluid, also known as coelomic fluid, aids in digestion and immune response. In addition, it is responsible for internal transport of dissolved nutrients, waste products, and coelomocytes (Smith, 1981). The perivisceral fluid surrounds the internal organs (Farmanfarmaian & Philips, 1962; Matranga, 2005) and contains coelomocytes.

The digestive tube of the sea urchin consists of a buccal pouch, a short pharynx, and an esophagus that enters the first convolution of the digestion tube (Figure 8). The stomach immediately follows the esophagus. The stomach then leads to the intestine that terminates in a short rectum opening to the anus. The stomach and the intestine each have five festoons (pouches or sacs linked together), also called F1 to F5. Digestion and absorption of food occurs primarily on the first day after consumption. The esophagus and the stomach have been found to be the main sites of digestion. Food remains in bite form and free of bacterial enrichment until the fourth festoon (F4 in the stomach, Figure 9). It is when the food passes through the fourth and fifth festoons of the stomach that the food is converted to bag form via enzyme action. It is postulated, that when urchins are continuously feeding, that most nutrients are

absorbed with the help of enzyme activity (in the stomach) and not bacterial activity (intestine). Only when food is a limiting factor, do nutrients get absorbed in the intestine. The main sites of nutrient storage are in the esophagus and F1 of the stomach (Farmanfarmaian and Philips, 1962). However, if the animal is starved, it will begin to resorb its gonads as an energy storage substitute.

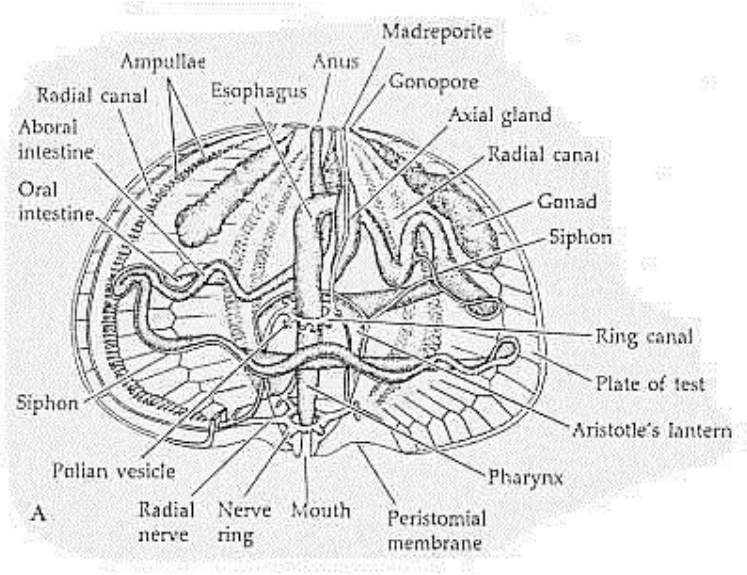


Figure 8: Internal anatomy schematic of a sea urchin (Internal anatomy of sea urchin, 2015).

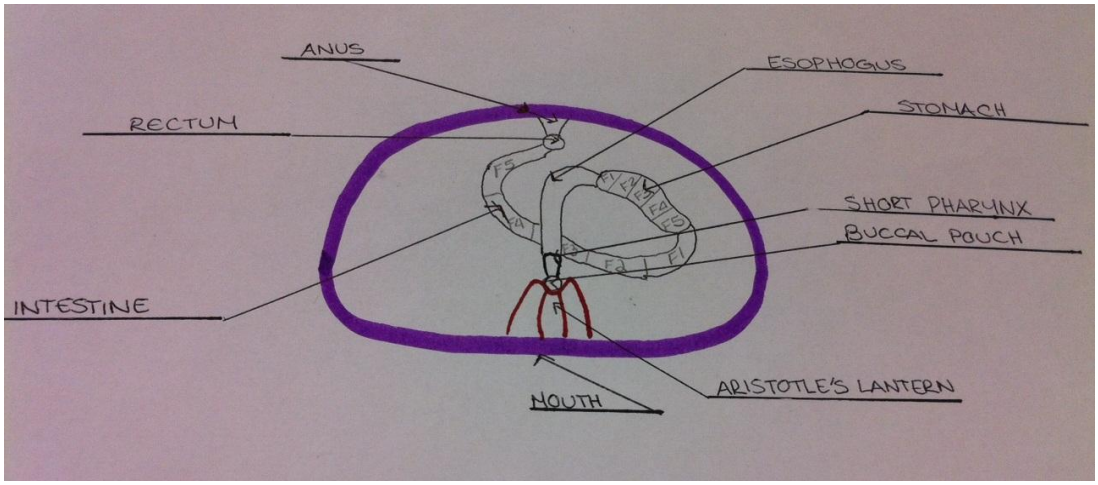


Figure 9: Diagram of sea urchin intestine (*Original Drawing*). This drawing is representative of the work completed by Farmanfarmaian and Philips (1962).

Reproduction

The reproductive cycle of *P. lividus* has an annual cycle consisting of a long period of mature gonads from January to August (Figure 10) and a short period of gonad regeneration and maturation during the rest of the year (Sánchez- España *et al.*, 2004). Spawning is largely dependent on the photoperiod and water temperature (Spirlet *et al.*, 1998; Sánchez- España *et al.*, 2004). Crapp & Willis (1975) found no significant differences between the reproductive cycles of male and female urchins in *P. lividus*; the gonad indices rose to peak values in late winter and late summer, with high gonad condition variation within each population. A single large spawning period from January to August has also been described for populations from Mediterranean Northeast Spain (Lozano *et al.*, 1995), Ireland (Byrne, 1990), and Atlantic Northern France (Spirlet *et al.*, 1998). However, a double spawning period was reported for some Irish populations in spring and summer (Crapp & Willis, 1975; Spirlet *et al.*, 1998) and Mediterranean French populations (Fenaux, 1968; Régis, 1979; Sánchez-España *et al.*, 2004).



Figure 10: Picture showing the roe of a dissected urchin (indicated with a green arrow) (Roe of dissected urchin, 2015).

Sea Urchin Immune System

The sea urchin has an innate immune system with the first line of defense being a physical barrier protecting the organism from the environment. When this barrier is penetrated, the second line of defense can be both cellular and humoral; however, it mainly involves a humoral response e.g. antimicrobial peptide production, where antimicrobial agents, released by phagocytes, bind to pathogens or other foreign agents in invertebrate species (Ganz, 2003). Lysozymes act to lyse bacterial cell walls, which lead to osmotic and mechanical stress in the bacterial cell and often death (Ganz, 2003). Lastly, the third line of defense is cellular defense (e.g. phagocytosis), which uses specialized immune cells, called coelomocytes, to engulf and digest the foreign agents (Matranga, 2005) (4 categories; Figure 11).

Coelomocytes are present as a heterogeneous population with varying quantities of the different cell types dependent on the individual's patho-physiological condition (Matranga *et al.*, 2006). Thus, relative quantities of immune cells can be used as an indicative method of assessing host response to pathogens and/or stressors:

a) **Red and colourless amebocytes or spherule cells (Kindred, 1924):** Fast-moving (0.5 μm s^{-1}) round (or ovoid) cells, ranging from 8–20 μm in diameter. These cells constitute 5–7% of the total cell population. Due to the fast-moving nature of these cells, it is believed that they are the first mobilization response responsible for clotting and encapsulation. In the red cells, the red pigment consists of napthaquinone compounds, known as echinochromes, which are thought to act as anti-bacterial agents. The cytoplasm of colourless amebocytes contains yellow or brown granules and some vesicular bodies (Smith, 1981; Matranga, 2005).

b) **Vibratile Cells:** Fast-moving, small (5–10 µm in diameter) round cells with a flagellum (20–50 µm), constituting 5–6% of the total cell population. The function of this cell type is currently unknown (Matranga, 2005), but it has been suggested that they have the same functional properties as the platelets in vertebrate blood (Bertheussen & Seljelid, 1978).

c-d) **Petaloid and Philopodial phagocytes:** Large cells (14–30 µm in diameter) and variable in shape, phagocytes are the most common immune cell type (50–80% of the total population). Characteristically, the cells exhibit two morphologically distinct phases: the petaloid form and the philopodial form. The petaloid form occurs when the cytoplasm is extruded into a number of petal-like flaps. The philopodial form occurs when the cytoplasm is extended into long fine filiform pseudopodia (Smith, 1981; Matranga, 2005). The transformation between the two forms is rapid, occurring within 5–10 minutes (Matranga, 2005). However, a majority of the cells found are in the petaloid form or in a transition state (Smith, 1981). It is thought that the transition state allows the cells to rapidly transform into the necessary cell for appropriate stimulation (Fontaine & Lambert, 1977; Edds, 1977a, b; Smith, 1981). The petaloid form is responsible primarily for phagocytosis, while the philopodial stage is responsible for clotting (Cuénot, 1891; Andrew, 1962; Endean, 1966; Smith, 1981).

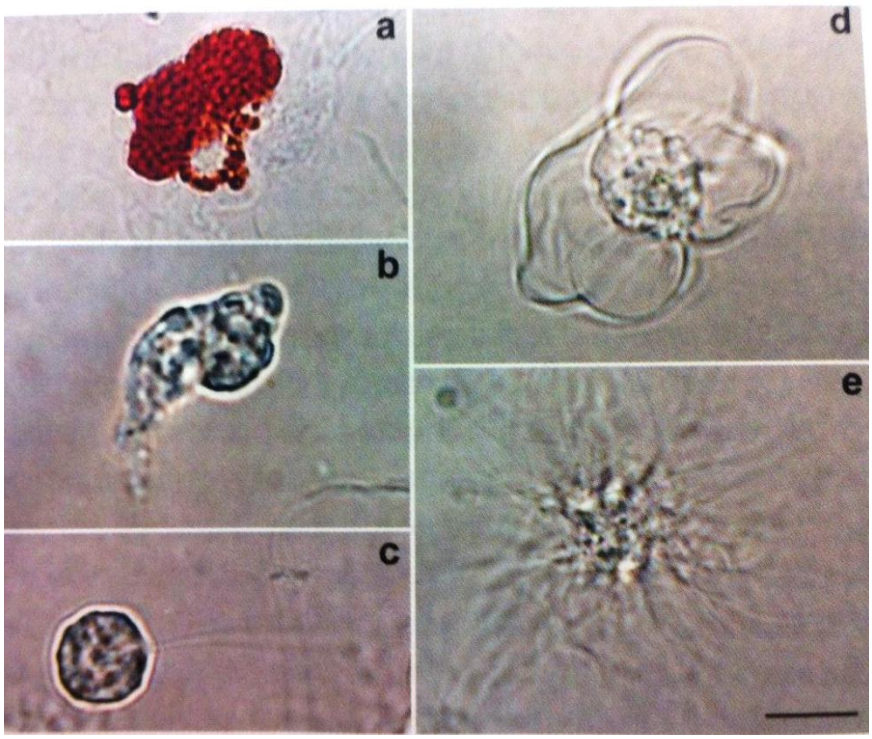


Figure 11: *P. lividus* immune cells: (a) red amebocyte; (b) colourless amebocyte; (c) vibratile cell; (d) petaloid phagocyte; (e) philopodial phagocyte (Matranga, 2005).

Problems faced when culturing sea urchins

Frequent outbreaks of infectious diseases in cultured animals have caused significant economic losses and have limited the development of aquaculture (Fan, 2002; Fan *et al.*, 2009). Requirements in the culturing of any organism are the development of mechanisms to reduce the impact of pathogens and disease, as well as stressors such as handling and transport. Traditionally for vertebrates, management of disease outbreaks by aqua-farmers has consisted of antibiotics; however, it is not possible to use antibiotics in invertebrates due to only having an innate immune system. Therefore, the use of immunostimulatory agents, which activate the innate immune system, have been used to boost cultured animals' immunocompetence, especially in invertebrates, in order to fight off pathogens more efficiently (Fan, 2002; Wang *et al.*, 2004; Gatlin *et al.*, 2006).

Importance of clearly defined terminology in immunology

In reviewing the literature from both an immunological and aquaculture perspective, it became clear that the definitions of an immunostimulant are sometimes used loosely. For example, "Immunostimulants are additives and adjuvants, and include probiotics and prebiotics. Probiotics play a role as common immunostimulants in improving the growth, survival, food conversion ratio (FCR) and immune responses…"(Van Hai & Fotedar, 2009). While some authors make a clear distinction, "in order to evaluate whether immunostimulants may act in synergy with pro- and prebiotics…" (Ringø *et al.,* 2012). Other studies acknowledge the confusion but do not elaborate on it: "Given that some definitions of probiotics include components of microbial cells (Salminen *et al.*, 1999) it is appropriate to discuss products that have been regarded as immunostimulants. Certainly, many studies have utilized whole or components of microbial cells as immunostimulants, specifically to stimulate the immune system against pathogens" (Sakai, 1999). It is the role of researchers to carefully define their results, companies to clearly and accurately label their products, and farming industries to understand the immunology of their animals and the effects of additives, such as immunostimulatory agents.

Defined terminology

a) Immunostimulants

Immunostimulants are non-nutritive compounds that cause up-regulation of host innate defense mechanisms against opportunistic pathogens in the environment (Kiser *et al*., 1956; Anderson, 1992; Galindo-Villegas & Hosokawa, 2004). This mechanism is dependent on the receptors on the target cells recognizing them as potential high-risk molecules thus triggering various defense pathways (Ringø *et al.,*

2012). Immunostimulants include β-glucans, peptidoglycans or lipopolysaccharides (LPS). In nature, β-glucans are widespread and have been characterized in microorganisms, algae, fungi, and plants (Volman *et al.,* 2008; Ringø *et al.*, 2012). Additionally animal derived products like chitin (Sakai *et al.,* 1991, Siwicki *et al.*, 1994), abalone extract (Sakai *et al.,* 1991), bacterial-derived products such as muramyl dipeptide (MDP), alginates (Fujiki & Yano, 1997) or spirulina (Duncan & Klesius, 1996) can also have immunostimulatory effects. The structure of immunostimulants consists of repeating single molecular forms (monomers) such as glucose in β-glucans and (deoxy) ribose in DNA/RNA, fatty acid chains in bacterial lipopolysaccharides (LPS) and certain lipoproteins (Ringø *et al.,* 2012). These patterns are also observed in microbial communities of prokaryotes, and can be termed pathogen-associated molecular patterns (PAMPs) if they initiate inflammatory responses (Ringø *et al.,* 2012). PAMPs are molecules associated with groups of pathogens that are recognized by the innate immune system. For example, bacterial lipopolysaccharide (LPS), an endotoxin found on the bacterial cell membrane of a bacterium, is considered the prototypical PAMP. Commensal microbiota also contains PAMP molecular patterns, which scientists now term, microbe-associated molecular pattern (MAMP) (Ringø *et al.*, 2012). Probiotics are considered MAMPs; therefore, sub-categorizing probiotics as "immunostimulants." For a full review on immunostimulants, please refer to Sakai (1999).

b) Probiotics

The word 'probiotic' is derived from the two Greek words 'προ' (pro) and 'βιοτος' (biotic), which translates as 'for life' (Zivkovic, 1999; Hamilton-Miller, 2003; Lee *et al.,* 2013). In 1908, Elie Metchnikoff reported the potential benefits of

microorganisms, thereby becoming the father of modern day probiotics (Tauber, 2003). However, over time the definition evolved to the World Health Organization's: probiotics are "live microorganisms, which when administered in adequate amounts confer a health benefit on the host" (Reid *et al.,* 2003). Probiotics either enhance the growth of beneficial bacteria or reduce the growth of other, potentially harmful, microbes via acidification and competitive exclusion (Boirivant & Strober, 2007; Lebeer *et al.,* 2008; Callaway *et al.,* 2008; Kim, 2012) within the gut (Nayak, 2010). Working in both the innate and acquired immune systems (Ringø *et al.,* 2012), probiotics appear to modulate immunity of the host by improving the mucosal barrier properties (Lebeer *et al.,* 2010; Bron *et al.,* 2012; Wells *et al.,* 2011; Remus *et al.,* 2011), stimulating antibody secreting cell responses (Kaila *et al.,* 1992; Panigrahi *et al.,* 2004, 2005, 2007; Salinas *et al.,* 2008; Oelschlaeger, 2010), enhancing the phagocytosis of pathogens, and modulating production of cytokines (Nayak, 2010).

Within the innate immune system, probiotics are suggested to directly interact with the different cell lineages that reside in the intestinal tract mucosa. This immunostimulatory interaction is said to involve direct molecular recognition of specific microbial components, known as Microbe Associated Molecular Patterns (MAMPS), by the Pattern Recognition Receptors (PRRs) expressed by the host cells (Lebeer *et al.,* 2010; Bron *et al.,* 2012; Kleerebezem *et al.,* 2010; Lee *et al.,* 2013); hereby, classifying it as an immunostimulant (please refer to section describing immunostimulants). Within the acquired immune system, probiotics are suggested to interact with the intestinal tract predominately contained within the gut-associated lymphoid tissues (GALT) of the mucus membrane of the intestine (Bron *et al.,* 2012; Wells *et al.,* 2011; Green-Johnson, 2012; Lee *et al.,* 2013). For a more thorough

examination of probiotic mechanisms, please refer to Lee *et al.* (2013). Moreover, although probiotics are MAMPs, they do not entirely fit the definition of an immunostimulant as they work both in the innate and acquired immune systems and they are nutritive live micrograms who work within the gut.

c) Prebiotics

Prebiotics are a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of specific health promoting bacteria that can improve the host health (Pharmaceutiques, 1995). In order to fit this definition, prebiotics have to 1) resist gastric acidity, hydrolysis by (mammalian) enzymes and GI absorption, 2) be fermented by intestinal microbiota, and 3) stimulate selectively the growth and/or activity of intestinal bacteria associated with health and well-being (Gibson *et al.*, 2004). Prebiotics mainly consist of oligosaccharides promoting beneficial bacterial growth within the GI tract (Yazawa *et al.*, 1978; Gibson *et al.*, 2003). According to Gibson *et al.*, (2004), only three oligosaccharides can be classified as prebiotics: inulin, transgalacto-oligosaccharides (TOS) and lactulose. However, recent studies by Roberfroid (2007) include fructooligosaccharides (FOS), Mannanoligosaccharides (MOS), a mixture of partially autolyzed brewers' yeast, dairy ingredient components, and dried fermentation products known as GroBiotic®-A, and galactooligosaccharides (GOS). Further information on different types of prebiotics can be found in Merrifield *et al.* (2010) and Ringø *et al.* (2010). Prebiotics are in fact immunostimulants due to the oligosaccharide chains and serve as food to probiotic bacteria.

d) Antibiotics and vaccines

Antibiotics are a type of antimicrobial agent used specifically against bacteria and are often used in medical treatment of bacterial infections (*"Antibiotics,"* NHS, 2015). They may either kill or inhibit the growth of bacteria. Several antibiotic agents are also effective against a number of fungi, protozoans and some are toxic to humans and animals, even when given in therapeutic dose. Antibiotics are not effective against viruses. The indiscriminate use of antibiotics has led to the appearance of antibiotic-resistant strains (Skjermo & Vadstein, 1999), contamination of commercial meat, and residuals left in the environment (Holmström *et al.*, 2003). Therefore, several countries have banned the use of antibiotics, such as chloramphenicol (FAO/WHO, 2002). *Vaccines*, on the other hand, are a biological preparation that improves immunity to a particular disease (*"Vaccines,"* NHS, 2015).

Problems addressed and objectives of this thesis

The overall objective of this thesis was to look at the immunostimulatory effects of different aspects of aquaculture on the host response of the edible sea urchin, *Paracentrotus lividus*, which are a prized delicacy (roe) in many Asian and Mediterranean countries.

Chapter 2: Evaluation of three tagging methods in the sea urchin, *Paracentrotus lividus* under both laboratory and field conditions

The individual identification of sea urchins is difficult due to the presence of spines and the structure of the delicate calcium carbonate skeletal-like test. In this chapter, passive integrated transponder (PIT) tags and two external methods (fingernail polish

and beads) were tested on *P. lividus* individuals to assess tagging capability, survival, and host response. Additionally, PIT-tagged individuals were released in an intertidal rock pool and monitored in order to test field application.

Chapter 3: Immunostimulatory effect of diet (*Laminaria digitata* and *Mytilus edulis*) in the edible sea urchin, *Paracentrotus lividus*

The objective of this study was to determine if using *Laminaria digitata* as a food source for *P. lividus* had an immunomodulatory effect on the sea urchin. This work was carried out in the context of planning for future trials to assess the impact of potential immunostimulants on the sea urchin's immune function. This algal food source was compared to an animal food source (*Mytilus edulis*) and a starved group over 28 days. A range of immune parameters were monitored in this study (nitric oxide, lysozyme activity, cell viability, and differential cell counts).

Chapter 4: The effects of three potential immunostimulants on the host response of the edible sea urchin, *Paracentrotus lividus*

The objective of the current study was to assess three potential immunostimulatory agents and observe whether there was a beneficial effect on the edible sea urchin, *P. lividus'* humoral (nitric oxide, lysozyme activity) and cellular, (cell viability and differential cell counts assays) immune response. The three candidates were (1) *Lactobacillus paracasei 336* PNZ, a gram-positive, non-spore forming microorganism; (2) GTF, a recombinant β-glucan-producing counterpart to PNZ; and (3) Zymosan A, an insoluble yeast cell wall extract prepared from *Saccharomyces cerevisiae*. PNZ and GTF, both in the development stage, were chosen based on

promising results shown in previous studies on mice, while Zymosan was chosen for its promising results shown in previous studies in oysters.

Chapter 5: The effects of Zymosan A on the host response of the edible sea urchin, *Paracentrotus lividus* during handling and storage

The objective of this study was to determine if Zymosan A has a beneficial effect on host response of *P. lividus* individuals which experienced handling and storage stress using cell viability, differential cell counts, nitric oxide, and lysozyme activity as measured immune parameters.

Chapter 6: The effects of *Vibrio anguillarum* on the edible sea urchin, *Paracentrotus lividus'* immune function

The objective of this study was to determine the impact of a pathogenic strain of *V. anguillarum* on *P. lividus* host response using several immune parameters. *V. anguillarum* was chosen based on previous unpublished research from our lab.

Chapter 7: Seasonality observed in the immune parameters in the edible sea urchin, *Paracentrotus lividus*

Seasonal observations of the physiological processes are essential in order to understand the natural variation that occurs within the populations and individual organisms. In this study, baseline (control) immune data taken from previous studies over two and a half years was collated to observe any differences over season or site in the sea urchin, *Paracentrotus lividus.* The compiled data were used to observe the impact of annual patterns on several immune parameters (e.g. cell viability, nitric oxide, and lysozyme activity) within this sea urchin species

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Chapter 2: Evaluation of three tagging methods in the sea urchin, *Paracentrotus lividus*, under both laboratory and field conditions

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Cipriano, A., Long, S., Culloty, S., & Burnell, G. (2014). Evaluation of three tagging methods in marking sea urchin, *Paracentrotus lividus*, populations under both laboratory and field conditions. *Journal of Aquaculture Research and Development*, *5*(6).

**Please refer to the *Published Papers* section (*Pg.* 197-204) for the complete published manuscript.

Abstract: The edible sea urchin, *Paracentrotus lividus,* is an Atlanto-Mediterranean species that is of commercial interest for its roe in European and Pacific/Asian countries. The individual identification of sea urchins is difficult due to the presence of spines and the structure of the delicate calcium carbonate skeletal-like test. However, a successful tagging technique is important for monitoring growth rate and survival of marked individuals in the laboratory and in the field. In addition, tagging can denote ownership, help in broodstock management, and allow for the tracking of animals in the market chain and in laboratory experiments. In this study, passive integrated transponder (PIT) tags, which are smaller than previously reported, and two external methods (fingernail polish and beads glued to the spines) were tested on *P. lividus* individuals to assess tagging capability, survival, and host response (namely through lysozyme activity, nitric oxide levels, and cell viability). Additionally, PIT-tagged individuals were released into an intertidal rock pool and monitored in order to test field application. Of the three different tagging methodologies, PIT tags were found to be the most successful in both studies carried out in the laboratory in regards to survival and tag retention. In the field, PIT-tagged individuals were released and recaptured successfully.

Introduction

Demand for high-quality seafood is increasing, resulting in the rapid growth of the aquaculture industry, which in turn reduces pressure on capture fisheries. Maximizing the potential of the aquaculture industry, though, requires innovation to refine existing techniques and apply new technologies. Individual identification in holding conditions is important to monitor growth, behavior, genetics, and population dynamics (Emery & Wydoski, 1987), and has applications in broodstock management, denotation of ownership, tracking animals in the market chain, and ecological studies (Reisser *et al.,* 2008) by serving as a marker within the population. The mechanism used to identify individuals must be easily distinguishable, be retained for long periods, and have minimal impact on growth and behavior if it is to have practical applications (Freilich, 1989; Purcell, *et al.,* 2008). However, many commonly farmed aquatic organisms, such as shrimp, sea urchins, and other marine invertebrates, have proven difficult to tag despite recent advances in tagging technology (Hagen, 1996; Houghton, *et al.,* 2006; Reisser *et al.,* 2008). Although individual tagging has been used for many years in different species (mainly vertebrates) (Jenkins & Smith, 1990), other studies using PIT tags on aquatic invertebrates and lower fish species include prawns, *Macrobrachium rosenbergii* (Caceci, *et al.*, 1999) freshwater signal crawfish, *Pacifastacus leniusculus* (Bubb *et al.,* 2002), pot-bellied seahorses, *Hippocampus abdominalis* (Woods, 2005), green sea urchins, *Strongylocentrotus droebachiensis* (Hagen, 1996; Duggan & Miller, 2001), the kina sea urchin, *Evechinus chloroticus*, sea cucumbers, *Holothuria whitmaei* and *Actinopyga miliaris* (Purcell *et al.*, 2008), freshwater pearl mussels, *Margaritifera margaritifera* (Wilson *et al.*, 2011), and eastern lampmussels,

Lampsilis radiata radiata (Kurth *et al.*, 2007) with varying success as viable markers.

The edible sea urchin, *Paracentrotus lividus,* is an Atlanto-Mediterranean species that is of commercial interest for its gonads (or roe) in Europe and Pacific/Asian countries (Turon *et al.*, 1995; Boudouresque & Verlaque, 2002). This commercial demand has placed pressure on wild sea urchin populations worldwide and has led to an increased need for aquaculture and hatcheries. In 2010, marine aquaculture produced an estimated 384,300 tons of echinoderms for consumption (FAO, 2012), with an estimated 88,000 tons of that being sea urchins alone (Carboni *et al.*, 2012), necessitating the establishment of more hatcheries. Individual tagging or identification of sea urchins is difficult due to the presence of spines and the structure of the skeletal-like test. Previous studies have focused on external markings to the spines and test (Moore, 1935; Sinclair, 1959; Agatsuma, 2001), drilling holes in the test (Fuji, 1963; Ebert, 1965; Lees, 1968; Olsen & Newton, 1979), fingernail polish plus dental adhesive (Agatsuma, 2001) internal markings, such as tetracycline injections (Ebert, 1977; Gage, 1992; Kenner, 1992; Ebert & Russell, 1993) and passive integrated transponders (PIT) (Prentice *et al.*, 1984; Hagen, 1996; Prentice *et al.*, 1990; Hagen, 1991; Galimberti *et al.*, 2000). However, many of these techniques are often invasive and result in altered behavior and high mortality rates (Gauthier & Le Maho,2001; Godfrey *et al.*, 2003).

The invasiveness of the tags challenges the individual and could lead to a compromised immune system and possible mortality. Factors affecting the immune system include diseases, condition, and diet (Matranga, 2005). Any factor that challenges an individual can elicit a host response. A tag, whether attached to the spine or test, or inserted into the coelomic cavity, may be treated by the sea urchin's

immune system as potential foreign agents. The sea urchin's immune system is defined by its immune effectors, which have the capacity to respond to injuries, host invasion, and cytotoxic agents (Matranga, 2005). Using immune parameters, there are two means of evaluating host response in echinoderms: 1) humoral components such as nitric oxide and lysozyme activity assays and 2) cellular components such as differential cell counts and the cell viability assay. The humoral responses use antimicrobial compounds as a first response to invaders. Nitric oxide, a nitrogen radical produced from L-arginine during phagocytosis, serves to fight off invasive pathogens (Tafalla *et al.,* 2003). Additionally, lysozyme levels demonstrate defense capabilities through antimicrobial properties and the enzymatic breakdown of pathogenic cell membranes (Cronin *et al.,* 2001). Cellular responses directly involve coelomocytes, circulating immune cells, located within the coelomic cavity. Therefore, cell viability and differential cell counts are important immune parameters, which allow insight into the effects of tagging on *P. lividus.*

In this study, two laboratory trials looked at the effect of internal implanted passive integrated transponder (PIT) tags and two external methods (fingernail polish and beads glued to the spines) on *P. lividus* individuals. The first study took place over a four-month period in order to assess individual tagging viability. The second study looked at host response to the different tag types. Additionally, PIT-tagged individuals were released and detected in the field in West Cork, Ireland, using a portable universal microchip reader. The overall aim of the study was to identify a tag that was the most suitable for identifying an individual based on (a) tag retention, (b) host response to tags, and (c) survival of *P. lividus* in the laboratory and in the field.

Materials and Methods

In this study, three trials were conducted on sea urchins. The first trial (Trial 1) assessed the retention of different tags and survival of the urchins post-tagging for 16 weeks (119 days) in the laboratory. The second trial (Trial 2) assessed host response to tagging over a 4-week period in the laboratory. The final trial (Trial 3) assessed the feasibility of utilizing PIT tags in the field.

All *P. lividus* individuals were sourced from Dunmanus Seafoods Ltd. sea urchin hatchery in Dunmanus Bay, Ireland. Both laboratory trials were carried out at ambient temperature (14.0 ± 1.0 °C; maintained with a PSA Aquaclim 10 reversible heat pump/chiller) with continuous water circulation (1000 L sump filled with fresh seawater every 3 days) in four 400 L black plastic circular tanks (Figure 1). Oxygen saturation (DO) and pH were monitored throughout the experiment to ensure water quality. Each tank (both trials) contained 4-5 plastic mesh baskets (3'x 6''x 2'); each basket had ten *P. lividus* individuals and underwent a different tag treatment from the other baskets in the tank (Figure 2). In order to acclimatize the sea urchins, they were held for seven days prior to trial commencement. No sea urchin mortality was recorded during the acclimation period. Animals were fed *ab libitum* with *Laminaria* spp.



Figure 1: Experimental setup of tagged urchins in 400 L tanks (*original photograph by Cipriano-Maack*).

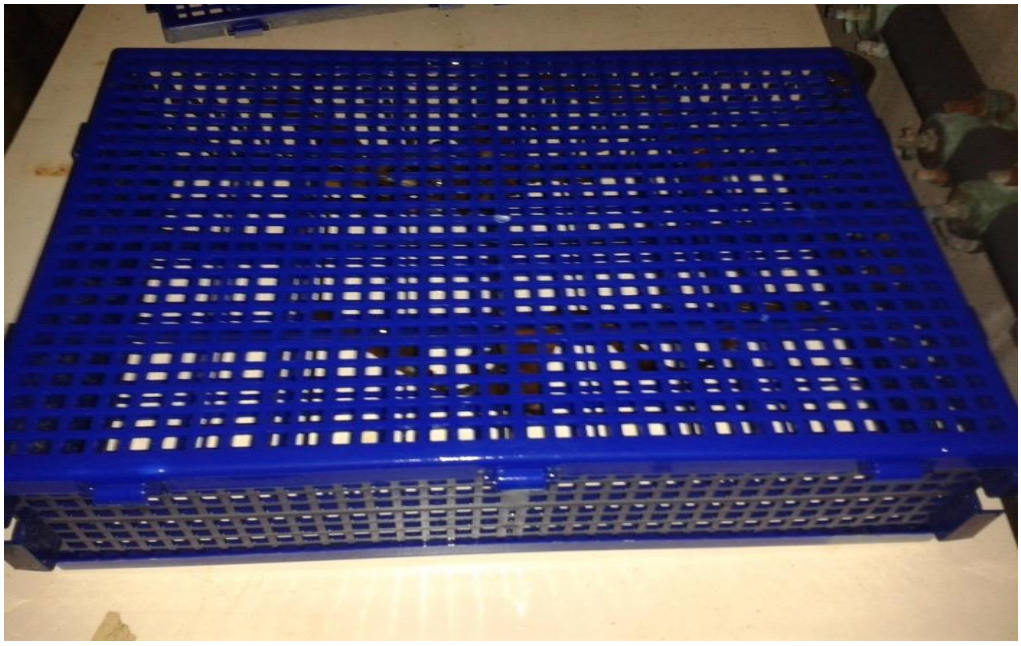


Figure 2: Plastic mesh basket used to separate differently tagged urchins in 400 L tank (*original photograph by Cipriano-Maack*).

Trial 1: Assessment of different tag options in sea urchins

(a) General Set-up

In each of the tanks, one basket held controls, one held 10 sea urchins tagged with fingernail polish (40 ± 5 mm individuals; test diameter ± SD), one basket held 10 urchins tagged with beads (40 ± 5 mm individuals; mature), and two baskets held sea urchins of one of two size classes (20 ± 5 mm (juvenile) or 40 ± 5 mm individuals) tagged with PIT tags. The control group consisted of 10 un-tagged sea urchins (40 ± 5 mm). The baskets were used to make the urchins easily retrievable.

(b) External Tagging

Two external tags were used. The first tag type was fingernail polish (Boot's Natural Collection®) applied to the top of an individual sea urchin's spines (approx. 20 spines) after drying the spines with cotton (Figure 3). The second tag type was 2 mm craft beads glued to five spines per sea urchin with a BISON non-toxic, non-drip super-glue gel (Figure 4).

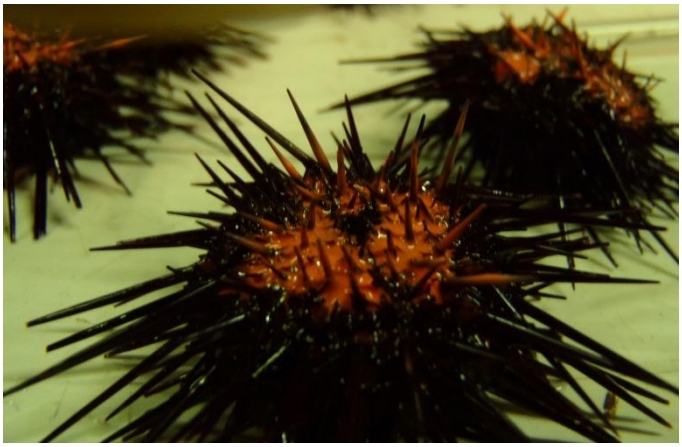


Figure 3: Sea urchins tagged with nail polish (*original photograph by Cipriano-Maack*).



Figure 4: Sea urchins tagged with 2 mm craft beads glued to five spines per sea urchin with a BISON non-toxic, non-drip super-glue gel (*original photograph by Cipriano-Maack*)).

(c) Internal Tagging

1.4 mm × 8 mm PIT tags (Trovan®) (Chips4Fish, Zoo Chip, UK) programmed with a unique 12-digit identification number (Roggers *et al.*, 2002) (Figure 5) were inserted through the peristomal membrane via syringe application (2mm horizontal needle diameter) (Woods & James, 2005) – one per urchin (Figure 6).

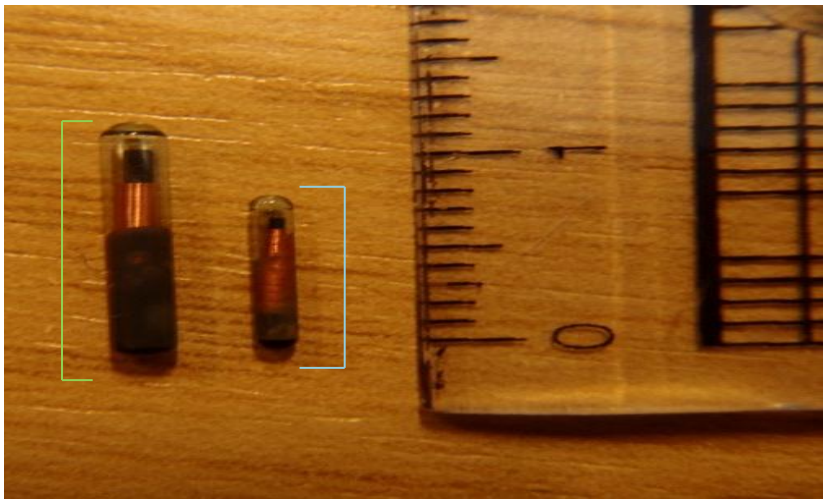


Figure 5: 1.4 mm × 8 mm PIT internal capsule (blue) compared to larger 1.4 mm x 12 mm PIT tag (green) programmed with a unique 12-digit identification number (*original photograph by Cipriano-Maack*).

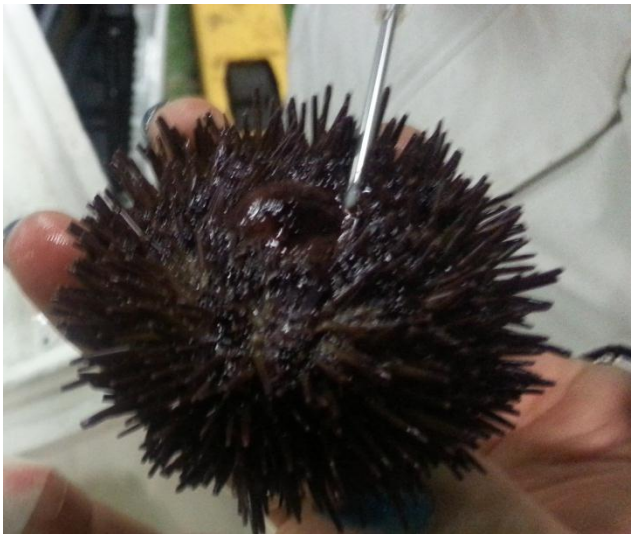


Figure 6: PIT tags were inserted through the peristomal membrane via syringe application (*original photograph by Cipriano-Maack*).

(d) Monitoring

Animals were monitored daily for 16 weeks (119 days). External tags (fingernail polish and beads) on each individual sea urchin were counted daily. Internal tags (PIT Tags) were scanned using a portable universal microchip reader (RealTrace® RT100) (Figure 7). Any dead sea urchins or urchins that had lost their tags (expelled PIT tag or lost all beads/ fingernail polish) were removed from the experimental system.



Figure 7: PIT Tags were scanned using a portable universal microchip reader (RealTrace® RT100) (*original photographs).*

After the 16-week experimental period, the remaining urchins still containing the PIT tag (eight individuals total, four 20 ± 5 mm and four x 40 ± 5 mm) were fixed with Davidson's Solution (Shaw & Battle, 1957). The Davidson's solution was first injected straight into the coelomic cavity via 2 ml syringe. 5 ml was injected into the 40 ± 5 mm individuals, while 3 ml was injected into the 20 ± 5 mm individuals. These animals were then stored in Davidson's solution for two days at 4˚C. Individuals were then opened carefully, and pictures were taken of the tags inside the animal (Figure 9). These tags with attached membrane tissues were then removed and placed into 70% alcohol for later cell identification analysis.

Trial 2: Host response from tagging of sea urchins in the laboratory

The treatments and trial set up was the same for *Trial 1* except that the smaller urchin size class (20 ± 5mm) was used for this trial, due to availability, over a 4-week period (28 days). Ten urchins were sampled and coelomic fluid was pooled per replicate tank at each sampling point; four replicate baskets existed per tag type and control group. Sampling occurred 2, 24, and 48 hours, and 7, 14, 21 and 28 days after tagging.

Host response measurements

At each sampling point, 30 µl of coelomic fluid was taken from each individual within each tag group and placed into a 2 ml eppendorf tube for pooling. Due to the small size of each individual urchin (20 ± 5 mm), samples were pooled from the ten individuals per tag type per replicate tank. Host response was monitored using lysozyme activity, nitric oxide, and cell viability assays. In total, forty individuals were analyzed per treatment.

(a) Cell Viability

100 µl of coelomic fluid, pooled from ten sea urchins per treatment, was incubated in a 96 well-plate for 30 min at room temperature (in triplicate of each sample). The supernatant was removed by overturning the plate. 100 µl of working neutral red solution (1/50 of stock solution: 0.02 g in 5 ml of filtered seawater (FSW), filter and maintain in dark) was added to each well and incubated for 2 hours. The supernatant was discarded by overturning. Samples were washed with FSW and discarded again. 100 µl lysis solution (1% Acetic acid and 50% Ethanol in distilled H_2O) was then added to each well. The 96 well plates were placed in a spectrophotometer reader (540 nm) after being shaken for 1 minute.

(b) Nitric Oxide (NO), The Griess Reaction

The nitric oxide production (Griess Reaction) was carried out as described Green *et al.* (1982). 100 µl of coelomic fluid, pooled from ten sea urchins per treatment, was incubated in a 96 well-plate for 30 min at room temperature (in triplicate of each sample). The same volume of filtered seawater (FSW) was added to the controls and left to incubate for 2 hours.

50 µl of supernatant was removed and transferred to a new plate. 50 µl of each of the Sodium Nitrite standards: 0.1, 0.5, 1, 5, 10, 50, 100 µM, was added to new wells. 100 µl of Solution A (1% sulphanilamide in 2.5% phosphoric acid) then 100 µl of Solution B (0.1% N-naphthyl-ethylenediamine in 2.5% phosphoric acid) was then added to all wells (samples, standards and blanks) and incubated for 5 minutes at room temperature. The 96 well plates were placed in a spectrophotometer reader (Elx808 Ultra Microplate Reader, BIO-TEK instruments, INC.) and read at 540 nm.

(c) Lysozyme Activity Assay

The lysozyme activity assay was carried out as described by Cronin *et al.* (2001), according to Carballal *et al.* (1997), a modification of Shugar (1952). 200 µl of coelomic fluid, pooled from ten sea urchins per treatment was immediately placed in a 2 ml eppendorf and placed on ice to prevent degradation of the samples. The samples were centrifuged at 3,000 rpm for 10 minutes to separate the cells from the serum. The supernatant was removed without disturbing the pellet formed at the bottom of the tube, placed into a clean 2 ml eppendorf and stored at -20 °C until analysis. The corresponding pellet was also frozen at -20 °C.

Lysozyme activity was measured using a 96 well plate reader at a wavelength (λ) of 450 nm which calculated the mean decrease in absorbance at T_0 (before tagging commenced), 1, 2, 3, and 4 minutes. Duplicate lysozyme standard solutions (30 µl) made from hen egg white lysozyme (SIGMA) were serially diluted, were included on each plate and consisted of seven concentrations 5.0 µg ml^{-1}, 2.5 µg ml^{-1}, 1.25 µg ml^{-1}, 0.625 µg ml^{-1}, 0.3125 µg ml^{-1}, 0.156 µg ml^{-1}, and 0.078 µg ml^{-1}. A corresponding number of blank wells, consisting of 200 µl phosphate buffer (0.1 M; pH 7.5), were included on each plate. 30 µl of the supernatant of each sea urchin

sample (in triplicate of each sample) was added to the wells of each plate. 170 µl of *M. lysodeikticus* suspension (pH 6.4) was added to the wells containing the standard solutions and the sample solutions on each plate (in order to measure lysozyme response) to make up to a total volume of 200 µl per well.

Trial 3: Individual tagging of sea urchins in the field

The remaining 44 PIT tagged sea urchins from *Trial 1 & 2* (sizes ranging from 20 ± 5 mm to 40 ± 5 mm) were ranched in shallow rocky tide pools near the Dunmanus Seafoods Ltd. sea urchin hatchery in Dunmanus Bay, Ireland from August to October 2013 for a total of six weeks. The urchins were simply released into the rock pool and monitored fortnightly at spring tides using a portable universal microchip reader (RealTrace® RT100).

Data Analysis

For both laboratory experiments (*Trial 1 & 2*), mortality rates were calculated in percentages. In *Trial 2*, post hoc analyses were conducted given the statistically significant ANOVA ($p<0.05$) for the cell viability and nitric oxide assay results on day 14 (last day where all treatments were still measured). Specifically, Tukey HSD tests were conducted on all possible pairwise contrasts. For the lysozyme activity assay results, individual Kruskal Wallis tests were used to test for significance on day 14 due to the missing data from later sampling points.

Results

Trial 1: Assessment of different tag options in sea urchins

pH and DO were maintained at 8.0 ± 0.05 mg L^{-1} and 8.0 ± 0.4 mg L^{-1}, respectively. The controls with no tags (urchin size: 40 ± 5 mm) had a 25 % cumulative mortality rate over the 16-week (119 days) study period (Figure 8). Within 24 hours, the individuals with fingernail polish on their spines (urchin size: 40 ± 5 mm) started to lift the entire epidermal layer off their tests and drop their spines. By day 29, 100 % mortality was observed in this group and all nail polish had fallen off. Although the bead methodology had 100 % survival, it was only successful in the short-term as sea urchins survived with the beads for only up to 29 days before all the beads fell off or the sea urchins dropped the spines holding the beads. Lastly, two size classes of sea urchins contained the PIT tags; small (20 ± 5 mm) and large (40 ± 5 mm). The large sea urchins showed a 52.5 % tag retention and the small urchins had a 22.5 % tag retention over the 16-week study period. All PIT tags remained operational throughout the experiment.

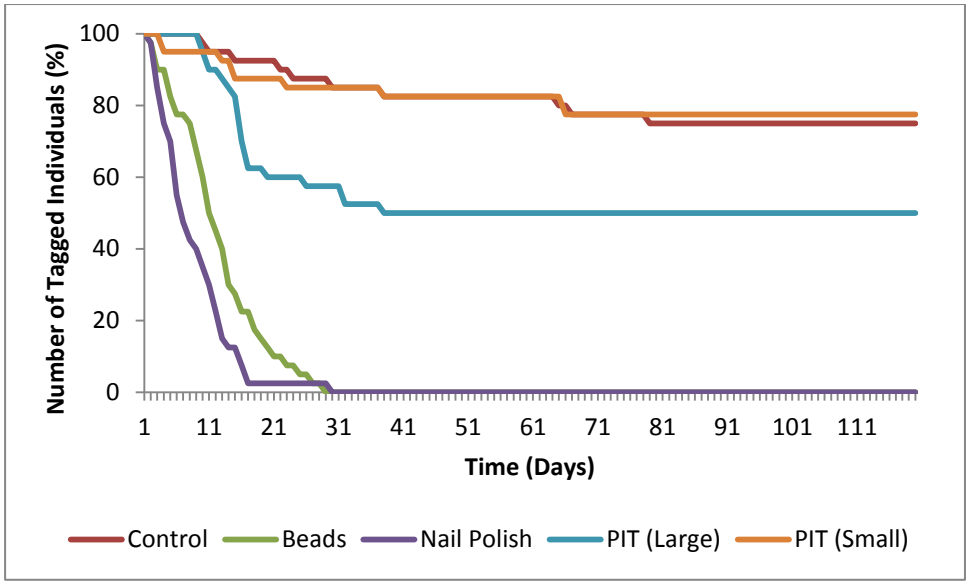


Figure 8: Percentage (%) of individuals with a retained tag over the 119-day trial period.

From the eight dissected PIT tagged individuals (Figure 9), it was observed that the tag had begun to be encased by the tissue lining of the test.

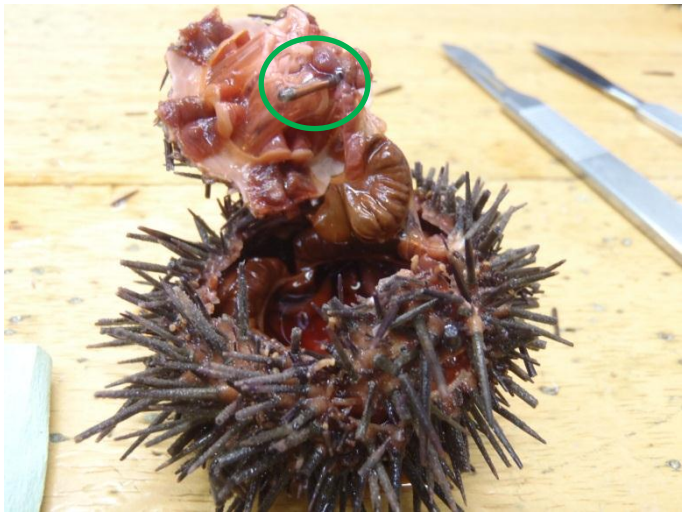


Figure 9: Images of mounted membrane tissue, which surrounded the tag (green circle), from PIT tagged sea urchins (*original photograph)*.

Trial 2: Host response from tagging of sea urchins in the laboratory

pH and DO were maintained at 8.0 ± 0.05 mg L^{-1} and 8.0 ± 0.4 mg L^{-1}, respectively. For *Trial 2*, only the small urchins (i.e. 20 ± 5 mm) individuals were used over a 4-week trial period (Figure 10). The controls showed a 20 % mortality rate. Within the first week, the individuals with fingernail polish started to lift the entire epidermal layer off their tests and dropped their spines with 90 % mortality. By day 21, 100 % mortality was observed. The bead methodology had a 90 % loss of tags by the end of the 28-day experiment. Lastly, urchins that contained the PIT tags had 60 % mortality over the 28-day trial period. The tag types were statistically compared on day 14 as it was the last sampling point when all tag types were still viable.

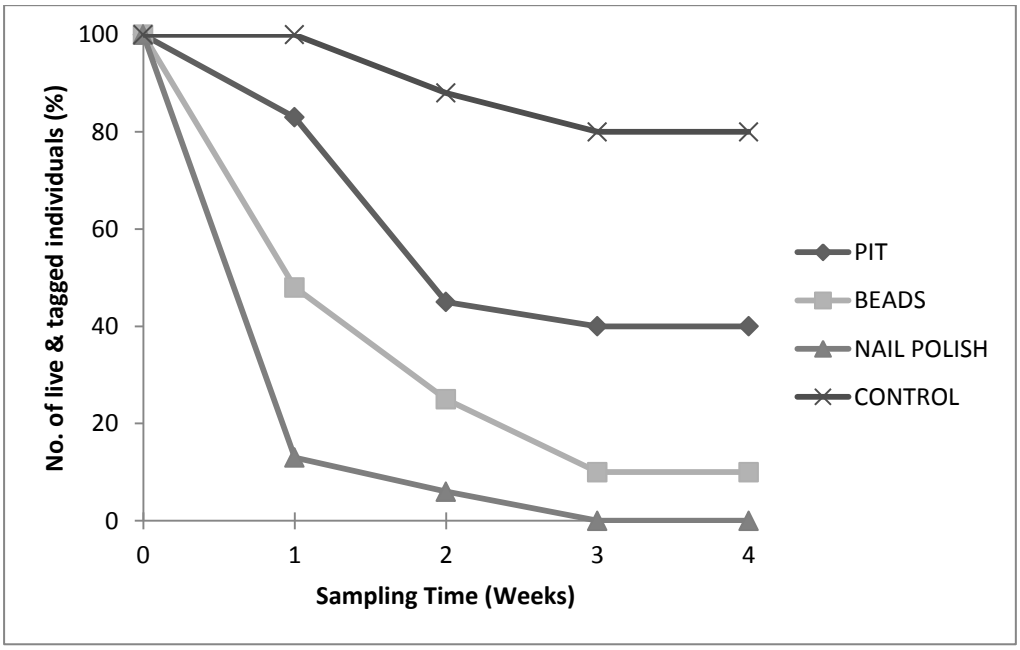


Figure 10: Number of individual sea urchins still alive for each treatment over the one month (28 days) trial period. Measured in percentage (%).

(a) Cell Viability

The cell viability measurements indicated an overall decrease for all treatments and the control (Figure 11). Two hours after tagging, the bead tagged individuals had a cell viability count of 0.58 OD_{540}, but decreased to 0.10 OD_{540} after 24 hours. The PIT tag treatment, nail polish treatment, and the control group all had similar cell viability counts from 0.36 OD_{540} after 2 hours and stabilized at 0.12 OD_{540} throughout the remainder of the trial. The fingernail polish tag type was significantly different ($p<0.05$) from other tag types on day 14. There was not enough sea urchin coelomocyte left over to sample until day 28 due to mortality in the nail polish and bead treatments.

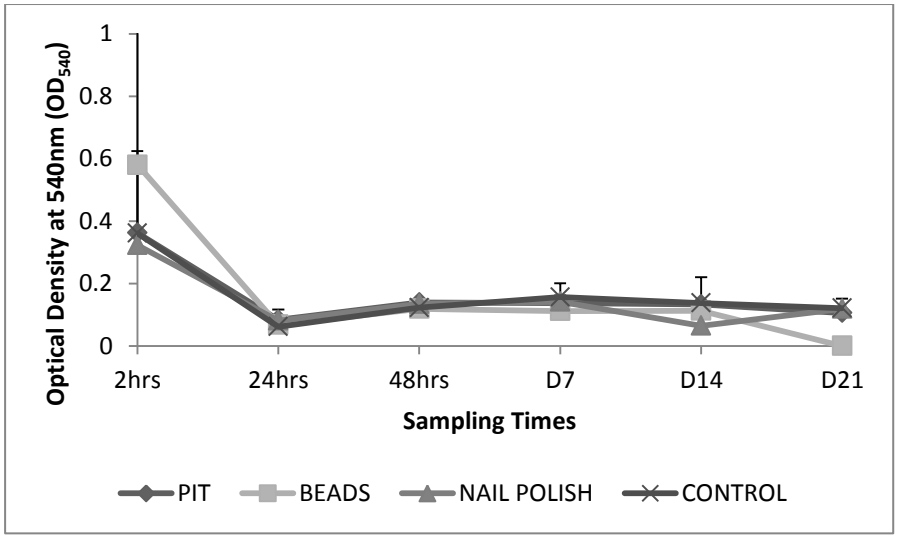


Figure 11: Cell viability, measured in optical density, for pooled *P. lividus* individuals tagged with different tagging options over a 28-day period.

(b) Nitric Oxide (NO), The Griess Reaction

Nitric oxide levels (Figure 12) showed an initial 48 hour decrease after tagging followed by general increases in all tag types and controls until the end of the experiment. The highest nitric oxide measurements occurred in the controls, after 2

hours measured, the NO measurements were 80.04 µM, which decreased to 43.3 µM after 48 hours and then increased to 117.78 µM on day 21. The three tag treatments had lower measurements which were similar to each other, the NO measurements were 62.08 ± 0.46 µM which decreased to 53.9 ± 1.16 µM after 48 hours and then increased to 107.28 ± 0.00 µM on day 21. The bead tag type was significantly different ($p<0.001$) from other tag types on day 14. There was not enough sea urchin coelomocyte left over in the nail polish treatment to sample until day 28 due to mortality.

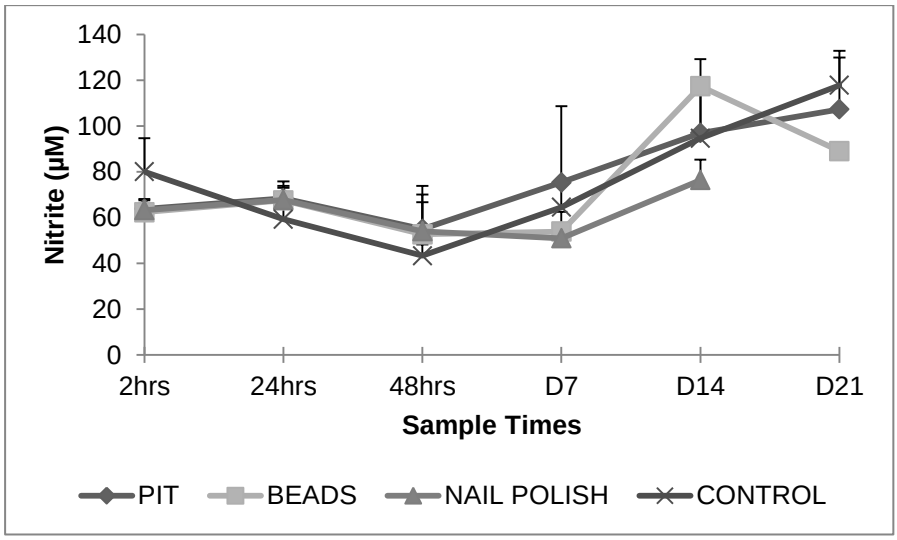


Figure 12: Mean nitric oxide levels (µM) ± SD for pooled *P. lividus* individuals tagged with different tagging options over a 28-day period.

(c) Lysozyme activity

Lysozyme activity (Figure 13) showed a general increase in all tag types and the control until day 7. The controls had the highest overall lysozyme activity level; after 2 hours levels were measured at 1.79 µg ml^{-1}, peaked at 12.42 µg ml^{-1} and measured 10.41 µg ml^{-1} at 21 days. The lowest activity levels were observed in the PIT tagged individuals; after 2 hours levels were measured at 1.37 µg ml^{-1}, peaked at 11.51 µg

ml^{-1} and measured 7.89 µg ml^{-1} after 21 days. The fingernail polish tag type was significantly different ($p<0.05$) from other tag types on day 14.

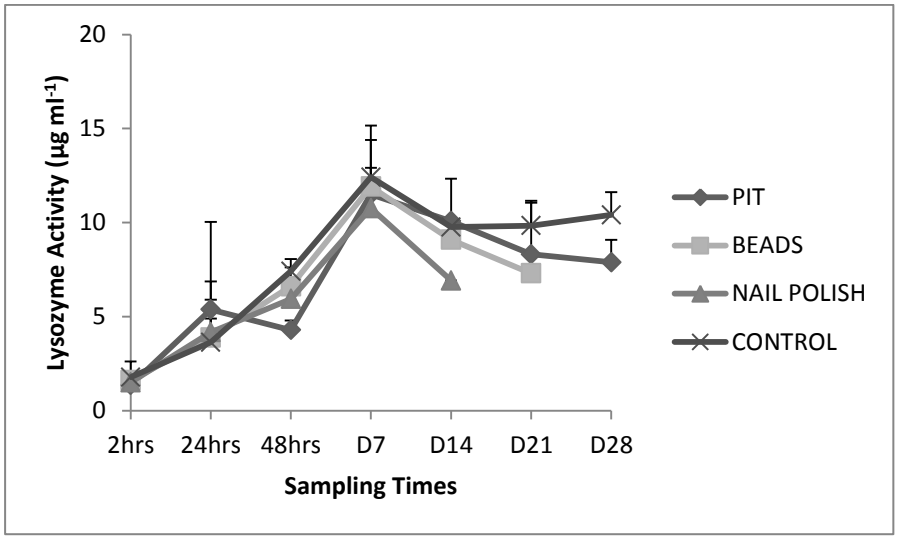


Figure 13: Mean lysozyme activity levels (µg ml^{-1}) ± SD of pooled *P. lividus* individuals tagged with different tagging options over a 28-day period.

Trial 3: Field detection of PIT tags

In the field, individuals (n=44; 20 ± 5 mm & 40 ± 5 mm) were monitored five times over a six week period. Tag identification numbers were recorded in order to observe individual occurrences and tag feasibility. One week after releasing the PIT tagged individuals into the rock pool, one of 44 individuals (1/44) was recaptured and identified. However, three weeks after the animals were released, 6/44 individuals were recaptured. 4/44, 9/44, and 2/44 individuals were caught and recaptured on weeks four, five, and six, respectively. Altogether twelve individuals (27 %) were identified and captured over the 6-week period, three of which were repetitive recaptures.

Discussion

The most successful tag in this study was the PIT tag with potential applications in denoting ownership and tracking individuals. Previous studies using PIT tagged aquatic invertebrates claim that PIT tagging does not adversely affect survival (Wiles & Guan, 1993; Hagen, 1996; Bubb *et al,* 2002; Woods, 2005; Kurth *et al,* 2007). Therefore, the higher mortality from this study could be due to sensitivity of *P. lividus,* condition of the individuals at the time of the experiment or the host response to the tags. Three tag types were used in this study: glued craft beads, nail polish and PIT tags. The glued crafts beads could be viable for short-term experiments that last less than two weeks and do not involve host response measurements. It was observed that the sea urchins dropped the spines with the beads, possibly as a defense mechanism. The fingernail polish resulted in 100% mortality, which could be due to the fact that a) the epithelial layer with the fingernail polish became detached within 24 hours of application layer making the urchin susceptible to infection and b) some urchins were observed to have a swollen peristomal membrane possibly indicating the fingernail polish, when applied, covered the anus preventing waste expulsion and possibly respiration (*personal observation*). The specific PIT tag used in this study was 4 mm, smaller than tags used in previous studies (Dutton *et al*, 1994; Hagen, 1996; Bubb *et al.,* 2002; Parker & Rankin, 2003; Christy, 2006). In our initial tag retention trial, the mortality rate observed was lower in the smaller individuals than in the larger individuals, possibly due to the life history and previous holding conditions of the urchins. Survival in the smaller sized urchins with PIT tags was the same as in the control group.

Additionally, upon the completion of the experiment, eight of the surviving PIT tagged animals were dissected in order to locate the PIT tag. It was observed that the

tag was starting to be encased in the tissue lining of the test (*personal observation*). This observation has not been reported before and warrants further investigation. In a study by Parker and Rankin (2003) on PIT tagging in Black Rockfish, it was suggested that the movement of tags could be the cause of observed mortalities. Christy (2006) observed PIT tags lodged in the outer peritoneum of two frogs (*Limnodynastes peronii).* The idea that PIT tags are either lodged into the individual's tissue or remain "floating" around may provide insight to the mortalities observed in PIT tagged urchins in this study. The PIT tags inserted into the coelomic cavity, where they could move around, could conceivably cause internal damage. Other studies using similar tagging techniques to this study, such as Agatsuma *et al.* (2000) reported successful tagging of *Strongylocentrotus nudus* spines with different colors of fingernail polish covered with a quick drying dental adhesive but only for trials lasting for shorter than three days. No studies to our knowledge have used craft beads glued to the spines.

There have been few studies on the effects of tagging on the host response, especially in invertebrates (Wilson *et al.*, 2011). This is the first study, to the best of our knowledge, to look at host response to tagging in *P. lividus* using immune parameters as indicators of host response. While both trials were carried out to assess tagging viability and mortality, the second trial tested immune parameters of the tagged host. All animals showed host response and higher mortality upon tagging when compared to the previous trial, including the controls, due to handling and sampling of the coelomic fluid via insertion of a syringe into the peristomal membrane. The overall decrease in cell viability within the treatment groups, as well as the controls, may be due to tag effects. With less viable cells, phagocytosis decreases as well; therefore, decreasing capability of an immune response. Upon

introduction of a stressor, the host will liberate oxygen and free radicals, such as nitric oxide, which is a potent antimicrobial agent (Kumar *et al.*, 1995; Pacelli *et al*., 1995; Wheeler *et al.*, 1997; Fang, 1997). NO measurements in this study indicate that the sea urchin coelomocytes produced NO with increased production at 48 hours; however, whether it was due to the tags or to the sampling is unclear and would need further verification. Lysozyme is an enzyme that can hydrolyze components of bacterial walls; therefore, aiding in immune defense and digestion (Cheng, 1986). Lysozyme results from this study indicate an increase in host response until day seven followed by a decrease in lysozyme activity in all treatments excluding the controls. Again, the initiation of the host response, whether it was due to the tags or to the sampling methodology, is unclear and would need further verification. Behavior studies should be developed to determine a less invasive ways of measuring stress and host response because the methodology used in this study challenged all individuals including the controls. Therefore, it is necessary to develop an alternative way of measuring host response (i.e. the activity of the podia and the configuration of the spines).

In the capture, monitor, and release study, surviving tagged individuals from the tag retention experiment were ranched in natural rock pools on the west coast of Ireland. The recaptured urchins had retained their PIT tags for at least five months (laboratory and field) and were easily scanned with a portable universal microchip reader (RealTrace® RT100) (standard in veterinary practices); however, there are two limitations to using PIT tags in the field: 1) sea urchins preferably hide in the crevices between rocks which limits accessibility and 2) the relatively short distance from which tags can be detected (also reported in Bubb *et al.*, 2002). One way to address these limitations is to apply a technique suggested by Bubb *et al.* (2002) and

Roussel *et al.* (2000), which calls for the use of a coil antenna or an 'open coil' antenna mounted on a pole to facilitate searching for tagged individuals in aquatic environments full of rocky crevices more easily.

PIT tagging permits repeated non-destructive sampling of individuals. The claim that PIT tagging is a technique, which has a theoretically indefinite life span, negligible tagging mortality, high tag retention, and no apparent long-term effects on growth and survival of tagged individuals (Bubb *et al.,* 2002), needs further verification in *P. lividus.* The PIT tags used in this study were a useful mechanism for individual sea urchin identification in the laboratory and in the subsequent field study. Additionally, this method showed that after three months in captivity to test for tag retention, animals can be usedto assess potential questions relating to the behavior, mobility, habitat use, broodstock management, and denotes ownership within the laboratory and in the field. Fingernail polish was the least successful tagging technique and caused 100% mortality. The bead technique is a temporary tagging solution but is highly stressful when compared to the control and PIT tagged individuals. Urchins released into rock pools were detected up to six weeks after release indicating that the use of these smaller PIT tags are a viable option in sea urchin culture.

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Chapter 3: Immunostimulatory effect of diet (*Laminaria digitata* and *Mytilus edulis*) in the edible sea urchin, *Paracentrotus lividus*

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Abstract: The edible sea urchin, *Paracentrotus lividus*, is an Atlanto-Mediterranean species that is of commercial interest for its gonads in Europe and Pacific/Asian countries. *Laminaria digitata*, a common food source for sea urchins, contains known compounds, which are beneficial to numerous organisms. The objective of this study was to determine if using *L. digitata* as a food source for *P. lividus* had an immunomodulatory effect on the sea urchin. This work was carried out in the context of planning for future trials to assess the impact of potential immunostimulants on the sea urchin's immune function. This algal food source was compared to an animal food source (*Mytilus edulis*) and a starved group over 28 days. Monitoring of a range of immune parameters in this study (nitric oxide (NO), lysozyme activity, cell viability (CV), and differential cell counts (DCC)) indicated that the main parameter that was impacted on by different diets was NO. *L. digitata* as a food source for *P. lividus* had a more significant effect on NO levels of the experimental animals compared to an alternative food source (*M. edulis*) or the starved group. Viable cell counts (cell viability) were not significantly different between the different treatments over the study period. However, in an assessment of different coelomocyte types and relative levels, amebocyte levels peaked much more rapidly in mussel-fed urchins compared to kelp-fed animals. Amebocytes are the first mobilized defense mechanism responsible for clotting and encapsulation and can contain an antibacterial agent known as echinochrome. In conclusion, this study demonstrates that foodstuffs, particularly *L. digitata*, being provided to sea urchins may impact on immune parameters and so should be considered when similar trials are being planned or data are being analyzed.

Introduction

The edible sea urchin, *Paracentrotus lividus*, is an Atlanto-Mediterranean species that is of commercial interest in European and Pacific/Asian countries (Turon *et al*., 1995; Boudouresque & Verlaque, 2002; Pais *et al*., 2007). This commercial demand has placed pressure on wild sea urchin populations worldwide and has led to an increased need for aquaculture and hatcheries to produce this species. In 2010, marine aquaculture produced an estimated 384,300 tons of echinoderms for consumption (FAO, 2012). *P. lividus* is often thought to be herbivorous, however, it has been observed to feed on artificial diets containing fish meal (Fernandez & Boudouresque, 2000) and naturally on sponges, hydrozoa, copepods, dead fish, and mussels (Boudouresque & Verlaque, 2007). Although many species are omnivorous scavengers (Lodeiros & García, 2004), traditionally aqua-farmers have fed their sea urchins solely on seaweeds found locally. One such seaweed, *Laminaria digitata*, is a brown seaweed located in the lower intertidal and shallow sub-tidal areas of Britain and Ireland (AlgaeBASE, 2014).

L. digitata contains polysaccharides such as laminarin (Smith *et al*., 2011), a seaweed derived (1-3) β-D glucan with a chemical structure consisting mainly of linear β-(1-3) linked glucans with some random β-(1-6)-linked side chains (Brown & Gordon, 2005). β-glucans bind to certain receptors (e.g. in humans and mice; Wu *et al*., 2010) in order to stimulate the innate immune system, resulting in enhanced phagocytosis and oxidative burst, cytokine production, activation of the alternative complement pathway and release of lysozymes (Tsoni & Brown, 2008; Goodridge *et al*., 2009; Soltanian *et al*., 2009). Laminarin exhibits beneficial properties on host health, such as suppressed apoptosis and up-regulated immunomodulatory genes in both vertebrates and invertebrates: e.g. pigs (Smith *et al*., 2011), rats (Devillé *et al*.,

2007), humans (Devillé *et al*., 2007), Mediterranean mussels, *Mytilus galloprovincialis* (Arumugam *et al*., 2000), crustaceans, the signal crayfish, *Pacifastacus leniusculus* (Söderhäll *et al*., 2003) and the fleshy prawn *Fenneropenaeus chinensis* (Yao *et al*., 2005).

The sea urchin has an innate immune system consisting of a series of barriers. The first barrier is a physical barrier (e.g. test and membranes) protecting the organism from the environment. When this barrier is penetrated, the second line of defense is humoral, mainly involving antimicrobial peptide production, where antimicrobial agents bind to pathogens or other foreign agents in invertebrate species (Ganz, 2003). One such antimicrobial enzyme is lysozyme, which is found in abundance in terrestrial invertebrates (Hoffmann *et al.*, 2003) and marine invertebrates. Lysozyme is now a widely accepted bio-defense effector in invertebrate innate immunity (Sotelo-Mundo *et al.*, 2003; Haug *et al.*, 2004; Ji *et al.*, 2009), especially by aquatic animals, against diverse microbial infections (Ding *et al.*, 2011). In echinoderms, Jollès and Jollès (1975) studied the biochemical properties of lysozyme in the sea star *Asterias rubens*. Canicatti and D'Ancona (1990) also confirmed the presence of lysozyme in the mucus of the sea star *Marthasterias glacialis.* In the sea cucumber, *Holothuria polii*, lysozyme-like lytic activity was found in coelomocytes (Canicatti & Roch, 1989). In sea urchins, lysozymes were identified in the coelomic fluid including coelomocytes of the sea urchin, *Strongylocentrotus intermedius*, using sodium dodecyl sulfateermedius, a urchin, coelomocytes (Canicatti & (~ 0.3 OD at 540nm) (Shimizu *et al.*, 1999). However, it was completely absent from the sea urchin, *S. droebachiensis* (Haug *et al.*, 2002). Additionally, in the sea urchin *Paracentrotus lividus*, antimicrobial activity, thought to be lysozyme, was found in

the coelomic fluid (Stabili *et al.,* 1996), jelly coat, seminal plasma (Stabili & Canicatti, 1994) and larval lysate (Stabili *et al.,* 1994).

Lastly, the third line of defense is cellular defense (e.g. phagocytosis), which uses specialized immune cells, called coelomocytes, to engulf and digest the foreign agents (Matranga, 2005). Sea urchins have four categories of coelomocytes all responsible for different aspects of host response: 1) red and colourless amebocytes, or spherule cells, (Kindred, 1924) are responsible for clotting and encapsulation, 2) vibratile cells whose function remains unknown but it has been suggested that they have the same functional properties as the platelets in vertebrate blood (Bertheussen & Seljelid, 1978), 3) petaloid phagocytes responsible for phagocytosis, and 4) philopodial phagocytes which are also involved in clotting (Matranga, 2005). Monitoring changes in concentrations of coelomocytes (differential cell counts) can indicate immune stimulation. For example, Jussila *et al.,* (1997) found that using differential hemocyte counts in the western rock lobsters (*Panulirus cygnus*), was a useful way of assessing stress or health status in lobsters, and possibly other invertebrates. Additionally, cell viability (CV) can be used as a proxy for health as it can be used to measure immune response to cytoxicity and other stressors in humans (Repetto *et al.*, 2008) and in invertebrates (Alvarez & Friedl, 1992).

In marine invertebrates, NO plays a part in several biological roles relating to feeding, defense, environmental stress, learning, metamorphosis, swimming, symbiosis, and haemocyte aggregation in marine invertebrates (Palumbo, 2005). NO is a nitrogen radical gas produced from L-arginine during phagocytosis and serves as a mechanism for fighting off invasive pathogens in the carpet shell clam, *Ruditapes decussatus* (Tafalla *et al*., 2003) and other invertebrates (Martínez, 1995). Reactive oxygen species (ROS), such as NO, have been shown to be involved in the immune

defense activities in molluscan hemocytes (Miller & Ratcliffe, 1994). These parameters are useful to determine the health status of fish (and invertebrates) and to evaluate the immunomodulatory substances in farming (Sahoo *et al*., 2005).

Research in aquatic species is focusing on immunostimulatory agents to suppress pathogen infection and disease development by boosting host response. In this context, it is important to assess traditional farming methods, because current food sources such as *Laminaria* spp. could themselves act as immunostimulatory agents. The objective of this study was to determine if using *L. digitata* as a food source for *P. lividus* had an immunomodulatory effect on the sea urchin prior to any future trials being carried out that assess immunostimulatory function of different agents.

Sea urchins were not fed 14 days prior to the start of the feeding experiment in order to minimize the quantity of residual food in the gut as according to several authors (Lawrence *et al.,* 1989; Siikavuopio *et al.,* 2007; 2008); the gut clearance takes up to 14 days in sea urchins. To assess the potential effect on the immune system, *L. digitata* was compared to a non-algal food source, *M. edulis*. Mussels, and other alternative food items, are often used in farms settings when access to *Laminaria* is limited due to natural availability. These two feeding regimes were compared to animals that were not fed during the trial. The following immune parameters were assessed in response to these varying feeding regimes: nitric oxide (NO), lysozyme activity, cell viability (CV), and differential cell counts (DCC).

Materials and Methods

Sea urchins (n = 180) were sourced from Daithi O'Murchu Marine Research (DOMMR) Station in Bantry, Co. Cork, Ireland in April 2014. Animals were 30 ± 5 mm in size (Test diameter ± SD). In order to remove any residual effects of previous diets, the sea urchins were not fed for two weeks prior to the trial commencing. Laboratory trials were carried out in nine 70 L aerated tanks. Three sets of three replicate tanks contained one of three treatments, each with 20 animals: starved animals (controls), animals fed with *L. digitata*, and animals fed on *M. edulis*. Both diet sources were collected fresh from the wild every three days. Feeding in the respective treatments consisted of no food, 150-200 g of algae (full stock) per tank or 10 mussels per tank (2'' ± 0.25''; opened and presented in the half shell) every three days after 50% water changes (old food was removed). With both diets, food was always in excess between feeding days and never fully consumed. Sampling points occurred at time zero (before food was added) and then on days 1, 3, 7, 14, 21, and 28. To measure the host response to the immunostimulants, cell viability (CV), nitric oxide (NO), lysozyme activity levels (*methods described in Chapter 2: pgs 51-53*) were measured. Histology was attempted; however, due to the complicated nature of sampling and processing the gut tissue, it was not used.

Differential Cell Counts (DCC)

Approximately 5-10 µl of collected coelomic fluid was placed onto a clean glass slide. The slides were incubated for 45 minutes in a moist chamber at 25 °C to promote cell adhesion prior to fixing in 100% methanol at room temperature for 10 min and staining in a Giemsa solution (Sigma) for 20 min. Glass slides were washed

in acetone, mounted with DPX and cover slipped. Differential cell counts, using light microscopy, were carried out using a Nikon YS2-146972 microscope at x4, x10 and x40 magnification. 100 cells were selected at random which then were categorized based on the four different immune cell types and the relative percentages of each were calculated. Slides were photographed using Nikon Eclipse 80i Microscope and NIS Elements Br 2.10 computer software and an Olympus Ax70 Provis Upright Microscope using Viewfinder Lite Software 1.00.

Data Analysis

Linear regression for nitric oxide production (NO) and cell viability (CV) through time for each treatment was conducted using Excel.

For the other analysis, SPSS software (IBM) version 23 was used. CV and NO (dependent variables) were compared between treatments for each sampling time. A mixed linear model was used, with treatment as a fixed effect and tanks within treatments as a nested random factor. This model accounts for data with unequal variances. A Least Significant Difference (LSD) post-hoc test was used for pairwise comparison among treatments when significance was observed. Wald-z tests were used to determine if the tanks within the treatments had a random effect in the model. Due to the sensitivity of the lysozyme assay, some samples were read as blanks by the spectrophotometer. Therefore, the low sample number for this variable made statistical analysis impossible.

For differential cell counts, all cell types were compared between treatments at the initial and final time point. If a difference was observed, difference among treatments of that cell type was tested for all sampling times. Normality plots were verified and

one-way ANOVA was used to assess differences between treatments. Welch correction was used when there were differences in the variances.

Results

Urchin survival during the trial and food availability

No urchin mortalities occurred during the acclimation period or throughout the experiment.

(a) Nitric Oxide Production (NO), Griess Reaction

NO levels in the starved group remained low across the entire 28-day trial (9.67 ± 0.45 µM) (Figure 1). The *L. digitata* fed group demonstrated a large increase in levels on day 1 in comparison with time zero. This difference reduced over 14 days, followed by an increase to 82.85 ± 50.01 µM on day 28. The group fed on *M. edulis* demonstrated a slow increase in NO levels from 8.04 ± 0.41 µM on day 1 to 78.77 ± 11.05 µM on day 28. There was a significant difference between the algal treatment and all others at day 1 ($F_{2,6}$=11.67; p=0.009). On day 3 and 7, this difference was no longer observed ($F_{2,6}$=1.76; p=0.25 and $F_{2,6}$=3.998; p=0.08). At day 14, the significant difference ($F_{2,6}$=23.37, p=0.001) was observed between mussels and other treatments. On day 21, there was no difference between treatments ($F_{2,5}$=5.41, p=0.056) and at day 28, the significant difference ($F_{2,5}$=8.01, p=0.028) was observed between the starved and other treatments (Figure 2). Random effect of the tanks within treatments was not significant for any sampling time (day 1: Wald Z=0.71, p=0.48; day 3: Wald Z= 1.36, p=0.17; day 7: Wald Z=1.47, p=0.14; day 14: Wald Z=0.175, p=0.86; time 21: Wald Z 0.32, p=0.75; time 28: Wald Z=1.48, p=0.14). Overall, there was an increase in NO production through time in *M. edulis* treatment (R^2=0.59). No correlation between time and NO production was observed in the other treatments (Figure 2).

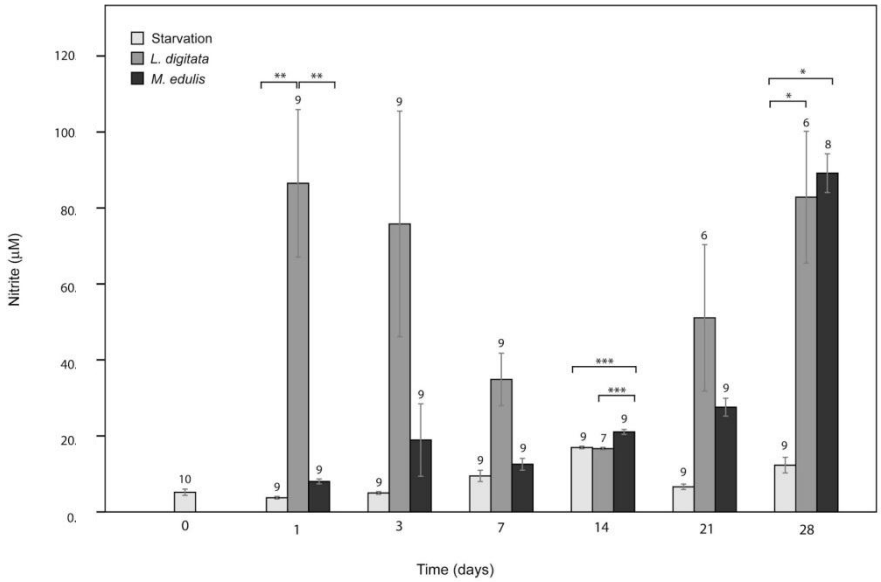


Figure 1: Nitric oxide levels in sea urchins undergoing different treatments over the 28-day trial period. Data are mean nitric oxide levels ± SE (µM). Numbers above error bars indicate sample sizes. Asterisks represent significant difference between treatments at each sampling time (*p<0.05, **p<0.01, ***p<0.001).

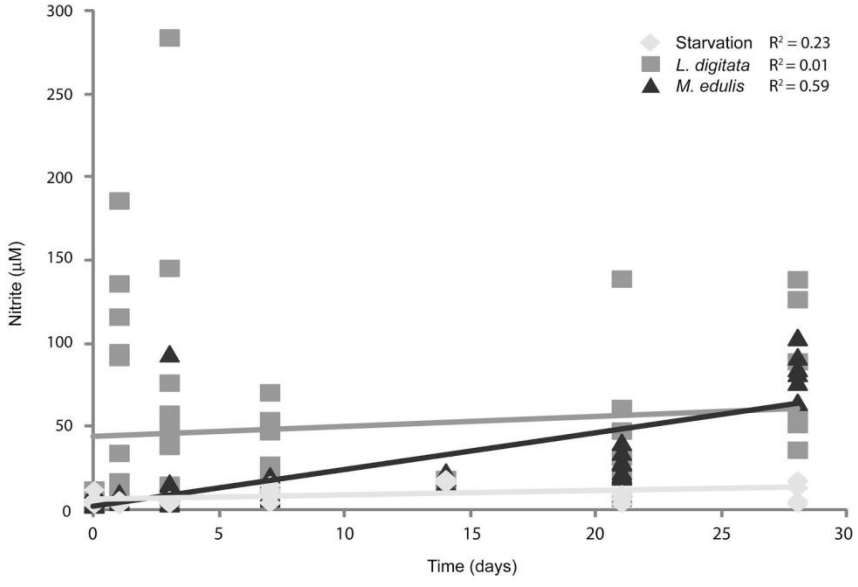


Figure 2: Linear regression of nitric oxide data levels in sea urchins over the 28-day trial period undergoing different treatments.

(b) Lysozyme Activity Assay

Lysozyme activity peaked on day 3 in all three treatment groups (Figure 3). Regarding the treatments, the highest peak was observed in the *M. edulis* fed individuals (8.67 ± 0.70 µg mL^{-1}), followed by the *L. digitata* individuals (7.79 ± 0.90 µg mL^{-1}) and the starved group (7.18 ± 0.69 µg mL^{-1}). Following there was a sharp decrease: starved group (0.78 ± 0.18 µg mL^{-1}), *L. digitata* fed individuals (0.75 ± 0.25 µg mL^{-1}); *M. edulis* fed individuals (0.37 ± 0.10 µg mL^{-1}). Sample size was reduced in late screening; therefore, statistical analysis was not possible.

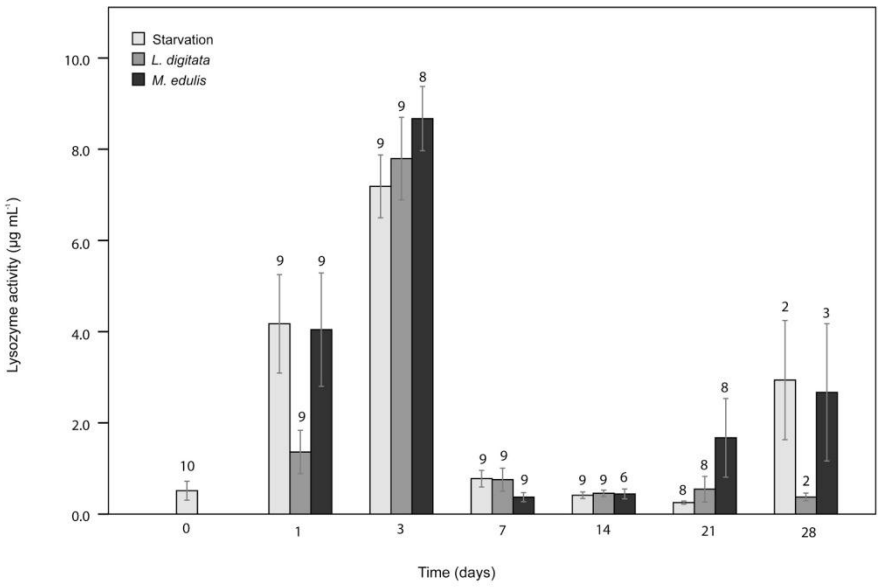


Figure 3: Lysozyme levels in sea urchins undergoing different treatments over the 28-day trial period. Data are mean lysozyme activity ± SE (µg mL^{-1}). Numbers above error bars indicate sample sizes.

(c) Cell Viability (CV)

There was no relationship between cell viability and time for any of the treatments (starved: R^2=0.03; *L. digitata*: R^2=0.01; *M. edulis*: R^2=0.01). Values varied from 0.28 ± 0.00 to 0.38 ± 0.04 OD_{540} for the starved group, 0.25 ± 0.01 to 0.40 ± 0.03 OD_{540} for the *L. digitata* fed group, and 0.16 ± 0.03 to 0.36 ± 0.06 OD_{540} for the *M. edulis* fed group.

After 1, 3 and 7 days, there was no significant difference between the treatment groups ($F_{2,24}$=1.225, p=0.31; $F_{2,6}$=1.10, p=0.39; $F_{2,6}$=0.07, p=0.93, respectively). The effect of tanks within treatments were not significant after 3 or 7 days (Wald Z=1.36, p=0.17; Wald Z=0.07, p=0.93) and it could not be calculated for day 1. However, after 14 days, there was a significantly lower CV level in the *M. edulis* fed animals in comparison with the other treatment groups. At this sampling time, there was an influence of the tanks within treatments (Wald Z=3.32, p=0.001). Days 21 and 28 had no significant difference between the treatment groups ($F_{2,5}$=0.18, p=0.843; $F_{2,5}$=0.43, p=0.68) or tanks within treatments (Wald Z=0.80, p=0.42; Wald Z=0.09, p=0.93, respectively) (Figure 4).

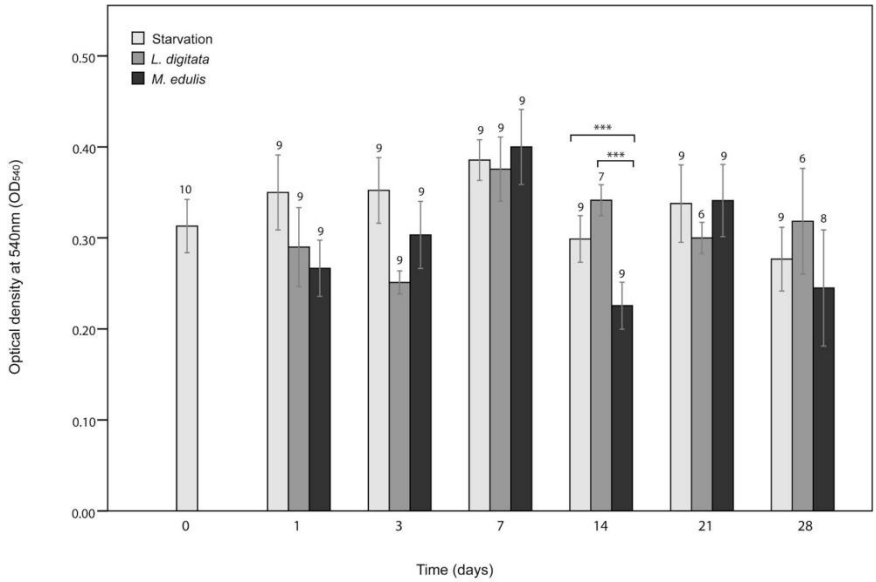


Figure 4: Cell viability levels in the sea urchins undergoing different treatments over the 28-day trial period. Data are mean cell viability levels ± SE (OD_{540}). Numbers above error bars indicate sample sizes. Asterisks represent significant difference between treatments at each sampling time (*p<0.05, **p<0.01, ***p<0.001).

(d) Differential Cell Counts (DCC)

Four different immune cell types (red/colorless amebocytes, vibratile cells, petaloid phagocytes, and philopodial phagocytes) were observed over the study period (*Please refer to Ch. 1 for a description of each cell type – pgs 22-24*). Analysis of variance of the counts of each cell type among treatments was performed at the first and last sampling time and amebocytes were the only cell type to differ between the diets. Therefore amebocytes were tested for all treatments and at each sampling time point.

In the starved treatment, levels remained low apart from a peak on day 14. As this was only based on a sample of three animals due to sample analysis, it may not be representative of the larger field population. In the mussel fed treatment, there was

an increase in amebocytes until day 14. In the *L. digitata* fed treatment, the peaks and troughs in levels were much more variable over the study period.

Individuals fed on *L. digitata* had varying levels of amebocytes over the 28 days, while amebocytes of individuals fed on *M. edulis* and the starved group peaked after day 14 (8.0 % and 14.7 %, respectively). The highest value of amebocytes observed for individuals fed on *L. digitata* was on day 21 (12.5 %). The *M. edulis* fed group was significantly different from the *L. digitata* fed group and the starved group ($F_{2,24}$=6.21; p=0.027) for day 1. Day 3 was not analyzed due to too few data points. Days 7 and 14 were not significantly different between the two diets and the starved group ($F_{2,20}$=0.79; p=0.47 and $F_{2,13}$=2.22; p=0.39, respectively). The *L. digitata* fed group was significantly different from the *M. edulis* fed group and the starved group ($F_{2,14}$=9.95; p=0.002) for day 21. Day 28 was not significant between the two diets and the starved group ($F_{2,16}$=0.49; p=0.62) (Figure 5).

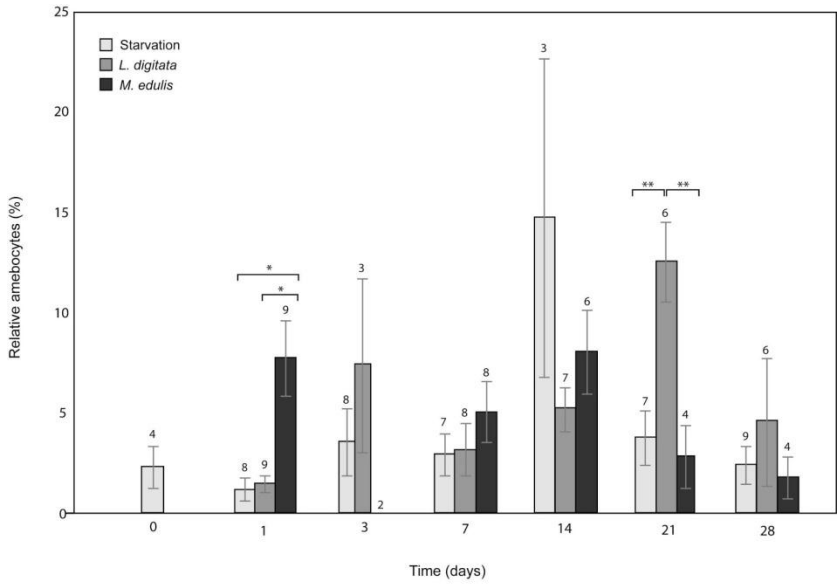


Figure 5: Mean relative percentage (%) of red/colourless amebocytes in the coelomic fluid of individuals fed on *M. edulis*, *L. digitata*, and a starved group over a 28-day period. Data are mean ± SE. Numbers above error bars indicate sample sizes. Asterisks represent significant difference between treatments at each sampling time (*p<0.05, **p<0.01, ***p<0.001).

Discussion

In this study, the potential effect of sea urchin diet on the animal's immune response was investigated. The study demonstrated that a starved group consisting of animals not fed over a period of six weeks (two weeks acclimation and a 4-week trial period) could be used to determine baseline levels in the experimental animals as this treatment had little impact on the stability of immune parameters measured. To our knowledge, this is the first study to look at effect of different feeding strategies on immune parameters in sea urchins. Furthermore, the algae and mussel diets affected the sea urchins in different ways over time and this could be observed by using the different immunoassays.

Diet had a more pronounced effect regarding NO production when compared to other measured parameters, as there was an observed difference between treatments in three of the sampling times. *L. digitata* fed individuals presented the highest values of NO in most sampling times. This shows the potential immunomodulatory gain that the urchins experience when fed on *L. digitata*. Previous literature observed that the dietary administration of β-glucans, another component of algae, to Asian catfish and zebrafish, served to enhance the macrophage oxidative burst activity (Kumari & Sahoo, 2006; Rodríguez *et al*., 2009).

The immunostimulatory effect of *Laminaria* diet in sea urchins can be observed in short term trials. NO levels presented a rapid increase within 24 hours of the study commencing. Furthermore, amebocyte levels were highly variable in the *L. digitata* fed animals over the experimental period, which possibly enhanced immunity over a longer period of time. This study used cell viability as a proxy for indicating how the immune system is responding and determined that urchin cell health was not adversely affected by diet in the short-term. Furthermore, the observed differences in

differential cell counts were in the amebocytes. Amebocytes are primarily responsible for the first mobilization response involving encapsulation and clotting (Matranga, 2005). A study by Delaporte *et al*. (2003) found that algal diets appear to have positive effect upon total haemocyte count, granulocyte percentage, phagocytic rate and oxidative activity of clam haemocytes. Further studies have shown that quality of the algal diet can affect the growth and development of molluscs such as the great scallop *Pecten maximus* (Delaunay *et al*., 1993; Soudant *et al*., 1996, 1998), *C. gigas* (Knauer & Southgate, 1997; Soudant *et al*., 1999, 2000), *O. edulis* (Berntsson *et al*., 1997), *R. decussatus* (Albentosa *et al*., 1997; Fernández-Reiriz *et al*., 1998, 1999) and *R. philippinarum* (Caers *et al*., 1999). While animals fed the *L. digitata* diet demonstrated the slowest response, urchins fed a mussel diet demonstrated a rapid increase in amebocyte levels until day 14. This immediate impact can be related to the fact that mussels are not a common source for sea urchins. Though mussels have a higher calorific value than *Laminaria* (5.47 ± 0.13 cal mg^{-1} versus 4.0 ± 0.09 cal mg^{-1} in other *Laminaria* spp.; *L. digitata* unknown (Paine & Vadas, 1969; Wacasey & Atkinson, 1987; Barkati & Ahmed, 1990)), their tissues are not easily accessible. This requires that the mussel shells are broken before being offered to animals in laboratory or a farm setting. In natural situations, the probability of sea urchins feeding on fresh mussels is low since the effort involved in accessing their soft tissue is high. Therefore, mussels are more likely to be scavenged and not a regular food item. This low occurrence in the diet makes it more likely for mussels to elicit an immune response in animals such as differentiated proportions of amebocytes as the mussel may contain unknown, rarely encountered, foreign agents.

Regarding lysozyme activity, there was a relatively stable trend in levels up until day 21 in the trial. As lysozyme, an antimicrobial agent, is a bio-defense effector in invertebrate innate immunity, this indicates that these foodstuffs did not elicit a response from this effector. Noga *et al.* (1996) reported haemolymph bactericidal activity in the blue crab, *Callinectes sapidus*, was highly inhibitory to gram negative bacteria. After 21 days, our results indicated a sharp increase observed in both diets. A study done by El-Boshy *et al.* (2010), also found that serum lysozyme in Nile tilapia, *Oreochromis niloticus*, treated with *S. cerevisiae* and laminarin significantly increased after the 21 days. Since the chances of having an infection during a trial increases in longer experiments, future studies looking at lysozyme activity should ensure that the study period lasts longer than three weeks to understand any impact.

Overall, an algal-based diet can provide sea urchins additional beneficial properties despite its lower calorific value. This was observed based on their immune response when compared to a mussel-based diet. Starvation was used as a control as lower calorific values could possibly interfere with the results. Furthermore, additional benefits of an algae based diet such as free radicals (observed increase in NO production) and essential vitamins and minerals outweigh the benefits of a higher calorific intake from a mussel diet (Heppleston, 1971; Hansson, 1976; Chambers & Milne, 1979; Zwarts & Wanink, 1993).

In conclusion, it is recommended that sea urchin diet should be taken into consideration when studies involving immunostimulatory agents are being carried out and that baseline impacts of diet should be determined to most accurately assess the impacts of immunostimulants.

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Chapter 4: The effects of three immunostimulant candidates on the host response of the edible sea urchin, *Paracentrotus lividus*

Abstract: Due to the increase in demand for sea urchin roe in American, Asian, and European markets, and the economic loss of high mortalities when faced with culturing challenges (e.g. disease, variable growth rates, handling and transport stress) the use of immunostimulants and probiotics has become a focal point for many researchers and industries as potential alternatives to support host immune function. Immunostimulatory agents are used to boost cultured animals' immunocompetence in order to fight off pathogens more efficiently. The objective of the current study was to assess three potential immunostimulatory agents and observe whether there was a beneficial effect on the edible sea urchin, *Paracentrotus lividus'* humoral (nitric oxide, lysozyme activity) and cellular, (cell viability and differential cell counts) immune response. The three candidates were (1) *Lactobacillus paracasei 336* PNZ, a gram-positive, non-spore forming microorganism; (2) GTF, a recombinant β-glucan-producing counterpart to PNZ; and (3) Zymosan A, an insoluble yeast cell wall extract prepared from *Saccharomyces cerevisiae*. PNZ and GTF, both in the development stage, were chosen based on promising results shown in previous studies on mice, while Zymosan A was chosen for its promising results shown in previous studies in oysters. PNZ and GTF had no advantageous effect on *P. lividus'* host response and high mortality was observed in all treatments thus were not studied further. In contrast, Zymosan A demonstrated a positive impact on host response with the higher doses of Zymosan A showing the greatest effect on sea urchin host response.

Introduction

Due to the increase in demand for sea urchin roe in American, Asian, and European markets, sea urchin culture has become a focal point for many researchers and industries. Sea urchin aquaculture is limited by stress and disease (Pais *et al.,* 2007) and is traditionally managed by selecting the healthiest animals for the market. The objective of the current study was to assess three potential immunostimulatory candidates and observe whether there was a beneficial effect on some immune parameters of the edible sea urchin, *Paracentrotus lividus'* humoral (nitric oxide, lysozyme activity) and cellular (cell viability and differential cell counts) immune response.

The first candidate, *Lactobacillus paracasei 336* PNZ (probiotic), is a gram-positive, non-spore forming microorganism (Hessle *et al.,* 1999). Probiotics are "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" (Reid *et al.,* 2003). Working in both the innate and acquired immune systems (Ringø *et al.,* 2012), probiotics appear to modulate immunity of the host by improving mucosal barrier properties (Lebeer *et al.,* 2010; Bron *et al.,* 2012; Wells *et al.,* 2011; Remus *et al.,* 2011), stimulating antibody secreting cell responses (Kaila *et al.,* 1992; Panigrahi *et al.,* 2004, 2005, 2007; Salinas *et al.,* 2008; Oelschlaeger, 2010); enhancing the phagocytosis of pathogens, and modulating production of cytokines (Nayak, 2010). Although little has been done in regards to sea urchin farming, there have been several studies that look at the effect of probiotics in other echinoderm species, such as the sea cucumber (Yasoda *et al.,* 2006; Zhou *et al.,* 2010; Liu *et al.,* 2012).

The second candidate, *Lactobacillus paracasei* 338 GTF (NFBC 338) (probiotic + immunostimulant), developed by researchers at Teagasc (Ireland) is a strain of

Lactobacillus paracasei which expresses the glycosyltransferase gene responsible for producing exopolysaccharides (*mechanism unpublished*). This means that the probiotic produces a β-glucan, which acts in synergy to enhance its function (London *et al.,* 2014). An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the host's resistance against stress and disease (Bricknell & Dalmo, 2005). Research has established that various inactivated natural microbes or microbial products, such as β-glucans, can stimulate the innate immune system by activating receptors, which trigger gene activation (Galindo-Villegas & Hosokawa, 2004). Glucans are composed of glucose building blocks, isolated from the cell walls of bacteria, algae, cereal grains, yeast, and fungi (Zekovic & Kwiatkowski, 2005; Ringø *et al.,* 2012). Kiseleva and colleagues (2014) found that the effect of brown algae 1,3 and 1,6-β-D-glucans, at the right concentrations, had a beneficial stimulating effects and increased survival rates in sea urchin, *Strongylocentrotus intermedius,* embryos. Since London *et al.* (2014) found GTF to be a successful strain in mice at 10^9 CFU; we were asked to test it in urchins against the original *Lactobacillus paracasei* strain (PNZ).

The last candidate, Zymosan A (SIGMA) (immunostimulant), is an insoluble yeast cell wall extract prepared from *Saccharomyces cerevisiae* (Jiang *et al.*, 2013) and is generally used to induce sterile inflammation, including pro-inflammatory cytokines, protein phosphorylation and inositol phosphate formation in the host organism. Zymosan A has previously been used in several oyster species (Costa *et al.,* 2009; Prado-Alvarez *et al.,* 2015). Due to the similar size of *Ostrea edulis* compared to *P. lividus,* previous work done by Costa *et al.* (2009) and Prado-Alvarez *et al.* (2015) was the basis of using Zymosan A in this study. Additionally, Zymosan A has previously been successfully used as an immunostimulant in many other

marine species including, the crayfish, *Pacifastacus leniusculus* (Persson *et al.*, 1987), Sydney rock oysters, *Saccostrea glomerata* (Aladaileh *et al.*, 2007), Eastern oysters, *Crassostrea virginica* (Bramble & Anderson, 1998), and the striped bass, *Morone saxatilis* (Bramble & Anderson, 1998).

Important molecules in innate immune parameters, such as lysozyme and superoxides, have often been used as indicators of aquatic stress response and disease resistance (Saurabh & Sahoo, 2008). Lysozyme levels demonstrate defense capabilities through the enzymatic breakdown of pathogenic cell membranes (Cronin *et al.*, 2001), through the measurement of the cellular release of lysozymes within the coelomic fluid. Superoxides, specifically nitric oxide (NO), is a nitrogen radical gas produced from L-arginine during phagocytosis and serves as a mechanism to fight off invasive pathogens (Tafalla *et al.*, 2003). In marine invertebrates, NO plays a part in several biological roles relating to feeding, defense, environmental stress, learning, metamorphosis, swimming, symbiosis, and haemocyte aggregation (Palumbo, 2005). Cell viability (CV) can serve as a proxy for organism health under a treatment on immune response by indicating the level of physiological functioning of cells. Furthermore, monitoring differential cell counts (DCC) can indicate immune response through the activity of the different cell populations and their specific functions (Jussila *et al.*, 1997; Fotedar & Evans, 2011). The objective of this study was to determine if PNZ, GTF, and Zymosan A had an effect on the immune function of *P. lividus* individuals using several immune parameters to measure immune response.

Materials and Methods

A series of five trials were performed focusing on different immunostimulatory agents, dosages and administration methods. Below the general method is described first and then the individual trial variations are detailed.

Experimental design

Sea urchins were sourced from Dunmanus Seafoods Ltd. in Dunmanus Bay, Ireland for *Trials 1* to *3* and from Daithi O'Murchu Marine Research (DOMMR) Station in Bantry, Co. Cork, Ireland for *Trials 4* and *5* (sea urchin harvest dates – Table 1). Animals were held in aerated tanks filled with fresh seawater sourced from West Cork, Ireland. The tanks were held in a constant temperature room at 12 ± 1 °C for *Trials 1* to *4* and at 15 ± 1 °C for *Trial 5*. Before the start of the experiment, animals were acclimated. Animals were fed *ab libitum* with *Laminaria* spp. for *Trials 1* to *4.* Based on results that became available from Chapter 3, animals were starved starting with *Trial 5.* This was for both the experiment and a two week acclimation period as suggested in previous feeding studies by Lawrence *et al.* (1989) and Siikavuopio *et al.* (2007, 2008), to remove any residual effects of previous diets.

Table 1: Sea urchin harvest dates per specified trial.

Trial	Month	Year
1	March	2013
2	March	2013
3	September	2013
4	September	2013
5	October	2014

Probiotics and immunostimulants methods

Suspensions of PNZ or GTF in 15% trehalose medium (London *et al.,* 2014) were provided at the requested concentrations, CFU of PNZ or GTF per volume, by Teagasc (Agriculture and Food Development Authority, Fermoy, Ireland). A 0.1 ml sample was either administered to each individual urchin daily via oral gavage (injection directly into the mouth via syringe), or was added directly to the tanks, once at time zero or daily, depending on the trial treatments.

For the Zymosan A particles stock solution, 40 mg of Zymosan A from *Saccharomyces cerevisiae* (Sigma) was suspended in 10 ml sterile artificial seawater (ASW), boiled for 30 min, washed twice, and then re-suspended in 10 ml sterile ASW and stored in aliquots at -20 °C (Lambert & Nicolas, 1998). A calculated volume of Zymosan A stock solution was added once at time zero directly to the tanks with known volumes to produce the desired tank concentration of Zymosan A to seawater.

Controls were either given no treatment or injected daily with 0.1 ml of 15% trehalose medium depending on the trial.

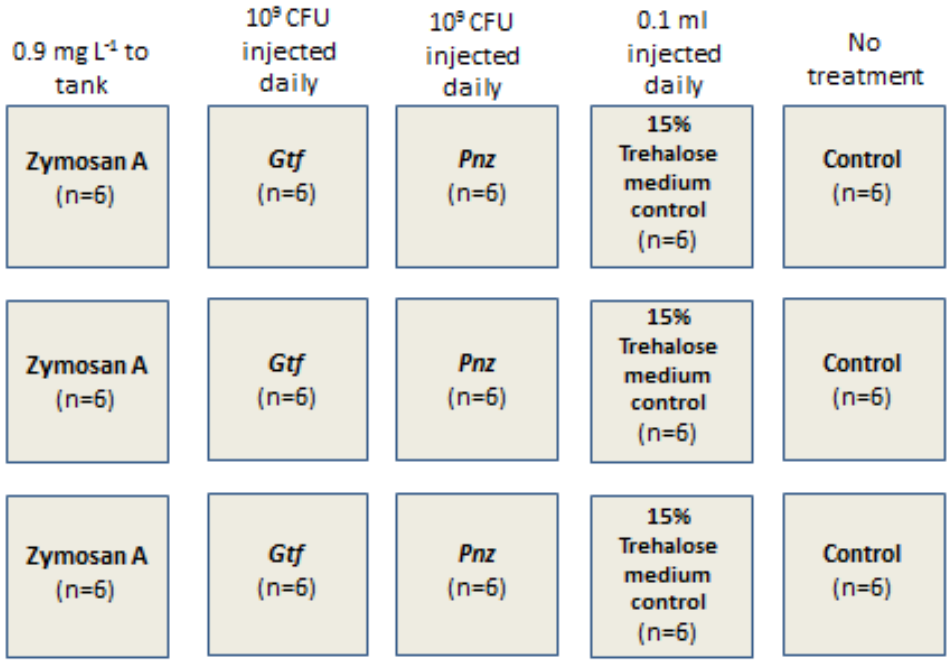
Host Response Measurement

Animals sampled at time zero were taken before any treatments. To assess host response, different trials measured different immune parameters. Cell viability (CV), nitric oxide (NO) and lysozyme activity levels were measured according to methods described in Chapter 2 (*pgs 51 – 53*) and differential cell counts (DCC) were done according to methods described in *Chapter 3* (*pgs 74 – 75*). Different immune parameters were used in different trials due to coelomic fluid availability and animal size.

Trial 1: Investigation of the effects of GTF (10^9 CFU injected daily), PNZ (10^9 CFU injected daily) and Zymosan A (0.9 mg L^{-1} once to tank at time zero) on P. lividus' immune function

Sea urchins (n=100; 50 ± 5 mm (mean test diameter ± SD)) were acclimated for 48 hours in two 100 L tanks. The experiment was carried out in fifteen 10 L tanks (Figure 1). Controls and treatments consisted of a control group with no treatment, a control group with 15% trehalose medium injections, 10^9 CFU PNZ injections, 10^9 CFU GTF injections, and 0.9 mg L^{-1} Zymosan A to seawater. Ten animals were sampled at time zero. One animal per tank (n=15) was sampled and sacrificed at the following time points: 7 and 24 hours and 2, 6, 9, and 14 days. Cell viability and nitric oxide levels were measured for each sampled animal.

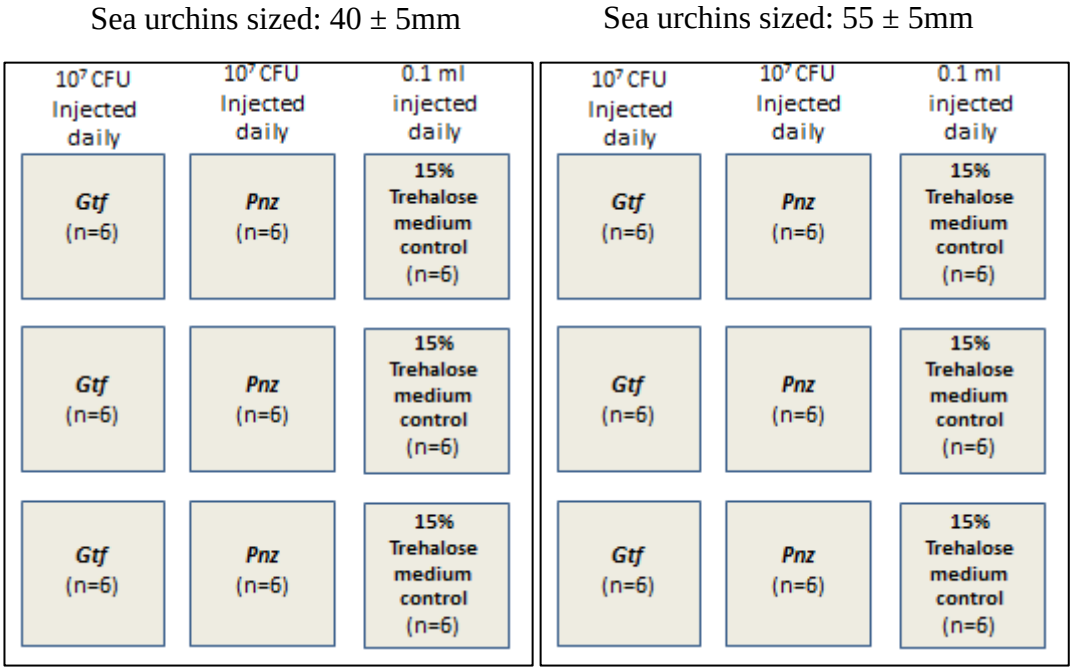
Figure 1: Schematic of *Trial 1* experimental design consisting of fifteen 10 L aerated tanks. Sets of three replicate tanks contained one of five treatments or controls; as outlined in the schematic below.



Trial 2: Assessment of the impact of a lower dose (10^7 CFU injected daily) of PNZ and GTF as potential immunostimulants on sea urchin's immune function

Sea urchins of two sizes (n=54 at 40 ± 5 mm and n=54 at 55 ± 5 mm; selected due to size availability within shipment) were acclimated for one week in two 100 L tanks. The experiment was carried out in eighteen 10 L tanks as (Figure 2). Controls and treatments consisted of a control group with 15% trehalose medium injections, 10^7 CFU PNZ injections, and 10^7 CFU GTF injections. One animal per tank (n=18) was sampled and sacrificed at the following time points: 0, 7, 24 and 48 hours. Cell viability and nitric oxide levels were measured for each sampled animal.

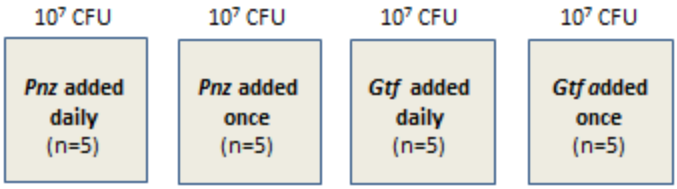
Figure 2: Schematic of *Trial 2* experimental design consisting of eighteen 10 L aerated tanks.



Trial 3: Assessment of the impact of two methods of dose administration for GTF and PNZ directly to the tank either once or daily

Sea urchins (n = 24; 30 ± 5 mm; selected due to size availability within shipment) were acclimated for 48 hours in one 100 L tank. The experiment was carried out in four 10 L tanks (Figure 3). Treatments consisted of adding 10^7 CFU PNZ or GTF directly to the tank either once at time zero or daily. One animal per tank (n=4) was sampled and sacrificed at the following time points: 0, 2, and 24 hours and at 2 and 7 days. Only lysozyme level was measured for each sampled animal.

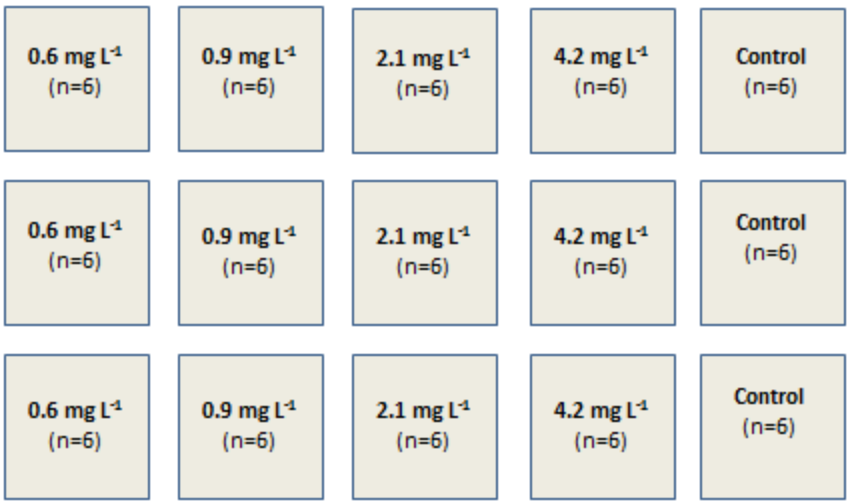
Figure 3: Schematic of *Trial 3* experimental design consisting of four 10 L aerated tanks.



Trial 4: Investigation of the effects of four different doses of Zymosan A (0.6 mg L^{-1}, 0.9 mg L^{-1}, 2.1 mg L^{-1} and 4.2 mg L^{-1}) on P. lividus' immune function

Sea urchins (n = 90; 30 ± 5 mm) were acclimated for one week in fifteen 10 L tanks. The experiment was carried out in fifteen 10 L tanks (Figure 4). Controls and treatments consisted of a control group with no treatment, and Zymosan A to seawater concentrations of 0.6, 0.9, 2.1 and 4.2 mg L^{-1}. One animal per tank (n=15) was sampled and sacrificed at the following time points: 0, 3, and 24 hours and at 2 and 7 days. Only lysozyme level was measured for each sampled animal.

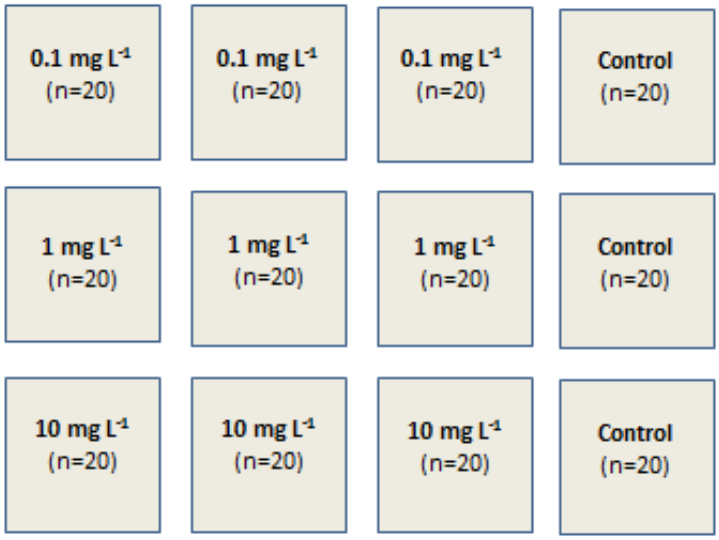
Figure 4: Schematic of *Trial 4* experimental design consisting of fifteen 10 L aerated tanks.



Trial 5: Investigation of the effects of three different doses of Zymosan A (0.1 mg L^{-1}, 1 mg L^{-1} and 10 mg L^{-1}) on P. lividus' immune parameters in a modified experimental setup

Sea urchins (n = 240; 30 ± 5 mm) were acclimated for two weeks in twelve 70 L tanks. The experiment was carried out in twelve 70 L tanks (Figure 5). Controls and treatments consisted of a control group with no treatment, and Zymosan A to seawater concentrations of 0.1, 1, and 10 mg L^{-1}. Twelve animals were sampled at time zero. Three animals per tank (n=36) were sampled and sacrificed at the following time points: 2 and 24 hours and 3, 7, 10, and 14 days. Cell viability (CV), nitric oxide (NO), lysozyme activity levels and differential cell counts (DCC) were measured for each sampled animal.

Figure 5: Schematic of *Trial 5* experimental design consisting of twelve 70 L aerated tanks.



Data Analysis

All statistics were compiled using SPSS software (IBM) version 23. If a statistical model could be applied, a detailed description is listed below under the trial headings. However, in some cases statistical models, such as a mixed linear model, that use predicted values could not be applied. This is due to the presence of too many data points that were influential (values that exceed 3x the standard deviation), but also not large enough to be eliminated as outliers (values that exceed 5x the standard deviation). Thus, any significance found with such a model could not necessarily be attributed to a population or treatment.

Trial 1-2: A statistical model was not possible.

Trial 3: Data presented normal distribution (normality test Shapiro-Wilk) ($p>0.05$). A t-test was used to compare PNZ added daily and PNZ added once for each

sampling time. For GTF, a 2-tailed t-test was used, as equal variances were not assumed since the Levene's test was significant.

Trial 4: Between treatments: Data presented normal distribution (normality test Shapiro-Wilk) ($p>0.05$) with one extreme case that exceeded five times the deviation, which was removed. A one-way ANOVA was used with Tukey as a post hoc test for each time point. Homogeneity of variances was tested and Welch correction or Dunnett T3 was used when homogeneity was not observed.

Treatments compared over time: A linear mixed model was applied. A Least Significant Difference (LSD) was used to compare interactions. For lysozyme activity, time was treated as a fixed effect and tanks were analyzed within that specific sampling point.

Trial 5: A statistical model could not be produced for both CV and lysozyme activity assays. For NO, a mixed linear model was used, with treatment as a fixed effect and tanks within treatments as a nested random factor. This model accounts for data with unequal variances. A Least Significant Difference (LSD) post-hoc test was used for pairwise comparison among treatments when significance was observed. Wald-z tests were used to determine if the tanks within the treatments had a random effect in the model. For differential cell counts, all cell types were compared between treatments at the initial and final time point. If a difference was observed, difference among treatments of that cell type was tested for all sampling times. Normality plots were verified and one-way ANOVA was used to assess differences between treatments. Welch correction was used when there were differences in the variances.

Results

Trial 1: Investigation of the effects of GTF (10^9 CFU injected daily), PNZ (10^9 CFU injected daily) and Zymosan A (0.9 mg L^{-1} once to tank at time zero) on P. lividus' immune function

Cell Viability (CV)

There was an initial impact on OD (optical density) after 7 hours in both the control, Zymosan A, and PNZ groups, followed by a decrease in all treatments and controls (Figure 6-7). Values over the study varied from 0.14 ± 0.06 OD_{540} for GTF to 1.29 ± 0.23 OD_{540} for the control. GTF and PNZ treatment animals did not survive past 9 days. No statistical model was performed; for an explanation please refer the *Data Analysis (pgs 103-104)* and for boxplot representation of the data please refer to the *Appendix* (*pgs 181-187*).

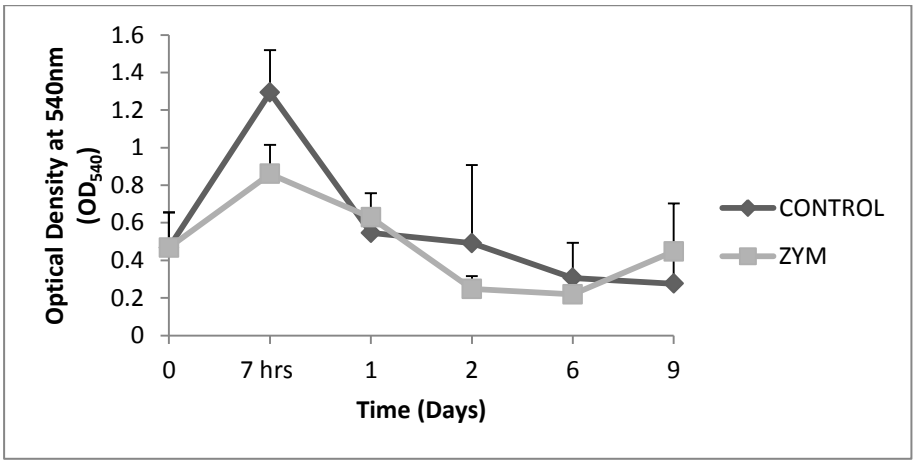


Figure 6: Mean cell viability levels ± SE (OD_{540}) for *P. lividus* individuals treated with 0.9 mg L^{-1} of Zymosan A, compared with a control group over a 9-day period.

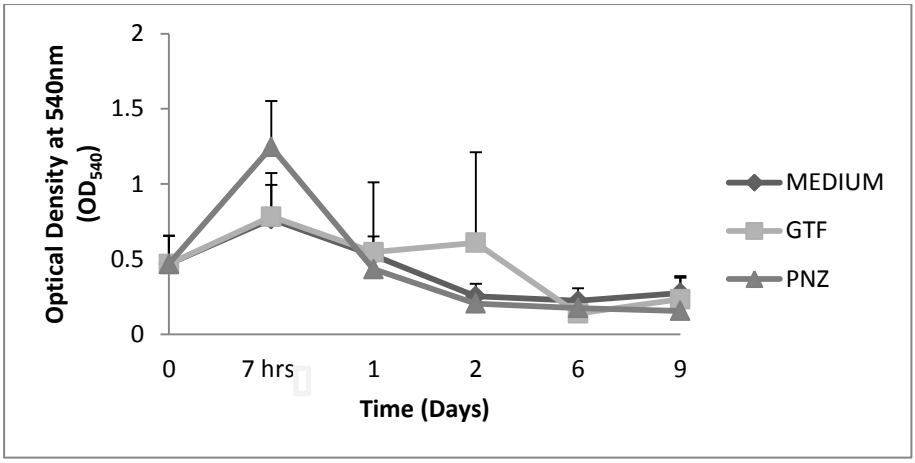


Figure 7: Mean cell viability levels ± SE (OD_{540}) for *P. lividus* individuals treated with 10^9 CFU of GTF and PNZ, compared with a 15% trehalose medium control group over a 9-day period.

Nitric Oxide (NO), The Griess Reaction

There was no impact between treatments and the controls. Nitric oxide levels varied over the study from 4.92 ± 0.86 µM for the Zymosan A treatment to 53.71 ± 0.31 µM for the control (Figures 8-9). GTF and PNZ treatment animals did not survive past 9 days. No statistical model was performed; for an explanation please refer the *Data Analysis (pgs 103-104)* and for boxplot representation of the data please refer to the *Appendix* (*pgs 181-187*).

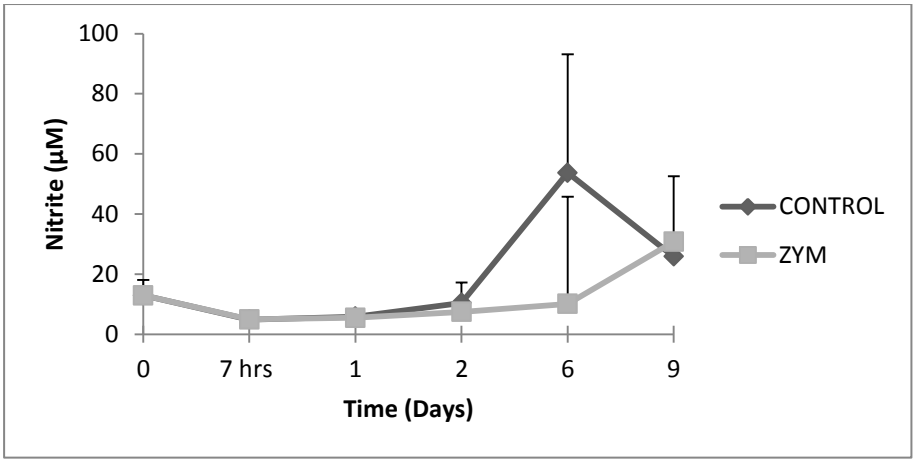


Figure 8: Mean nitric oxide levels ± SE (µM) for *P. lividus* individuals treated with 0.9 mg L^{-1} of Zymosan A, compared with a control group over a 9-day period.

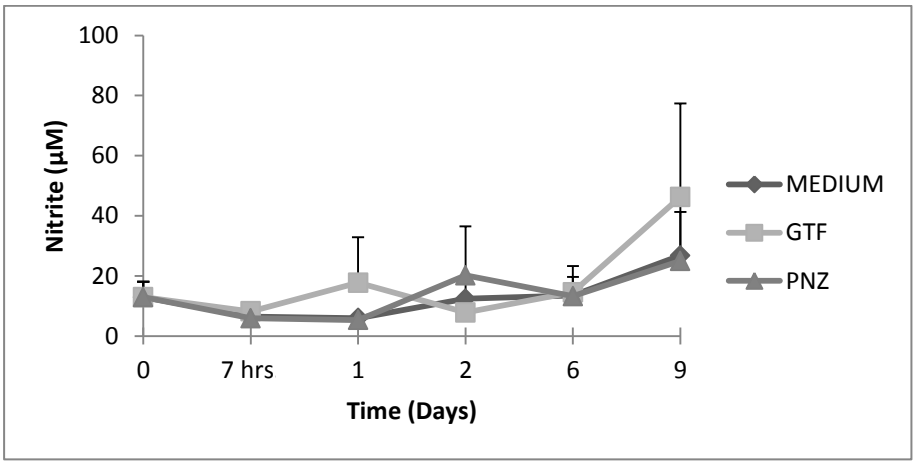


Figure 9: Mean nitric oxide levels ± SE (µM) for *P. lividus* individuals treated with 10^9 CFU of GTF and PNZ, compared with the 15% trehalose medium control group over a 9-day period.

Trial 2: Assessment of the impact of a lower dose (10^7 CFU injected daily) of PNZ and GTF as potential immunostimulants on sea urchin's immune function

Cell Viability

In the smaller animals (40 ± 5 mm) all treatments and controls showed a decrease in OD over the experimental period (Figure 10). Values over the study varied from 0.14 ± 0.06 OD_{540} for the GTF group to 1.25 ± 0.31 OD_{540} for the PNZ group. Animals did not survive after 48 hours. No statistical analysis was performed due to low number of data points per treatment.

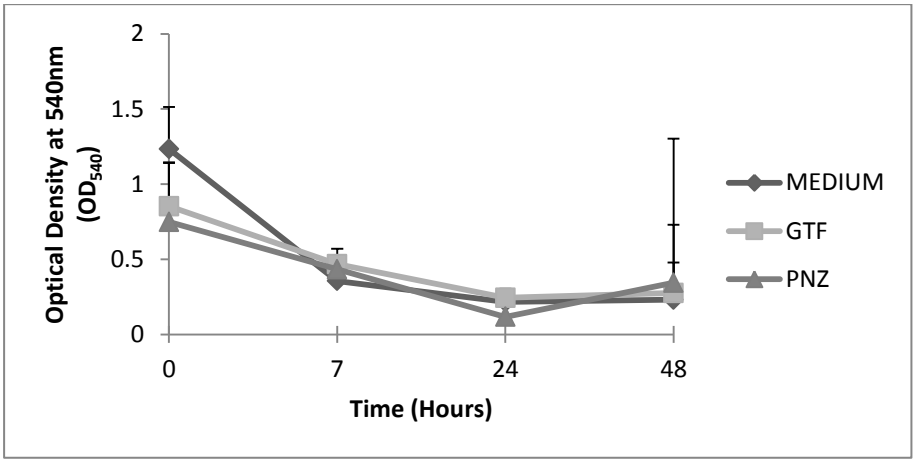


Figure 10: Mean cell viability levels ± SE (OD_{540}) for *P. lividus* individuals (40 ± 5 mm) treated with a 10^7 CFU of GTF and PNZ, compared with a 15% trehalose medium control group over 48 hours.

With larger animals (55 ± 5 mm) all treatments and controls showed a decrease in OD over the experimental period. *PNZ* had the greatest impact and induced the highest level at time zero (Figure 11). Values over the study varied from 0.18 ± 0.03 OD_{540} for the 15% trehalose medium control to 1.18 ± 0.35 OD_{540} for the PNZ

group. Animals did not survive after 48 hours. No statistical analysis was performed due to low number of data points per treatment.

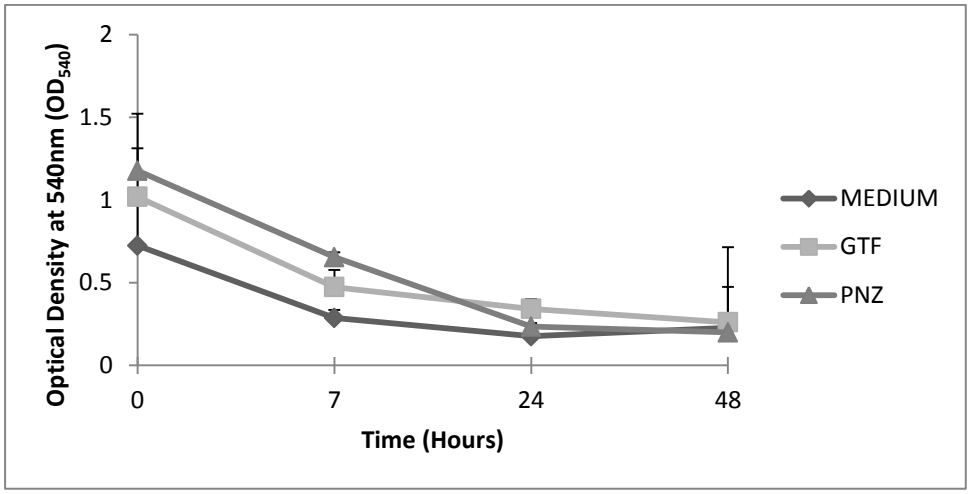


Figure 11: Mean cell viability levels ± SE (OD_{540}) for *P. lividus* individuals (55 ± 5 mm) treated with a 10^7 CFU of GTF and PNZ, compared with a 15% trehalose medium control group over 48 hours.

Nitric Oxide (NO), The Griess Reaction

With the smaller animals (40 ± 5 mm) all levels were the same as the controls; therefore, GTF and PNZ had no impact on NO (Figure 12). Values over the study varied from 18.77 ± 14.37 µM for the PNZ group to 82.23 ± 4.92 µM for the 15% trehalose medium control. Animals did not survive after 48 hours. No statistical analysis was performed due to the low number of samples per tank.

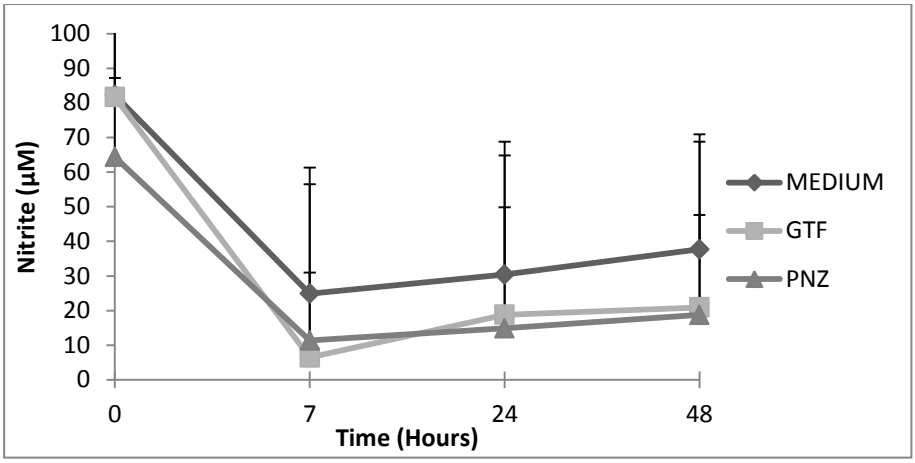


Figure 12: Mean nitric oxide levels ± SE (µM) for *P. lividus* individuals (40 ± 5 mm) treated with a 10^7 CFU of GTF and PNZ, compared with a 15% trehalose medium control group over 48 hours.

Larger animals (55 ± 5mm) all levels were the same as the controls, therefore, GTF and PNZ had no impact on NO (Figure 13). Values over the study varied from 18.34 ± 9.98 µM for the GTF group to 82.28 ± 17.07 µM for the PNZ group. Animals did not survive after 48 hours. No statistical analysis was performed due to the low number of samples per tank.

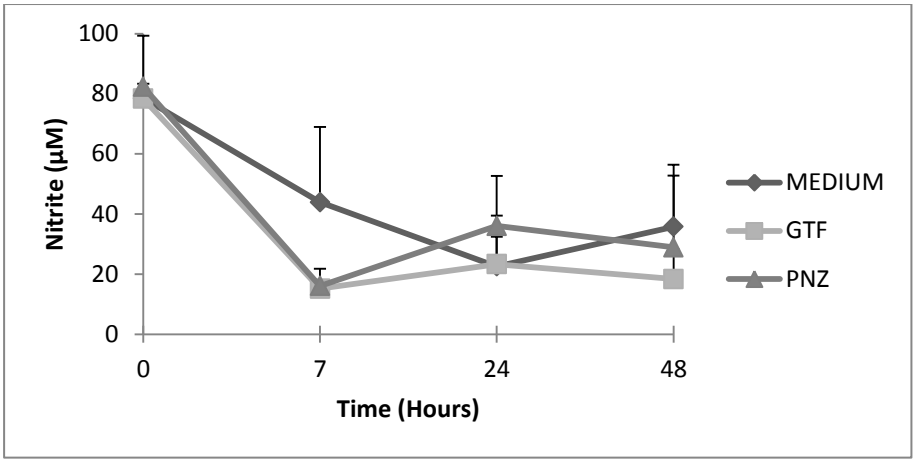


Figure 13: Mean nitric oxide levels ± SE (µM) for *P. lividus* individuals (55 ± 5mm) treated with a 10^7 CFU of GTF and PNZ, compared with a 15% trehalose medium control group over 48 hours.

Trial 3: Assessment of the impact of two methods of dose administration for GTF and PNZ directly to the tank either once or daily

Lysozyme Activity

There was an impact on lysozyme levels in animals treated daily with PNZ (Figure 14). Between time zero and 48 hours there was a significant difference observed ($p \geq 0.03$). There was not enough data to compare 24 and 72 hours. Animals treated once with PNZ at the beginning of the experiment did not survive past 24 hours, which was after a decline in lysozyme activity.

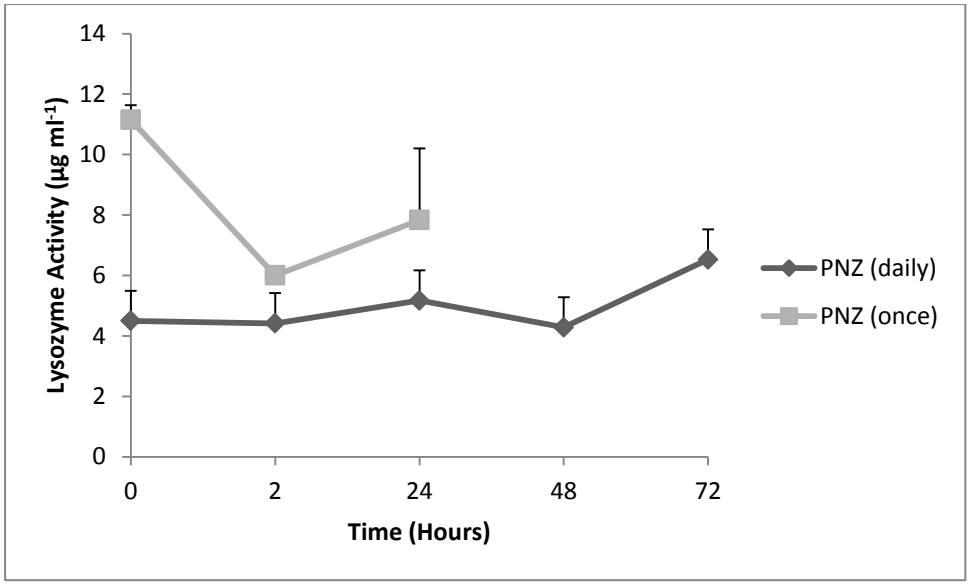


Figure 14: Mean lysozyme activity levels ± SE (µg ml^{-1}) for *P. lividus* individuals (40 ± 5 mm) treated with a 10^7 CFU dose of PNZ, which was added once at the beginning of the experiment, or daily, and monitored over 72 hours. PNZ (added once) animals did not survive past 24 hours.

The *P. lividus* individuals treated once with GTF at the beginning of the experiment had a greater initial impact on lysozyme activity than animals treated daily with GTF (Figure 15); however, animals did not survive after 48 hours. There was no difference between GTF added once or added daily at time zero ($p \geq 0.411$) or at 2 hours ($p \geq 0.478$). There were not enough data points to compare 24, 48 and 72 hours.

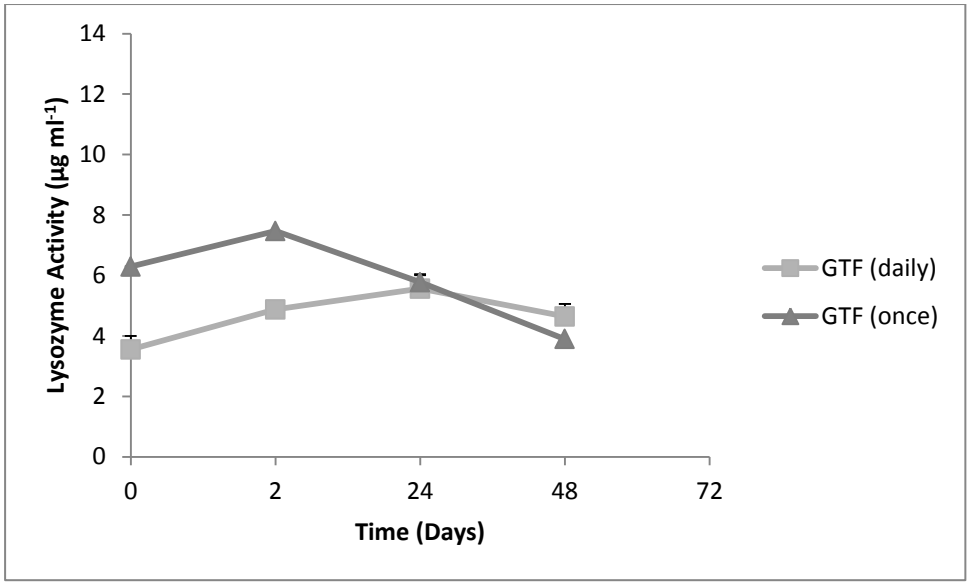


Figure 15: Mean lysozyme activity levels ± SE (µg ml^{-1}) for *P. lividus* individuals (40 ± 5 mm) treated with a 10^7 CFU dose of GTF, which was added once at the beginning of the experiment, or daily, and monitored over 72 hours. Animals did not survive past 48 hours.

Trial 4: Investigation of the effects of four different doses of Zymosan A (0.6 mg L^{-1}, 0.9 mg L^{-1}, 2.1 mg L^{-1} and 4.2 mg L^{-1}) on P. lividus' immune function

Lysozyme Activity

Lysozyme activity for the sea urchins prior to the addition of the treatments (T_0) had a mean of 20.07 ± 11.84 µg ml^{-1} (Figure 16). Values over the study varied from 2.37 ± 0.88 µg ml^{-1} in the 0.6 mg L^{-1} dose of Zymosan A to 43.16 ± 1.75 µg ml^{-1} in the 2.1 mg L^{-1} dose. At T_0, there was a significant difference between the 0.6 mg L^{-1} and 0.9mg L^{-1} doses ($p=0.033$). Two hours after dosing, there were significant differences observed between the control and the different treatments: 0.9 mg L^{-1} ($p<0.05$), 2.1 mg L^{-1} ($p<0.05$), and 4.2 mg L^{-1} ($p<0.01$). For the 24 and 72 hours.

sampling points, significant difference was observed ($F_{4,7.175}$=4.627, p=0.047 and $F_{4,7.290}$=3.861, p=0.055, respectively); however due to the marginal difference, the post hoc test couldn't detect a difference among doses. Lastly, after 168 hours, there was no significant differences observed between doses ($F_{4,11}$=0.392, p=0.810).

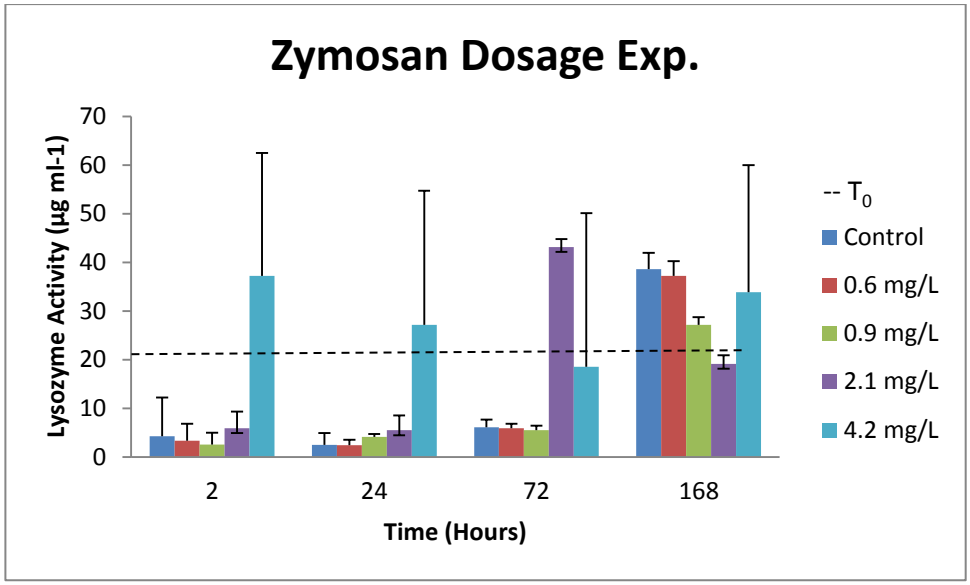


Figure 16: Mean lysozyme activity levels ± SE (µg ml^{-1}) for *P. lividus* individuals (40 ± 5mm) treated with Zymosan A compared with a control group over 7 days.

Trial 5: Investigation of the effects of three different doses of Zymosan A (0.1 mg L^{-1}, 1 mg L^{-1} and 10 mg L^{-1}) on P. lividus' immune function in a modified experimental setup

Cell Viability

The greatest impact on OD measurements was observed in the control, which varied over the study from 0.15 ± 0.07 OD_{540} to 0.61 ± 0.17 OD_{540} (Figure 17). Values over the study varied from 0.22 ± 0.06 OD_{540} for the 0.1 mg L^{-1} dose of Zymosan A to

0.78 ± 0.38 OD_{540} for the 10 mg L^{-1} dose. No statistical model was performed; for an explanation please refer the *Data Analysis (pgs 103-104)* and for boxplot representation of the data please refer to the *Appendix* (*pgs 181-187*).

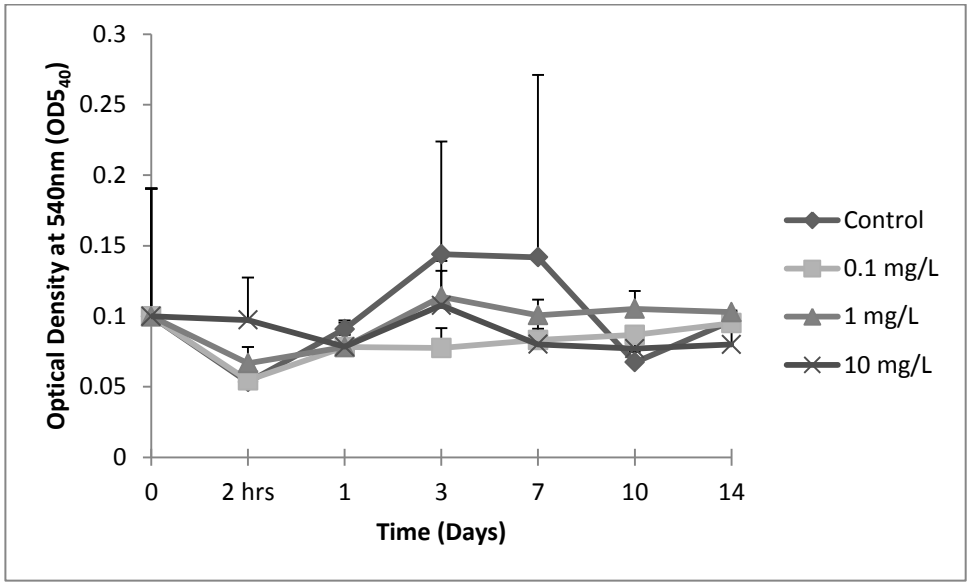


Figure 17: Mean cell viability levels ± SE (OD_{540}) for *P. lividus* individuals (30 ± 5 mm) treated with different doses of Zymosan A (0.1 mg L^{-1}, 1 mg L^{-1}, and 10 mg L^{-1}) compared with a control group over 14 days.

Differential Cell Counts (DCC)

Philopodial phagocytes were the main immune cell type in all groups (Figure 18); however, there was no difference observed between the different doses and the control. The 0.1 mg L^{-1} group had a lower relative percentage of amebocytes cells (0.56 %) when compared to the control group (1.22 %; $p \leq 0.26$; df = 26) and the 1 mg L^{-1} and 10 mg L^{-1} group: 1.5 % and 1.67 %, respectively ($p \leq 0.4$; df = 26; $p \leq 0.32$; df = 26). There were no differences observed in either the petaloid phagocytes or vibratile cells between treatments.

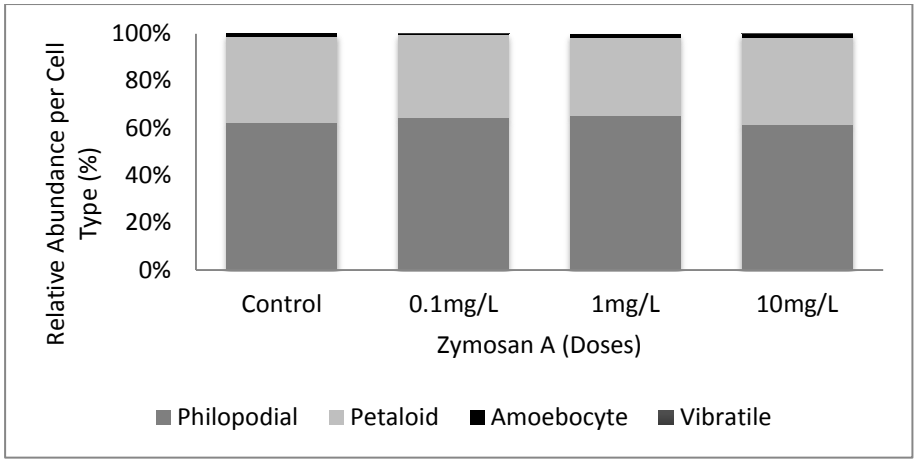


Figure 18: Relative percentages (%) of the four different coelomocytes found in each group of sea urchin individuals: control and Zymosan A doses of 0.1 mg L^{-1}, 1 mg L^{-1}, and 10 mg L^{-1}.

Red/ colourless amebocytes were low in all treatments with no significant difference between doses ($p \leq 0.74$; df = 3) over the 14 day trial period ($p \leq 0.63$; df = 5) (Figure 19). Levels of vibratile cells were low in all treatments with no significant difference between doses ($p \leq 0.09$; df = 3) over the 14-day period ($p \leq 0.25$; df = 5). Philopodial phagocytes showed significant difference between doses ($p \leq 0.15$; df = 3) but did not vary significantly over the 14-day trial period ($p \leq 0.005$; df = 5). Petaloid phagocytes showed no significant difference between doses ($p \leq 0.27$; df = 3) but did vary significantly over the 14-day trial period ($p \leq 0.005$; df = 5).

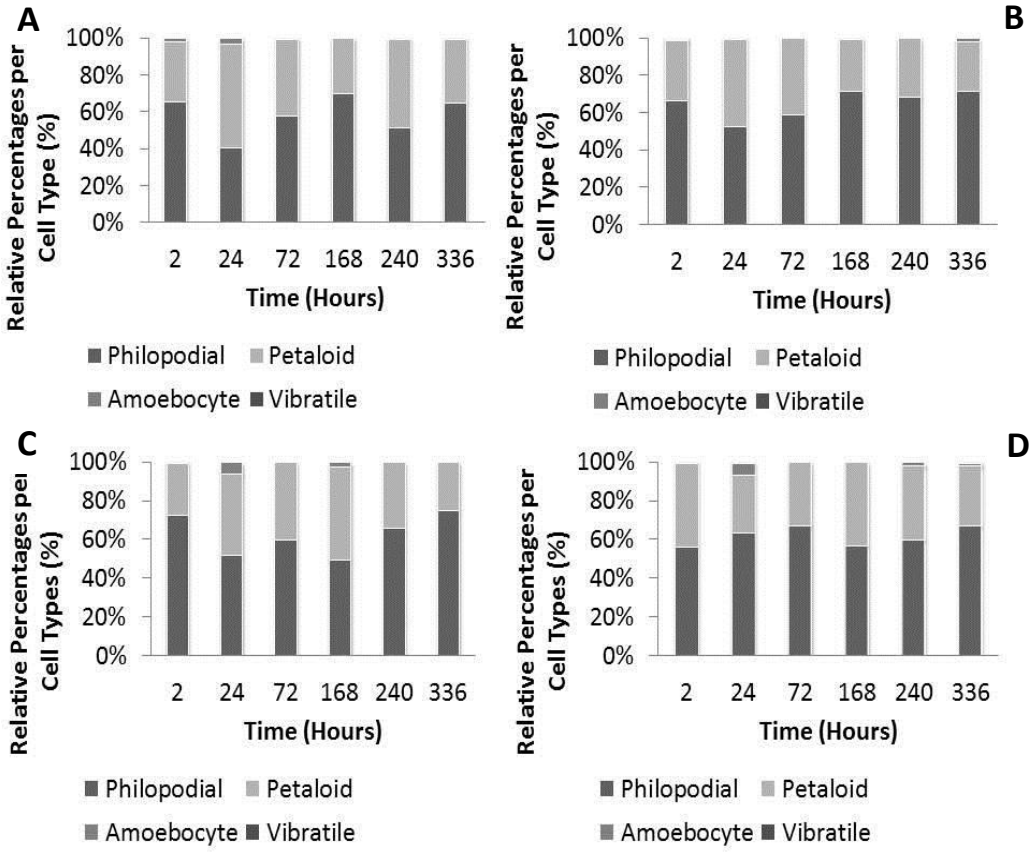


Figure 19: Abundance percentages (%) of the four different immune cell types found in each group of sea urchin individuals: control (A) and Zymosan A doses of 0.1 mg L^{-1} (B), 1 mg L^{-1} (C), and 10 mg L^{-1} (D).

Nitric Oxide (NO), The Griess Reaction

The initial baseline NO measurement, prior to the commencement of the trial, was 63.88 µM (Figure 20). Doses of 0.1 mg L^{-1} Zymosan A and 1 mg L^{-1} and the control groups' levels all remained low. The 10 mg L^{-1} treatment group measured 164.49 ± 22.28 µM throughout the experiment with significant differences observed at 24 hours ($F_{8,3}$=6.025, p=0.019; control (p=0.007), 0.1mg L^{-1} (p=0.026), and 1mg L^{-1} (p=0.006)), 72 hours ($F_{8,3}$=5.249, p=0.027: control (p=0.012), 0.1mg L^{-1} (p=0.042), 1mg L^{-1} (p=0.007)), and 168 hours ($F_{8,3}$=5.00, p=0.031: control (p=0.017) and 1mg L^{-1} (p=0.007)).

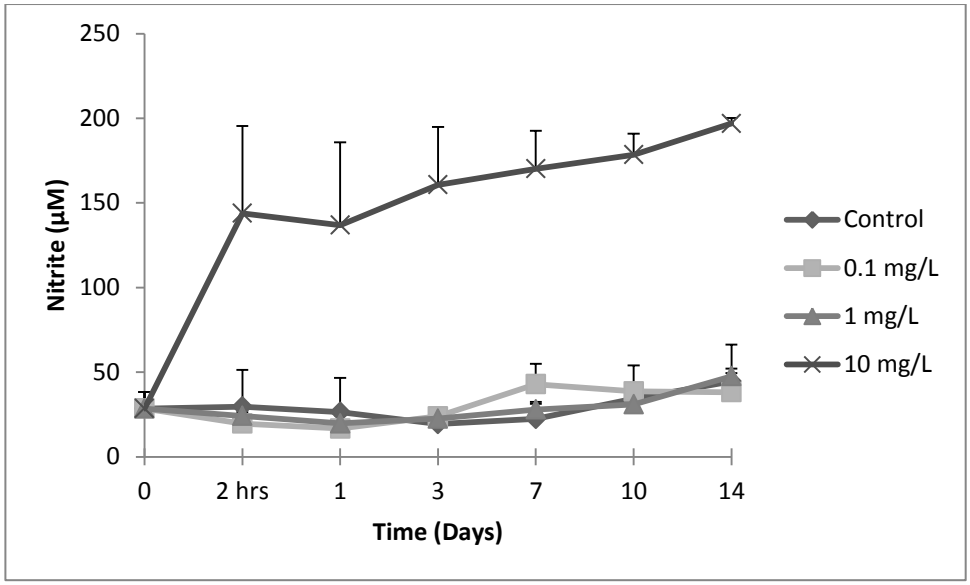


Figure 20: Mean nitric oxide levels ± SE (µM) for *P. lividus* individuals (20 ± 5 mm) treated with different doses of Zymosan A (0.1 mg L^{-1}, 1 mg L^{-1}, and 10 mg L^{-1}) compared with a control group over 14 days.

Lysozyme Activity

Lysozyme activity in the control group showed stability at 0.4 ± 0.16 µg ml^{-1} throughout the experiment (Figure 21). The 1 mg L^{-1} dose peaked on day 3 with high variability at 1.39 ± 0.33 µg ml^{-1}. Dose treatment 10 mg L^{-1}peaks on day 7, at 0.97 ± 0.55 µg ml^{-1}, and steadily declined until day 14. No statistical model was performed; for an explanation please refer the *Data Analysis (pgs 103-104)* and for boxplot representation of the data please refer to the *Appendix* (*pgs 181-187*).

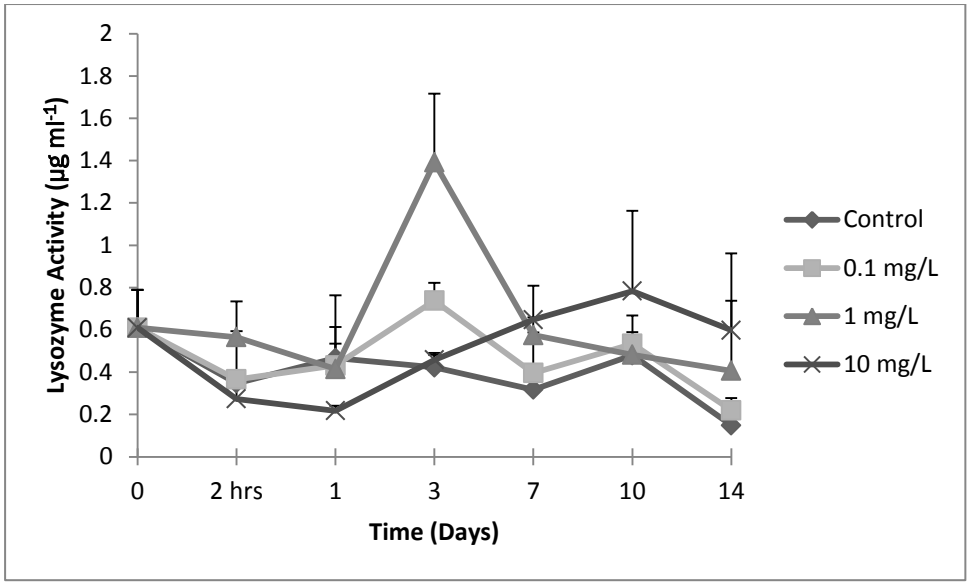


Figure 21: Mean lysozyme activity levels ± SE (µg ml^{-1}) for *P. lividus* individuals (30 ± 5 mm) treated with different doses of Zymosan A (0.1 mg L^{-1}, 1 mg L^{-1}, and 10 mg L^{-1}) compared with a control group over 14 days.

Discussion

The objective of this study was to determine if the probiotic PNZ, the immunostimulant producing probiotic GTF, and the immunostimulant Zymosan A had an immunostimulatory effect on the host response of *P. lividus* individuals. It is important to note that the different sized animals used in trials 1-3 were due to what arrived in the shipment, which showed a general tank effect across the different parameters.

The animals dosed with PNZ and GTF (10^9 CFU) experienced 100 % mortality before the end of each trial even at a lower CFU (10^7) dose and different administration method. Although the tested strains of PNZ and GTF in this study have never been tested in invertebrates, other *Lactobacillus* spp. have been used to significantly improve immune parameters and disease resistance in other aquatic animals such as the freshwater prawn, *Macrobrachium rosenbergii* (Dash *et al.*, 2015), the white shrimp, *Litopenaeus vannamei* (Chiu *et al.*, 2007), the Japanese pufferfish, *Takifugu rubripes* (Biswas *et al.*, 2013), and the European Sea Bass, *Dicentrarchus labrax* (Silvi *et al.*, 2008). To the best of our knowledge, *L. paracasei*, has never been studied in marine invertebrates; therefore, it is unknown if *L. paracasei* is compatible with the immune function of *P. lividus*, or other invertebrates at any CFU dosage. Due to ethical reasons, as well as the fact that PNZ and GTF are not strains available commercially and access to the strains was limited, we decided to move forward assessing the impact of Zymosan A (SIGMA).

Zymosan A, successfully studied in previously in tunicates (Raftos *et al*, 1992), the mussel, *Mytilus galloprovincialis* (Torreilles & Guérin, 1999), and in oysters (Samuni *et al.*, 1988; Lynch, *personal communication from preliminary work on the BEADS project*), is a harmless immunostimulant known to elicit a phagocytic

response in marine invertebrate hemocytes (Anderson, 2001). Upon ingestion of Zymosan A in an invertebrate, phagocytes engulf (or encapsulate) the particles and treat them as a possibly dangerous foreign agent; thereby, alerting the immune system to be on standby for future dangers (Anderson, 2001), such as *Vibrio* spp. Furthermore, reactive oxygen species, such as NO, are cytotoxic agents produced by phagocytic cells in response to immune defense perturbations and are used to defend against infectious diseases via their antimicrobial properties (Anderson, 2001). Significantly increased NO levels were observed in the animals dosed with the highest dose of Zymosan A possibly causing a significant release of NO in order to fight off the foreign agent.

Lastly, there were observed differences in differential cell counts, specifically in the amebocytes. Amebocytes are primarily responsible for the first mobilization response involving encapsulation and clotting (Matranga, 2005). As mentioned above, Zymosan A particles are encapsulated during phagocytosis as an immune defense mechanism. If there are more amebocytes present as a first response during a possible threat, the immune system has a greater chance of defending itself. Also, if the threat is greater (i.e. a higher amount of particles present in a higher dose), more red amebocytes (which contain the antimicrobial agent, echinochrome) will be generated (Smith, 1981; Matranga, 2005).

In conclusion, Zymosan A was shown to elicit a positive effect on the immune response of *P. lividus*, especially at higher doses. Therefore, it is recommended that further testing be completed to determine if Zymosan A, with the addition of a stressor such as transportation (physical stressor) and *Vibrio* infection (biological stressor), provides a beneficial effect on the immune function of *P. lividus.*

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Chapter 5: The effects of Zymosan A on the host response of the edible sea urchin, *Paracentrotus lividus*, during stimulated handling and storage

Abstract: Handling and transportation are both adverse stimuli that may cause physiological stress and immune response in organisms. Due to expense and regulation of antibiotics, the use of immunostimulants has become a focal point for many researchers and industries in invertebrates. An immunostimulant called Zymosan A, used in previous studies, was observed to have an impact upon the immune response of *P. lividus.* Zymosan A is an insoluble yeast cell wall extract prepared from *Saccharomyces cerevisiae*. The objective of this study was to determine if Zymosan A has a beneficial effect on host response of *P. lividus* individuals which experienced handling and simulated storage stressors, using cell viability, differential cell counts, nitric oxide and lysozyme activity as measured immune parameters. The results indicate that the higher doses of Zymosan A elicited elevated levels of amebocytes. Additionally, Zymosan A suppressed NO production when compared to the levels observed in the controls. Lastly, both the 1 mg L^{-1} and 10 mg L^{-1} doses suppressed lysozyme activity throughout the experimental period. In conclusion, simulated storage elicited a response in the CV (cellular), NO (humoral), and lysozyme activity (humoral) immune parameters observed by increased levels. However, the Zymosan A treatments minimized the production of NO and lysozyme parameters, resulting in a reduction of the inflammatory response to storage stress.

Introduction

Commercially important aquatic organisms are exposed to a variety of stressors during transport including air exposure, handling and physical disturbance, temperature fluctuations and exposure to adverse environmental conditions including high levels of dissolved ammonia, altered salinity (Le Moullac & Haffner, 2000), low oxygen levels, and poor water quality (Erikson *et al.*, 1997). Stress from handling, transport, and storage can affect metabolism, growth, molting, immunity and survival (Le Moullac & Haffner, 2000). Additionally, sea urchin aquaculture is limited by stress and disease, often associated with transportation, handling, storage, and over-crowding mortalities (Pais *et al.*, 2007).

Finding immunostimulants and probiotics that improve health and condition of these animals under the influence of transport (including handling and storage) conditions is crucial to sustained success in the live export market. In the previous study conducted (Chapter 4), Zymosan A was observed to have a positive impact on levels of *P. lividus*' immune parameters in laboratory holding conditions.

Seafood is highly perishable; however, live fish/shellfish are in particularly high demand in Asia and other niche markets. Industries and fisherman use technology, such as specially designed or modified tanks, containers and transport vehicles equipped with aeration or oxygenation facilities (Fotedar & Evans, 2011) to transport and store live seafood in order to reduce mortality. Transport and handling stress has been well documented in several invertebrate species such as the sea cucumber, *Holothuria scabra* (Purcell *et al.*, 2006), crustaceans (Fotedar & Evans, 2011), the blue crab, *Callinectes sapidus* (Welsh & Sizemore, 1985), and New Zealand abalone, *Haliotis iris* and *Haliotis australis* (Wells & Baldwin, 1995). Within these studies, it has been observed that survival and stress levels are largely influenced by stocking

density, the transport medium (including water quality) and container, oxygen levels, handling, and temperature (Purcell *et al.*, 2006).

Due to the level of stress an organism experiences while being handled or transported, which can be quite significant, mortalities from disease are high. The objective of this study was to determine if Zymosan A, an immunostimulant, had a beneficial effect on the immune response of stored (simulated) *P. lividus* individuals. With this in mind, the overall aim was to determine if Zymosan A is a successful immunostimulant in suppressing immune response to storage and handling stressors; thereby reducing the amount of stress the organism experiences.

Materials and Methods

Sea urchins (n=240; 30 ± 5mm) were sourced from Daithi O'Murchu Marine Research (DOMMR) Station in Bantry, Co. Cork, Ireland in April, 2014.

(a) Preparation of Zymosan A

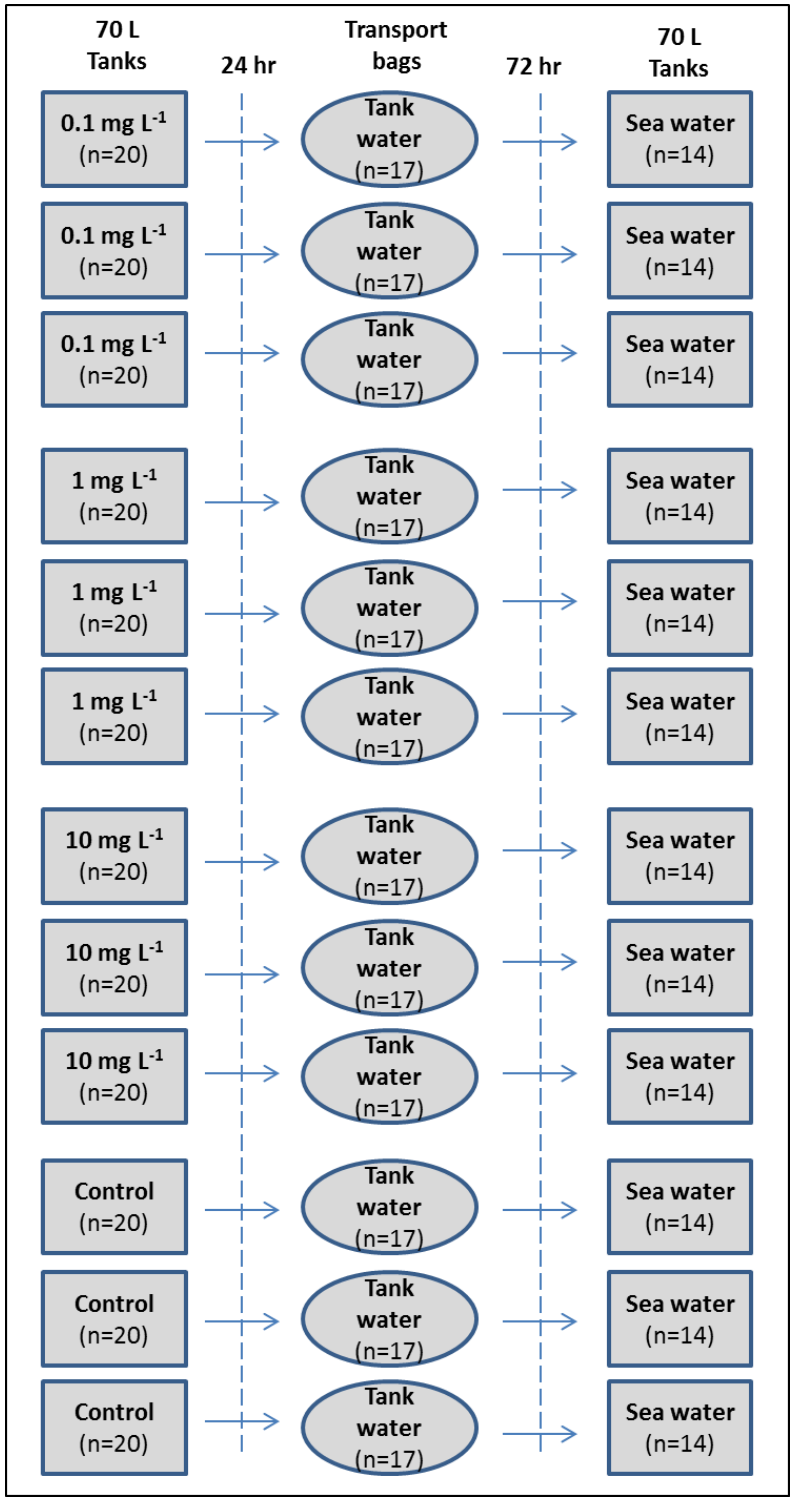
Zymosan A stock solution (*pg. 100*) was added directly to the tanks at time zero to produce tank concentration of 0.1 mg L^{-1}, 1 mg L^{-1} and 10 mg L^{-1} Zymosan to seawater. These tank concentrations were previously shown (*Chapter 4*) to provide a range host response from little to no effect, intermediate, and an extreme dose.

(b) Experimental design

Laboratory trials were carried out in twelve 70 L aerated tanks stocked with 20 sea urchins each. All tanks contained fresh seawater sourced from West Cork, Ireland and were held in a constant temperature room at 15 ± 1 °C. Animals were acclimated for two weeks. Sea urchins were not fed 14 days prior to the start of the experiment in order to minimize the quantity of residual food in the gut as according to several authors (Lawrence *et al.*, 1989; Siikavuopio *et al.*, 2007; 2008); the gut clearance takes up to 14 days in sea urchins. After acclimation, the trial consisted of three phases: treatment with Zymosan A from 0 to 24 hours, simulated storage from 24 hours to 3 days and subsequent monitoring of urchins from 3 to 14 days (schematic of tank setup in Figure 1). At the following time points, three animals per replicate tank (n=36) were sampled and sacrificed: 24 hours (before simulated storage), 3 days (directly after simulated storage) and at 4, 5, 7, and 14 days. No measurements were taken at time zero because the general effect of Zymosan A had been already

examined (*Chapter 4*). During the treatment phase, four sets of three replicate tanks contained either a control with no treatment or addition of Zymosan A with one of three tank concentrations: 0.1 mg L^{-1}, 1 mg L^{-1} and 10 mg L^{-1}. Simulated storage began by packing remaining animals after sampling into polyethylene "fish" bags (one bag per replicate treatment with n=17 animals) with 500 ml of seawater from individual replicate tanks, and enriched with oxygen. Bags were sealed and placed randomly into two Styrofoam shipping boxes, sealed with packing tape, and placed in a constant temperature room at 13 ± 1 °C for 48 hours (Ocaño-Higuera *et al.*, 2015) as recommended by our supplier based on their shipping policies. Temperature was monitored using a min/max. temperature tracker. After 48 hours within the boxes, animals were unpacked. Tanks were refilled with fresh seawater and remaining animals after sampling (n=14) were returned to their respective tanks. To measure host response to Zymosan A and storage, cell viability (CV), nitric oxide (NO), lysozyme activity levels (*methods described in Chapter 2: pgs 51 to 53*) and differential cell counts (DCC) (*methods described in Chapter 3: pgs 74 to 75*) were used as immune parameters.

Figure 1: Schematic of the experimental design of treatment, storage and monitoring periods.



(c) Data Analysis

A statistical model could not be applied to NO, CV, and lysozyme activity due to the presence of too many data points that were influential (values that exceed 3x the standard deviation), but also not large enough to be eliminated as outliers (values that exceed 5x the standard deviation). Thus, any significance found with such a model could not necessarily be attributed to a population or treatment.

For differential cell counts, all cell types were compared between treatments at the initial and final time point. If a difference was observed, difference among treatments of that cell type was tested for all sampling times. Normality plots were verified and one-way ANOVA was used to assess differences between treatments. Welch correction was used when there were differences in the variances.

Results

Urchin survival during the trial and food availability

No urchin mortalities occurred during the acclimation period or throughout any of the treatments.

Cell Viability (CV)

The CV measurements (Figure 2) indicated that over the 48 hour period (time between day 1 and 3) that the animals were stored, an overall decrease in CV levels was observed in all groups. After this initial decrease, an increase was observed in all groups. However, the levels began to increase again after day 3 within the control group and the 0.1 mg L^{-1} treatment group showing the largest increases up to 0.12 ± 0.02 OD_{540}. After 14 days, all treatments and the control had higher OD measurements than the initial measurement at day 1, with the 1 mg L^{-1} being the highest (0.13 ± 0.02 $OD_{540)}$. No statistical model was performed; for an explanation please refer the *Data Analysis (pg. 133)* and for boxplot representation of the data please refer to the *Appendix* (*pgs 188-191*).

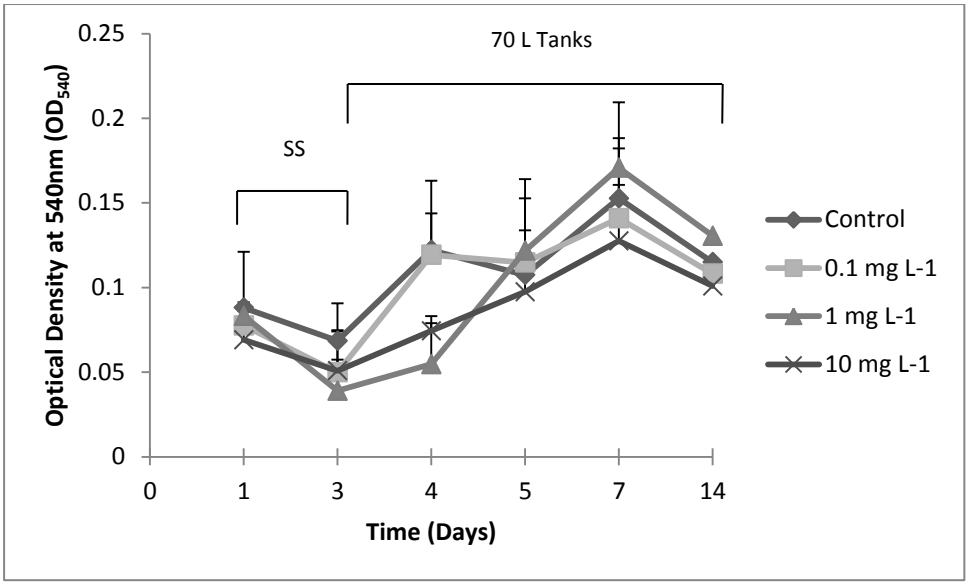


Figure 2: Mean cell viability levels ± SE (OD_{540}) for stored *P. lividus* individuals (30 ± 5mm) treated with different concentrations of Zymosan A (0.1 mg L^{-1}, 1 mg L^{-1}, and 10 mg L^{-1}) compared with a control group over 14 days**.** First measurement was taken 24 hours (1 day) after Zymosan A was initially added. SS = Simulated Storage.

Differential Cell Counts (DCC)

Four different coelomocyte types (red/colorless amebocytes, vibratile cells, petaloid phagocytes, and philopodial phagocytes) were observed (*Please refer to Ch. 1: pgs 22-24 for a description of cell types*). Philopodial phagocytes were the main cell type observed in all groups (Figure 3). In the control group, 78.61 % of the immune cells were philopodial phagocytes, 29 % were petaloid phagocytes, 0.39 % were amebocytes, and there were no vibratile cells. The 10 mg L^{-1} group had a higher relative percentage of amebocytes (1.22 %) when compared to the control group (0.39 %; $p \leq 0.2$; df = 26) and the 0.1 mg L^{-1}: ($p \leq 0.03$ df = 26) and 1 mg L^{-1} groups: ($p \leq 0.41$; df = 26). There was no significant difference observed between the different cell types over the 14-day experimental period (Figure 4): philopodial

phagocytes ($p \leq 0.8$; df = 5), petaloid phagocytes ($p \leq 0.78$; df = 5), amebocytes ($p \leq 0.07$; df = 5), and vibratile cells ($p \leq 0.99$; df = 5).

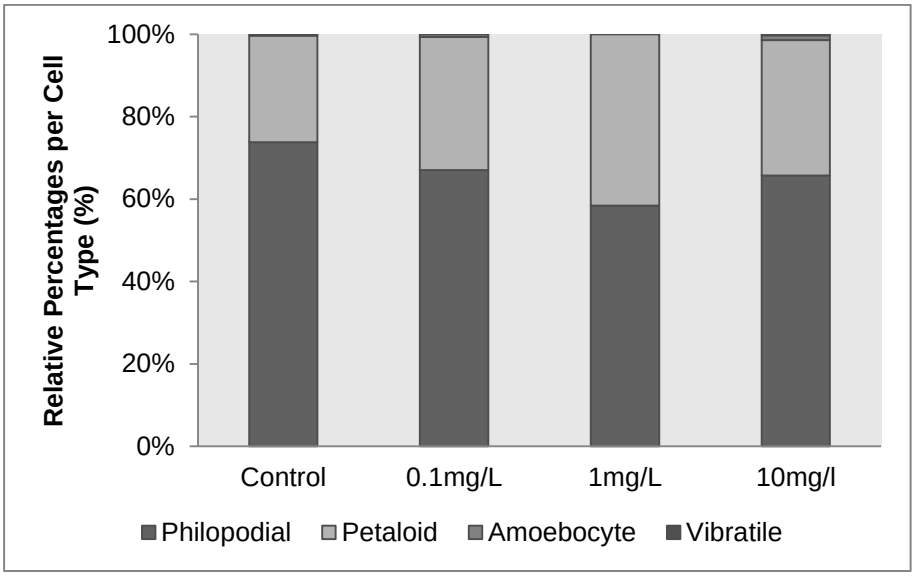


Figure 3: Relative abundance (%) of the four different coelomocyte type found in each group of stored sea urchin individuals: control and Zymosan A concentrations of 0.1 mg L^{-1}, 1 mg L^{-1}, and 10 mg L^{-1}.

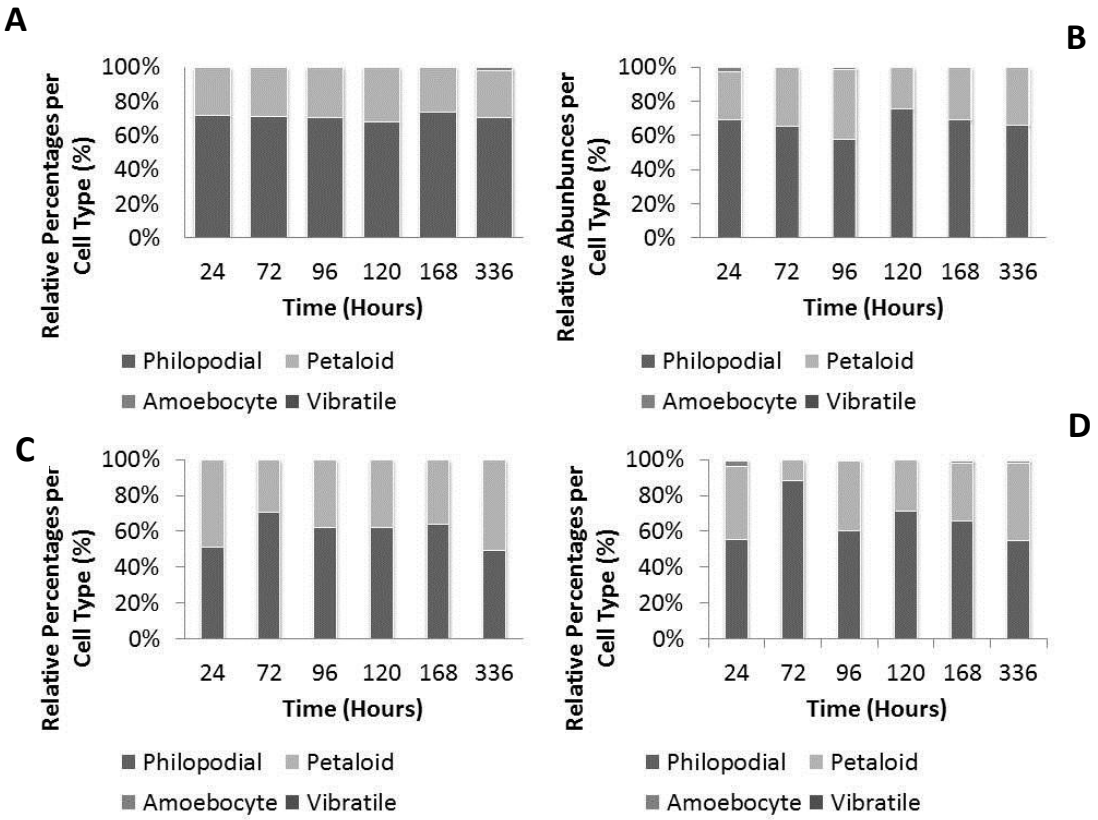


Figure 4: Abundance percentages (%) of the four different coelomocytes found in each group of sea urchin individuals: control (A) and Zymosan A concentrations of 0.1 mg L^{-1} (B), 1 mg L^{-1} (C), and 10 mg L^{-1} (D).

Nitric Oxide (NO), The Griess Reaction

After storage, all animals treated with Zymosan A had lower NO levels than controls from day 1-3 (Figure 5). Upon returning to the tanks, NO levels fell back down and remained stable. All animals showed elevated NO levels during the simulated storage; however, Zymosan A treatment minimized the NO production particularly in the 1 mg L^{-1} treatment until day 4 ($p \leq 0.13$; df = 3). No statistical model was performed; for an explanation please refer the *Data Analysis (pg. 133)* and for boxplot representation of the data please refer to the *Appendix* (*pgs 188-191*).

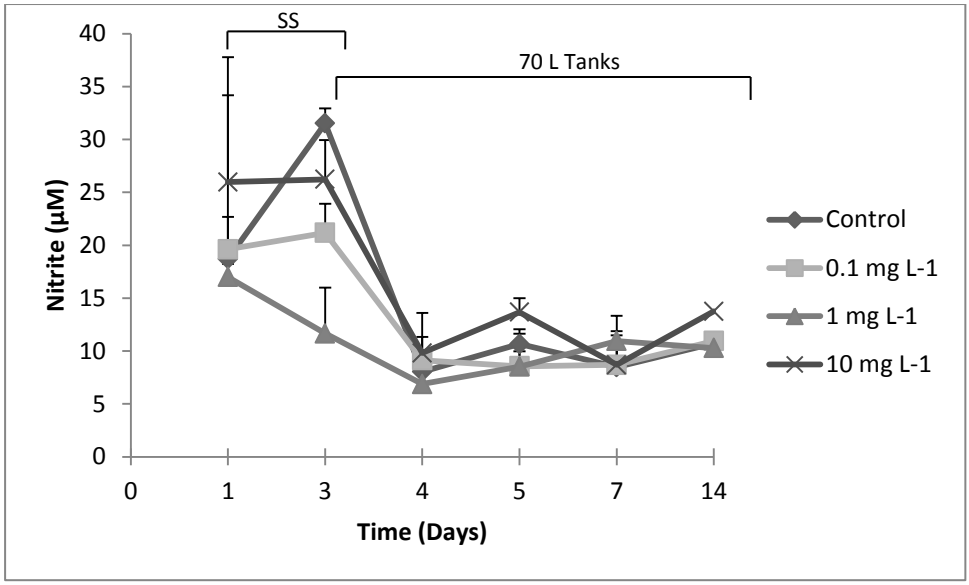


Figure 5: Mean nitric oxide levels ± SE (µM) for stored *P. lividus* individuals (20 mm ± 5 mm) treated with different concentrations of Zymosan A (0.1 mg L^{-1}, 1 mg L^{-1}, and 10 mg L^{-1}) compared with a control group over 14 days. First measurement was taken 24 hours (1 day) after Zymosan A was initially added. SS = Simulated Storage.

Lysozyme Activity

For the control urchins and the urchins administered low levels of Zymosan A, there were increased lysozyme levels during the storage period (Figure 6) and culminated at day 4, followed by a decrease. The 1 mg L^{-1} concentration was relatively stable throughout the experiment (2.49 ± 2.96 µg ml^{-1}), while the 10 mg L^{-1} concentration showed a similar response to the 1 mg L^{-1} concentration, except for a peak at day 5 (7.13 ± 2.99 µg ml^{-1}). No statistical model was performed; for an explanation please refer the *Data Analysis (pg. 133)* and for boxplot representation of the data please refer to the *Appendix* (*pgs 188-191*).

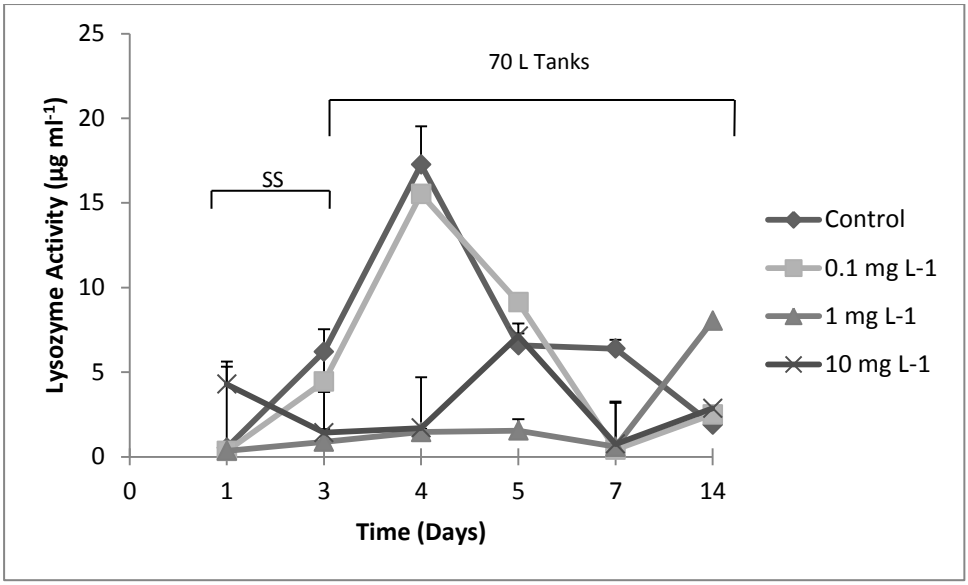


Figure 6: Mean lysozyme activity levels ± SE (µg ml^{-1}) for stored *P. lividus* individuals (30 ± 5 mm) treated with different concentrations of Zymosan A (0.1 mg L^{-1}, 1 mg L^{-1}, and 10 mg L^{-1} compared with a control group over 14 days. First measurement was taken 24 hours (1 day) after Zymosan A was initially added. SS = Simulated Storage.

Discussion

The results of this study indicated that treatment with Zymosan A, prior to storage, reduced the measured levels of some immune parameters relative to the control which may reduce the amount of stress the animals experience. Furthermore, the results indicate that handling and storage elicited an immune response in *P. lividus* as the OD measurements increased in all treatments and the control over the experimental period. If there is an increase in CV levels observed, the immune system is in a successful defense mode.

The highest dose of Zymosan A showed elevated levels of amebocytes. Amebocytes are primarily responsible for the first mobilization response involving encapsulation and clotting (Matranga, 2005). Zymosan A particles act as "harmless" foreign agents that put the immune system on alert for a possible threat. Also, if the threat is greater (i.e. a higher amount of particles present in a higher dose), more red amebocytes (which contain the antimicrobial agent, echinochrome) will be generated (Smith, 1981; Matranga, 2005), which could explain our results.

In addition, the two higher doses of Zymosan A reduced the amount of lysozyme produced. Demers and Bayne (2010) reported that, following exposure to a handling stressor, lysozyme activity was significantly increased in rainbow trout. This was also observed in this study in both the control and lowest dose of Zymosan A treatment. Further suggesting that the higher concentrations of Zymosan A actually suppressed lysozyme levels during storage. Lysozyme activity is dependent on the degree of stress, intensity and its duration and type of stressors (Yildiz, 2006). Therefore, changes in lysozyme activity are considerably influenced by the potency and the type of immunostimulants (Kumari & Sahoo, 2006).

Lastly, all animals showed elevated NO levels during storage; however, treatment with Zymosan A minimized NO production. Although very little work has been conducted in relation to nitric oxide and transport stress, Storey (1996) determined that there is a close relationship between environmental stress and the rate of cellular reactive oxygen and nitrogen species (ROS/RNS) generation in the organism. ROS/RNS are a particularly transient species due to their high chemical reactivity and can react with DNA, proteins, carbohydrates and lipids in a destructive manner (Storey, 1996). Therefore, if NO levels are too high, it could potentially be fatal to the organism; therefore, finding an immunostimulant with the ability to reduce NO production is highly beneficial.

It is well established that the immune system of fish, and other marine species, can be severely affected by various stress conditions, including transport and handling (Saurabh & Sahoo, 2008) and are eventually immunosuppressive. In conclusion, simulated storage elicited a response in the CV (cellular), NO (humoral), and lysozyme activity (humoral) immune parameters observed by increased levels. However, the Zymosan A treatments minimized the production of NO and lysozyme parameters, resulting in a reduction of the inflammatory response to storage stress. The 1 mg L^{-1} showed the most effect when compared to the other concentrations and control. This is a highly positive outcome as it may lead to a reduction in the amount of stress experienced by *P. lividus* throughout storage duration.

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Chapter 6: The effects of *Vibrio anguillarum* on the edible sea urchin, *Paracentrotus lividus*' immune function

Abstract: Vibriosis, a prevalent disease especially in marine invertebrates and fish, is caused by bacteria belonging to the genus *Vibrio.* Bacterial diseases, especially caused by *Vibrio* spp., in sea urchins have been documented worldwide and are known to cause dramatic mass mortalities both in the wild and in aqua-farms. *V. alginolyticus,* has previously been recorded to cause vibriosis in the edible sea urchin, *Paracentrotus lividus*. However, another species of *Vibrio*, *V. anguillarum,* has been shown to be pathogenic towards the edible sea urchin from previous unpublished studies. *V. anguillarum* is a gram-negative, curved-rod bacterium with one polar flagellum. The objective of this study was to determine the impact of a pathogenic strain of *V. anguillarum* on *P. lividus*' immune function using several immune parameters. The results indicated that the DCC baseline measurement was composed of mainly vibratile cells and amebocytes; however, after 10 days, the PBS control was composed of most petaloid phagocytes and amebocytes. Additionally, the 10^9 CFU dose contained mostly amebocytes as did the other doses but decreased with decreasing CFU. NO measurements indicate a general decrease for all doses and the control from the baseline, so injection of PBS and the *V. anguillarum* suppressed the immune system. Lysozyme activity increased rapidly with the presence of *V. anguillarum.* In conclusion, while *V. anguillarum* did not induce mortalities during the expected time scale for the trial, it did affect the host's immune function.

Introduction

Vibriosis, a prevalent disease in marine invertebrates and fish, is caused by a bacteria belonging to the genus *Vibrio* (Larsen, 1983). Different *Vibrio* spp. have been associated with vibriosis outbreaks in fish and marine invertebrates (molluscs, crustacean, and echinoderms) (Sindermann, 1990; Liu *et al.,* 1996; Lee *et al.,* 2001; Morris & Acheson, 2003). A major importance to the fish culture industry is *Vibrio anguillarum*. *V. anguillarum* is a gram negative curved and rod shaped bacterium with a single polar flagellum (Larson, 1983).

Bacterial diseases, especially caused by *Vibrio* spp., in sea urchins have been documented worldwide and are known to cause dramatic mass mortalities both in the wild and in culture settings (Becker *et al.,* 2008). Bacterial infections in sea urchins are characterized by body wall lesions, where spines, podia, pedicellariae and epidermis are lost (Becker *et al.,* 2008), and is referred to as the "bald sea urchin disease". First described by Johnson (1970) in *Strongylocentrotus franciscanus,* bald sea urchin disease was reported in several species of echinoids in the Mediterranean Sea (Höbaus *et al.,* 1981), along the French coasts of the Atlantic Ocean and the English Channel (Maes & Jangoux, 1984), and in *Strongylocentrotus intermedius* in Japan (Tajima *et al.,* 1997). Furthermore, Gilles and Pearse (1986) isolated 14 different strains of bacteria, including species of *Flavobacterium* and *Pseudomonas,* from epidermal lesions in the sea urchin, *S. purpuratus,* from Monterey Bay, California, USA but found that only *V. anguillarum* and *Aeromonas salmonicida* could produce lesions experimentally.

Previous unpublished studies (Ryan, *unpublished*) conducted on *P. lividus* found that *V. anguillarum* was virulent towards this sea urchin species. These studies were conducted on a visual small-scale observation system; therefore, the objective of this

study was to determine the impact of a pathogenic strain of *V. anguillarum* on *P. lividus* using several immune parameters, including cell viability (CV), differential cell counts (DCC), nitric oxide (NO), and lysozyme activity. CV can be used as a proxy for health status of an organism by determining if the immune system is responding effectively to a stressor or not (Alvarez & Friedl, 1992). DCC measures cellular activity based on cell type. Changes in concentrations of cell types can give insight into immune response (Jussila *et al.,* 2013). NO fights off pathogens, causes inflammation, and increase energy levels (Shinde *et al.,* 2000). Lastly, lysozymes, antimicrobial agents, are released by phagocytes upon exposure to a foreign agent (Hoffman, 2003).

The objective of this study was to determine the impact of a pathogenic strain of *V. anguillarum* on *P. lividus*' immune function using several immune parameters.

Materials and Methods

Trial 1: Preparation of V. anguillarum and inoculation test

Sea urchins (n = 300; 30 ± 5 mm) were sourced from Daithi O'Murchu Marine Research (DOMMR) Station in Bantry, Co. Cork, Ireland in May, 2015.

(a) Preparation of Vibrio anguillarum

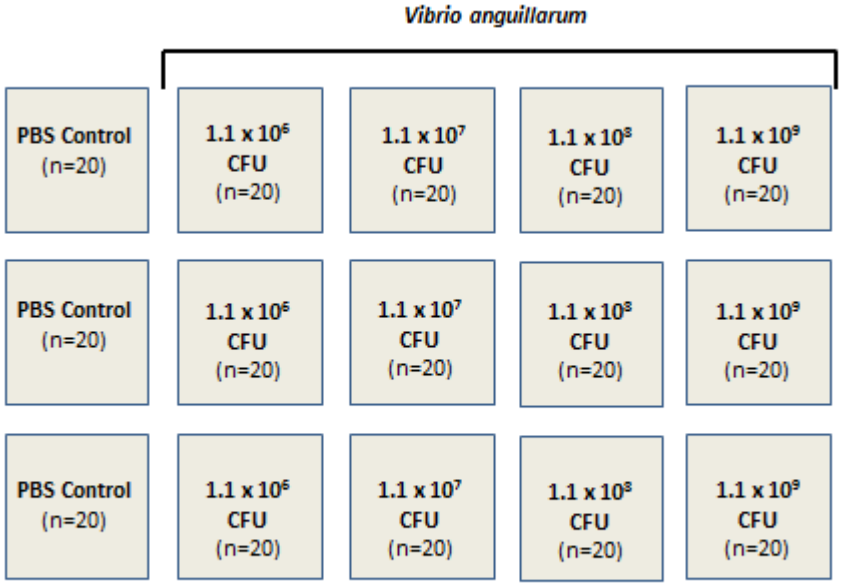
The *V. anguillarum* (CM31) was provided by Dr. Merrifield's group at Plymouth University, UK. The provided pathogen was sub-cultured in a 10 ml tris-buffered saline (TBS) 2% NaCl broth and allowed to grow for a 48-hour period at 25°C, as described by Larsen (1984). Next, 2 ml of the sub-culture was transferred to a 100 ml TBS broth and left to grow for a 48-hour period at 25°C. On the day of inoculation, 20 ml of the 100 ml broth was centrifuged at 3,000 rpm for 10 minutes. The *Vibrio* settled at the bottom and TBS was removed. 20 ml of PBS was added to the bacterial mass and agitated to re-suspend the bacteria. 0.1 ml of the serial dilutions (10^9, 10^8, 10^7, 10^6 CFU) of *V. anguillarum* and the PBS solution were injected into the coelomic cavity through the peristomal membrane.

(b) Experimental design

Laboratory trials were carried out in fifteen 70 L aerated tanks stocked with 20 sea urchins each. All tanks contained fresh seawater sourced from West Cork, Ireland and were held in a constant temperature room at 15 ± 1 °C. Animals were acclimated for two weeks. Sea urchins were not fed 14 days prior to the start of the experiment in order to minimize the quantity of residual food in the gut as according to several authors (Lawrence *et al.*, 1989; Siikavuopio *et al.*, 2007; 2008); the gut clearance takes up to 14 days in sea urchins. Animals in sets of three replicate tanks received

one of five doses: a control injected with PBS and injection with four concentrations of *V. anguillarum* PBS stock solution: 1.1 x 10^9, 1.1 x 10^8, 1.1 x 10^7, and 1.1 x 10^6 CFU (schematic of tank setup in Figure 1). At the following time points, three animals per replicate tank (n=45) were sampled and sacrificed: 0 and 10 days. Animals sampled at time zero were taken before any treatments. Animals were visually assessed daily for mortality. To measure the host response to *V. anguillarum*, cell viability (CV), nitric oxide (NO), lysozyme activity levels (*methods described in Chapter 2: pgs 51 – 53*) and differential cell counts (DCC) (*methods described in Chapter 3: pgs 74 – 75*) were used as immune parameters.

Figure 1: Schematic of the *Trial 1* experimental design consisting of fifteen 10 L aerated tanks represented. Sets of three replicate tanks contained one of five treatments: a control injected with PBS and injection with four concentrations of *V. anguillarum* PBS stock solution: 1.1 x 10^9, 1.1 x 10^8, 1.1 x 10^7, 1.1 x 10^6 CFU.



(c) Data Analysis

All statistics were compiled using SPSS software (IBM) version 23. For NO and lysozyme assays, data presented normal distribution (normality test Shapiro-Wilk) ($p>0.05$) with extreme outliers that exceeded 5 times the deviation, which were removed. A one-way ANOVA was used with Tukey as a post hoc test for each of the doses. Homogeneity of variances was tested and Welch correction or Dunnett T3 was used when homogeneity was not observed. For the CV assay, no statistical analysis was performed due to the negative values observed.

Trial 2: Different preparation method for *V. anguillarum* and inoculation test

In order to verify the results from *Trial 1*, a second microbiology laboratory at Teagasc Food Research Centre in Cork, Ireland prepared another batch of the *V. anguillarum* strain used above (prepared by the UCC Microbiology department).

Sea urchins (n = 10; 30 ± 5 mm) were sourced from Daithi O'Murchu Marine Research (DOMMR) Station in Bantry, Co. Cork, Ireland in May, 2015.

(a) Preparation of Vibrio anguillarum

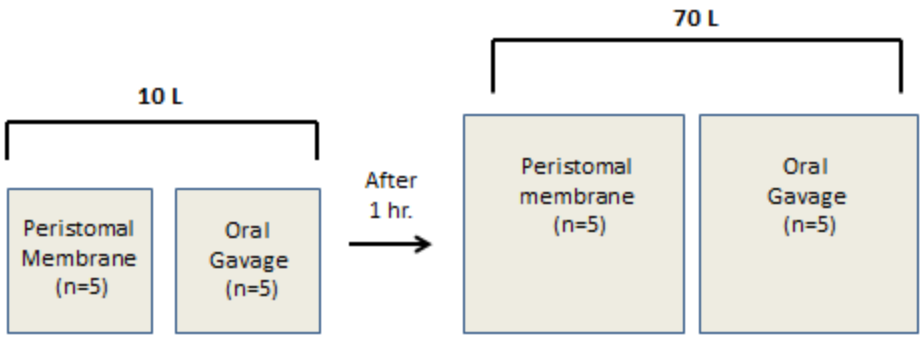
The *V. anguillarum* (CM31) was provided by Dr Merrifield's group at Plymouth University, UK. The provided pathogen was sub-cultured in a 10 ml tris-buffered saline (TBS) 2% NaCl broth and allowed to grow for a 48-hour period at 25°C plated and stored (Ryan, *personal communication*). Then following Harper *et al.*, 2011, a single colony was streaked onto blood agar (with adjusted salinity) to "induce high

virulence" and incubated at room temperature for 72 hours. Next, a single colony from one of the blood plates was taken and both 5 ml brain heart infusion (BHI) media and lysogeny broth (LB) (with adjusted salinity) were inoculated. Both cultures were allowed to grow under the same conditions. Growth occurred best in the BHI broth; therefore, it was sub-cultured in 20 ml of BHI. Once the stationary phase was reached, the culture was spun down and washed twice with PBS. The cells were then re-suspended to an $OD_{600,}$ equivalent to ~10^9 CFU for a 0.1 ml dose.

(b) Experimental design

All tanks contained fresh seawater sourced from West Cork, Ireland and were held in a constant temperature room at 15 ± 1 °C. Animals were acclimated for two week in one approximately 500 L aerated tank where they were fed *ab libitum* with *Laminaria* spp. Laboratory trials were carried out in a set of tanks stocked with five sea urchins each: two 10 L aerated tanks from time zero to one hour for closer monitoring and in two 70 L aerated tanks from 1 to 48 hours. Animals of the two sets of tanks received 1.1 x 10^9 CFU *V. anguillarum* PBS stock solution injection at time zero either via oral gavage (injection directly into the mouth via syringe as in Chapter 4, *Trial 1* and *2*) or injection into the peristomal membrane (as in Chapter 6, *Trial 1*) (schematic of tank setup in Figure 2). Animals were visually assessed for health (i.e. drooped spines and absence of pseudopodial activity) and mortality. Visual assessment and photo documentation occurred every 10 minutes from time zero to 1 hour, every 30 minutes from 1 to 2 hours, every hour from 2 to 5 hours and every 12 hours from 5 to 48 hours.

Figure 2: Schematic of the *Trial 2* experimental design consisting of two aerated tanks. Animals in the two sets of tanks received 1.1 x 10 ^{9} CFU *V. anguillarum* PBS stock solution was injected at time zero either via oral gavage (injection directly into the mouth via syringe) or injected into the peristomal membrane.



Results

Trial 1: The impact of V. anguillarum on exposed P. lividus individuals

No urchin mortalities occurred during the acclimation period or throughout any of the treatments. No urchin showed signs of abnormal appearance.

Differential Cell Counts (DCC)

The baseline cell counts had 16.6% philopodial phagocytes, 38% were petaloid phagocytes, 39.2% were amebocytes, and 6.2% were vibratile cells (Figure 4). In the PBS control group, there was an increase to 50% in the philopodial phagocytes and a decrease to 2.67% in the petaloid phagocytes. Upon inoculation with the 10^9 CFU *V. anguillarum* dose, there was a large decrease in both philopodial phagocytes (0.33%) and petaloid phagocytes (4.67%) and a large increase in amebocytes (95%) when compared with the baseline and PBS control. The percentage of amebocytes decreased with decreasing dose 10^8 CFU (64.33%), 10^7 CFU (76%), and 10^6 CFU (57.33%).

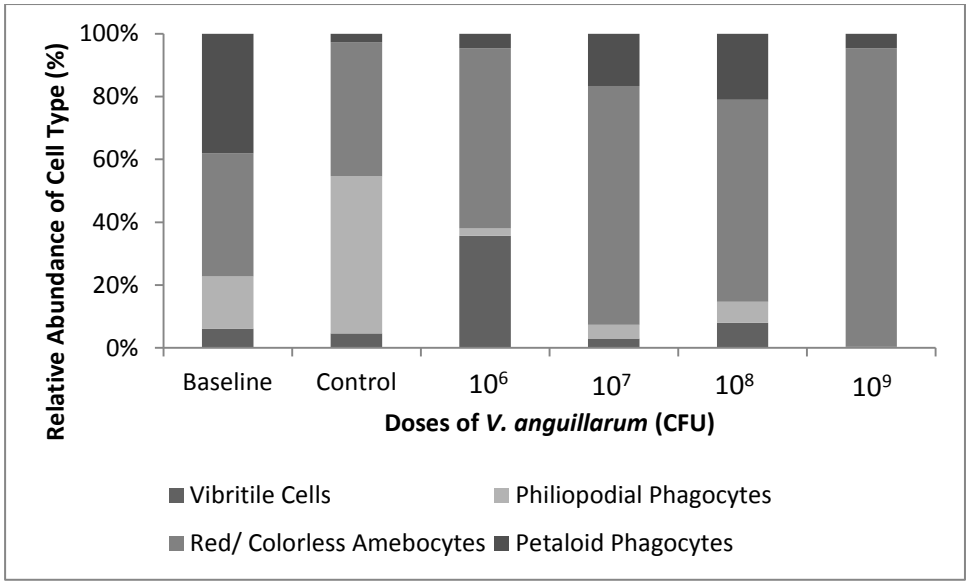


Figure 4: Relative abundance (%) of the four different coelomocytes found in each group of inoculated *P. lividus* individuals (30 ± 5 mm) with different concentrations of *V. anguillarum* (CFU) after 10 days.

Nitric Oxide (NO), The Griess Reaction

There was a baseline (time zero) measurement of 12.26 ± 8.03 µM (Figure 5). Ten days after inoculation with the *V. anguillarum*, NO levels were the lowest in the PBS group (7.34 ± 2.05 µM) and the highest in the 10^8 CFU dose (11.97 ± 4.21 µM). There were significant differences observed between the PBS control and the 10^9 CFU ($p=0.04$) dose and also the 10^9 CFU and the 10^6 CFU dose ($p=0.03$).

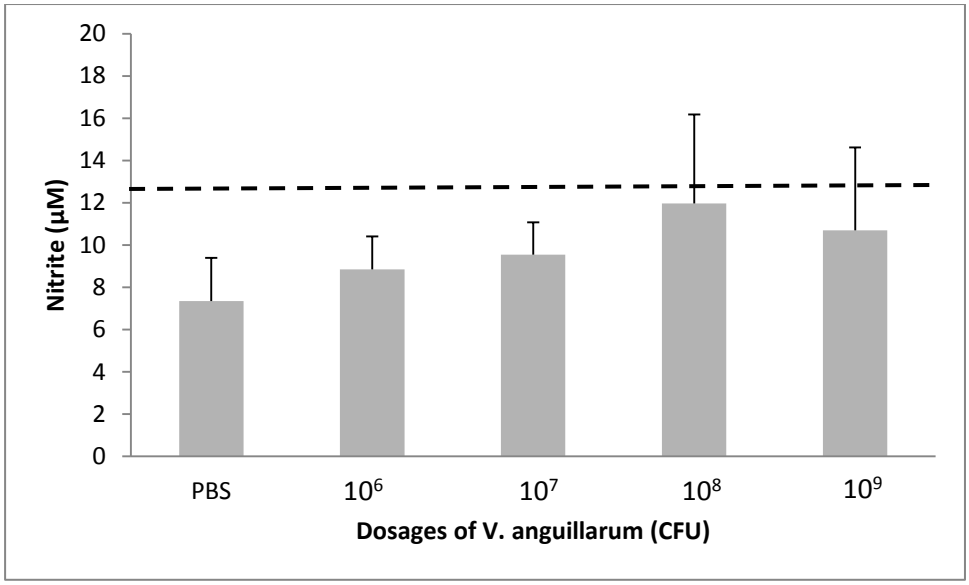


Figure 5: Mean nitric oxide levels ± SE (µM) for inoculated *P. lividus* individuals (30 ± 5mm) with different concentrations of *V. anguillarum* (CFU) after 10 days. Black dotted line represents a baseline measurement taken before inoculation.

Lysozyme Activity Assay

Lysozyme activity levels (Figure 6) indicated that there was a baseline measurement of 0.11 ± 0.13 µg ml^{-1}. After day 10, the lysozyme activity was the lowest in the PBS group (0.23 ± 0.05 µg ml^{-1}) and the highest in the 10^6 CFU ml^{-1} dose (1.33 ± 1.06 µg ml^{-1}). There were significant differences observed between the PBS control and the 10^7 CFU ($p=0.037$) and 10^6 CFU doses ($p=0.033$).

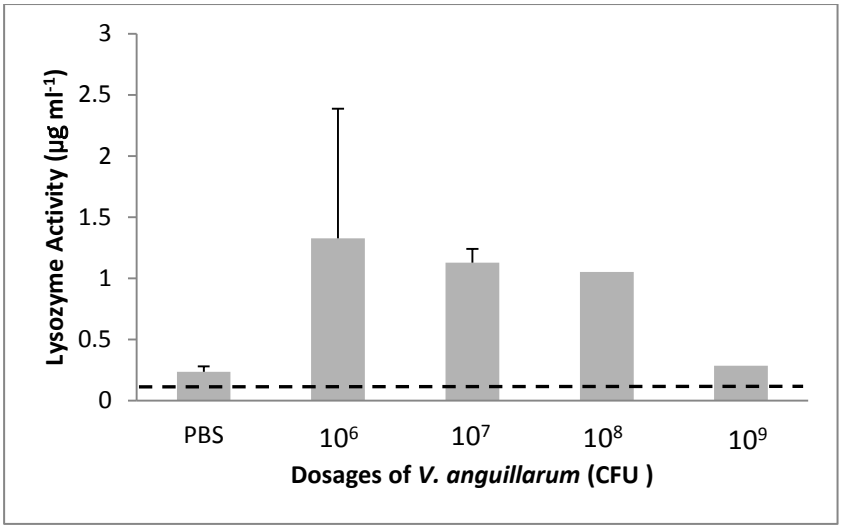


Figure 6: Mean lysozyme activity levels ± SE (µg ml^{-1}) for inoculated *P. lividus* individuals (30 ± 5 mm) with different concentrations of *V. anguillarum* (CFU) after 10 days. Black dotted line represents a baseline measurement taken before inoculation.

Discussion

Upon testing *V. anguillarum*, we found that our strain does have an impact on the immune system of *P. lividus*, but does not kill them as previously found by colleagues (Ryan, *personal communication*). The urchins, inoculated with the highest dose of 10^9 CFU, remained externally healthy (e.g. erect spines, podia movement, bright color) throughout both *Trial 1* and *2*. In *Trial 1*, the CV measurements had negative values; therefore, there may have been so many bacteria within the coelomic fluid sample that it masked the lysis from the cells. Due to these results, CV data were not reported, as negative CV values are not possible.

The DCC results indicated that the baseline measurement was composed of mainly vibratile cells and amebocytes; however, after 10 days, the PBS control was composed of most petaloid phagocytes and amebocytes. Additionally, the 10^9 CFU dose contained mostly amebocytes as did the other doses but decreased with decreasing CFU. Amebocytes are responsible for clotting and encapsulation (Matranga, 2005); therefore, the drastic increase in amebocytes with CFU indicates a response to a possible infection or a general response to the injection itself. Jussila *et al.* (1997) found that using differential hemocyte counts in the western rock lobsters (*Panulirus cygnus*), was a useful way of assessing stress or health status in lobsters, and possibly other invertebrates.

NO measurements indicated a general decrease for all doses and the control from the baseline, so injection of PBS and the *V. anguillarum* suppressed the immune system. There were slightly higher NO levels observed in the two higher *V. anguillarum* doses, but not significantly different. However, due to the increasing trend observed as doses of *V. anguillarum* increased, there is an indication that *V. anguillarum* did have an impact on NO production. NO plays a part in several biological roles

relating to feeding, defense, environmental stress, learning, metamorphosis, swimming, symbiosis, haemocyte aggregation, and regulation of blood pressure (Palumbo, 2005).

Lysozyme activity increased rapidly with the presence of *V. anguillarum.* However, the concentrations over 10^6 CFU seemed to overwhelm the immune system and not as much lysozyme was produced. Lysozyme activity is dependent on the degree of stress, intensity and its duration and type of stressors (Yildiz, 2006). Roed *et al.* (1993) observed a negative phenotypic correlation between serum lysozyme activity of Atlantic salmon and survival in a challenge test with vibriosis. Additionally, previous studies show the ability of haemolymph to inhibit bacterial growth through antibacterial activity (Noga *et al.,* 1994; Ueda *et al.*, 1994; Tsvetnenko et al., 2001); therefore, suggesting that low antibacterial activity is associated with lowered immunity. Perhaps the higher levels of lysozyme production in the 10^6 CFU inoculation, when compared to the production observed in the higher dosages, was showing a greater available effort from the immune response as it was not as overwhelmed.

In conclusion, *V. anguillarum* did elicit an immune response within the parameters measured but did not cause mortality as previously reported. Furthermore, due to the short experimental periods, it is possible that over time these doses would have led to vibriosis and death in the animals. In addition, it would be beneficial to re-isolate the strain from the coelomic fluid in order to confirm its presence and infection status using PCR methods. Furthermore, it is suggested to look at the effect of an immunostimulant (Chapter 4 and 5) on the different doses of *V. anguillarum*, since there was a variation observed in the immune response to *V. anguillarum.*

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Chapter 7: Seasonal (and site) observations of immune parameters in the edible sea urchin, *Paracentrotus lividus*

Abstract: Sea urchin aquaculture is limited by disease and handling associated mortalities; therefore, it is important to understand the sea urchin immune system and their ability to resist the impact of stressors and pathogens. As the urchin circulatory system is semi-open to the environment, changes in the environment can also strongly affect the organism. It is known that marine organisms can vary widely in the production of chemical defenses associated with physical factors, biological factors, and season. Seasonal observations of the physiological processes are essential in order to understand the natural variation that occurs within the individual organisms and populations. In this study, baseline (control) immune data taken from previous studies over two and a half years was collated to observe any differences over season or site in the sea urchin, *Paracentrotus lividus.* The compiled data were used to observe the impact of annual patterns on several immune parameters (e.g. cell viability, nitric oxide, and lysozyme activity) within this sea urchin species. In conclusion, different physiological and environmental variables need to be taken into account when conducting seasonality studies; however, the differences observed in this study were most likely due to collection site and culture techniques.

Introduction

Marine organisms can vary widely in the production of chemical defenses associated with physical factors (e.g. temperature, light, salinity, pH), biological factors (e.g. grazing pressure, reproduction), and season (Cajaraville *et al.,* 1996; Hégaret *et al.,* 2003a, b; Hellio *et al.,* 2004; Gagnaire *et al.,* 2006).

A study conducted by Beninger & Stephan (1985) observed seasonal variation regarding reproduction in two types of clam, *Tapes decussatus* and *T. philippinarum.* Furthermore, Wootton *et al.* (2003) used season variation and immune cell counts (and differentiation) in *M. edulis, Cerastoderma edule,* and *Ensis siliqua* to determine the effects of an oil spill. In an overview of innate immunity in fish, Magnadóttir (2006) presented the idea that innate immune parameters, in particular the phagocytosis, lysozyme and hemolytic activity, have been used as indicators of the effects of factors such as seasonality (Zapata *et al.,* 1992; Audet *et al.,* 1993; Collazos *et al.,* 1995), temperature (Alcorn *et al.,* 2002; Nikoskelainen *et al.,* 2004), pollution (Siwicki *et al.,* 1998; Köllner *et al.,* 2002), handling (Davis *et al.,* 2002), diets (Duncan & Klesius, 1996; Jeney *et al.,* 1997; Ortuno *et al.,* 2001), immunostimulants and probiotics (Vadstein, 1997; Sakai, 1999; Gatesoupe, 1999) as well as the effects of diseases (Balfry *et al.,* 2001) on the immune system. Using organisms as bio-indicators without a full understanding of natural variables in physiological parameters (e.g. seasonal variation) would prove extremely difficult.

While some previous research has looked at immune parameters in sea urchins (Coteur *et al.,* 2001; de Faria & da Silva, 2008; Branco *et al.,* 2013), other echinoderms (Wang *et al,* 2008; Dupont & Thorndyke, 2012), and a range of marine invertebrates (Coteur *et al.,* 2004; Mydlarz *et al.,* 2006), other studies have focused on how immune parameters change over time (seasonality; Magnadóttir *et al.,* 1999;

Kumari *et al.,* 2006; Duchemin *et al.,* 2007). Further still, others looked at seasonality in other marine invertebrate species (Clarke, 1988; Olive, 1992; Bowden *et al.,* 2009). To the best of our knowledge, only two previous studies have showed seasonality of immune parameters in sea urchins (de Faria & da Silva, 2008; Malanga *et al.,* 2009). de Faria and da Silva (2008) observed the innate immune response in the sea urchin, *Echinometra lucunter*, specifically at the coelomocytes, under varying temperatures. Malanga (2009), on the other hand, looked at the seasonal effect on oxidative metabolism in the sea urchin *Loxechnus albus.* In contrast, the objective of this study was to observe the impact of annual patterns on several immune parameters (e.g. cell viability (CV), nitric oxide (NO), and lysozyme activity) in the edible sea urchin, *Paracentrotus lividus,* by analyzing a composite of baseline (control) data taken from previous studies (2.5 year period) conducted by this laboratory.

Materials and Methods

Baseline (control) data from *P. lividus* individuals were taken from past studies conducted in this laboratory since 2013 (Table 1). Sea urchins were sourced from either Daithi O'Murchu Marine Research Station (DOMMRS) in Bantry, Co. Cork, Ireland or Dunmanus Seafood (DS), Dunmanus Bay, Ireland (both located in southwest Ireland) (Figure 1). DS sourced sea urchins were held in hatchery conditions, while DOMMRS sea urchins were harvested from the wild. Please refer to *Chapter 2, pgs 51-53* for the methods description of cell viability (CV), nitric oxide production (NO), and the lysozyme activity assays, which were all analyzed and compared in this chapter. Due to the varying elements in the different experimental designs, statistical analysis would not have been appropriate.

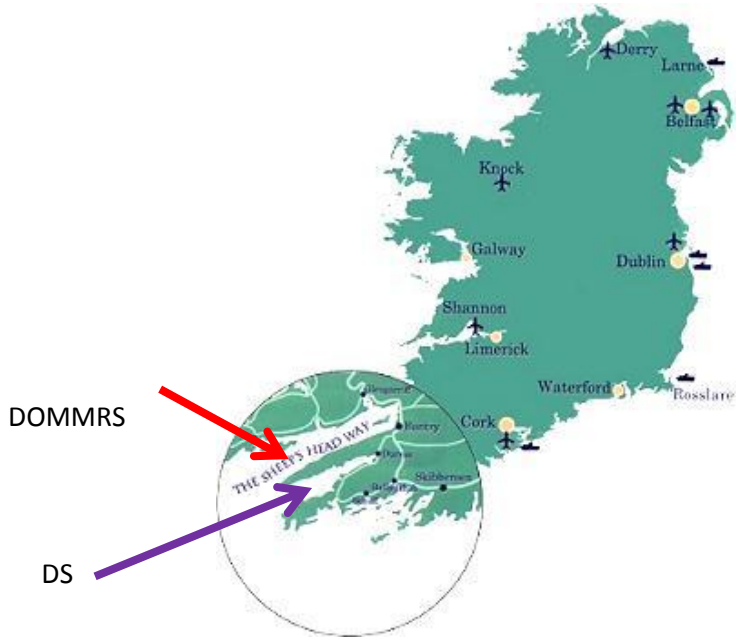


Figure 1: Map of Ireland. The red arrow indicates the location of the Daithi O'Murchu Marine Research Station (DOMMRS). The purple arrow indicates Dunmanus Seafood Ltd. (DS) (Map of Ireland, 2015).

Study	Month – yr.	Size	Acclimation period	Fed (Y/N)?	Sourcing Sites
1	Mar-13	50±5mm	48 hr	Y	DS
2	Apr-13	40±5mm/50±5mm	2 weeks	Y	DS
3	Jul-13	30±5mm	1 week	Y	DS
4	Aug-13	20±5mm/40±5mm	2 weeks	Y	DS
5	Sept-13	30±5mm	48 hr	Y	DS
6	Oct-13	25±5mm	48 hr	Y	DS
7	May-14	30±5mm	2 weeks	N	DOMMRS
8	Oct-14	30±5mm	2 weeks	N	DOMMRS
9	Nov-14	25±5mm	1 weeks	N	DOMMRS
10	Dec-14	30±5mm	2 weeks	N	DOMMRS
11	Mar-15	25±5mm	48 hr	N	DOMMRS
12	May-15	30±5mm	2 weeks	N	DOMMRS

Table 1: Baseline (control) data from *P. lividus* individuals were taken from past studies conducted in this laboratory since 2013. Sea urchins were sourced from either Daithi O'Murchu Marine Research Station (DOMMRS) or Dunmanus Seafoods Ltd. (DS), both located in southwest Ireland.

Results

Cell Viability (CV)

The mean (±SD) of three different sea urchin individuals were taken per sampling point over a two year period (n=24) in order to observe seasonal variation of measured cell viability levels (Figure 2). In 2013, the lowest levels observed in March (0.47 ± 0.21 OD_{540}) with the highest levels observed between April (0.93 ± 0.48 OD_{540}) and July (0.97 ± 0.58 OD_{540}). This was followed by a decrease in August (0.36 ± 0.22 OD_{540}). In 2014, May had levels of 0.31 ± 0.05 OD_{540} followed by a decrease in October (0.1 ± 0.09 OD_{540}) and December (0.09 ± 0.01 OD_{540}). In 2015, the only data collected was for May (0.00 ± 0.03 OD_{540}).

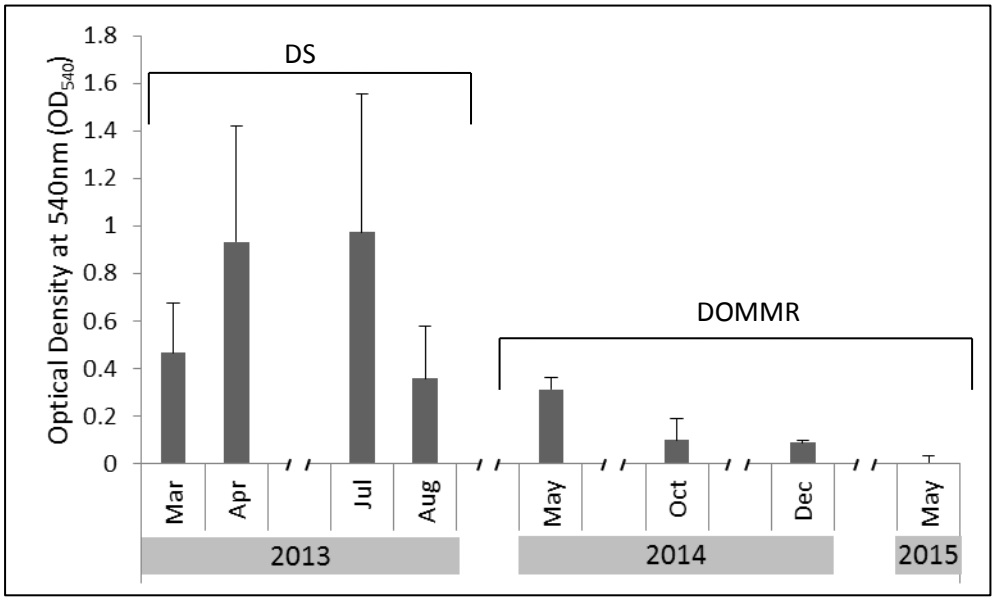


Figure 2: Mean cell viability levels (OD_{540}) ± SD of baseline (control) data from *P. lividus* individuals taken from past studies conducted in our lab since 2013. The // symbolizes missing data. Sea urchins were sourced from either Dunmanus Seafood Ltd (DS) or Daithi O'Murchu Marine Research Station (DOMMRS), both located in southwest Ireland.

Nitric Oxide (NO), the Griess Reaction

The mean (±SD) of three different sea urchin individuals were taken per sampling point over a two year period (n=24) in order to observe seasonal variation of measured nitric oxide production (Figure 3). In 2013, the lowest levels were observed in March (12.95 ± 5.48 µM) with the highest levels observed throughout the summer: April (77.69 ± 11.48 µM), July (79.72 ± 9.2 µM), and August (80.04 ± 18.57 µM). In 2014, the lowest levels were observed in May (5.17 ± 2.62 µM) followed by a peak in October (28.51 ± 9.86 µM) with a dip in December (13.64 ± 0.58 µM). In 2015, the only data collected was for May (12.26 ± 8.03 µM).

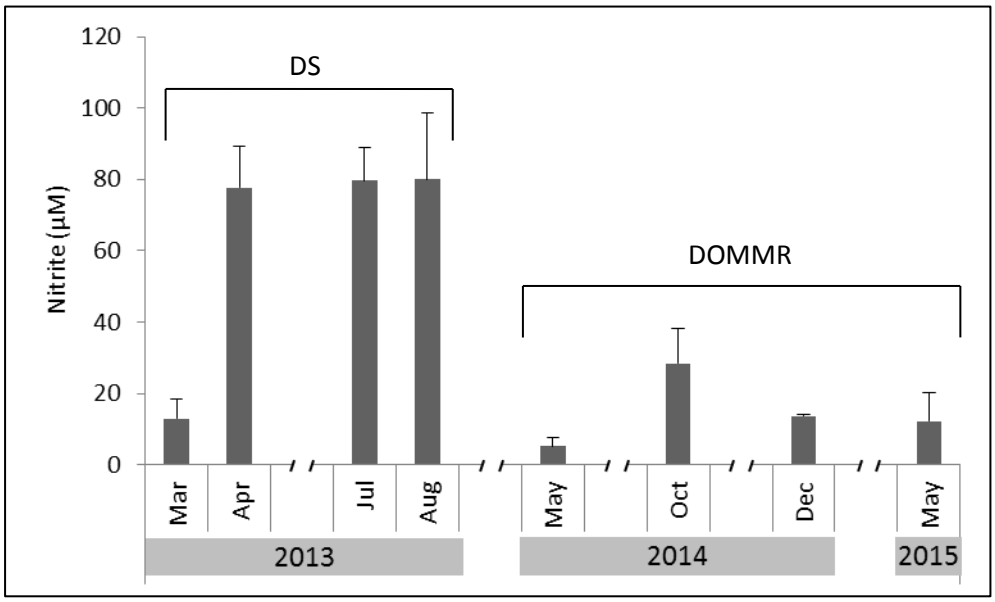


Figure 3: Mean nitric oxide levels (µM) ± SD of baseline (control) data from *P. lividus* individuals taken from past studies conducted in our lab since 2013. The // symbolizes missing data. Sea urchins were sourced from either Dunmanus Seafood Ltd (DS) or Daithi O'Murchu Marine Research Station (DOMMRS), both located in southwest Ireland.

Lysozyme Activity

The means (±SD) of three different sea urchin individuals were taken per sampling point over a two year period (n=36) in order to observe seasonal variation of measured lysozyme activity levels (Figure 4). In 2013, the lowest levels observed in March (0.33 ± 0.15 µg ml^{-1}) with the highest levels observed between September (4.23 ± 2.4 µg ml^{-1}) and October (4.31 ± 0.0 µg ml^{-1}). In 2014, there was a peak seen in December (6.21 ± 0.21) with the lowest levels observed in October (0.34 ± 0.03 µg ml^{-1}). In 2015, the only data collected were for March (0.94 ± 0.22 µg ml^{-1}) and May (0.54 ± 0.45 µg ml^{-1}).

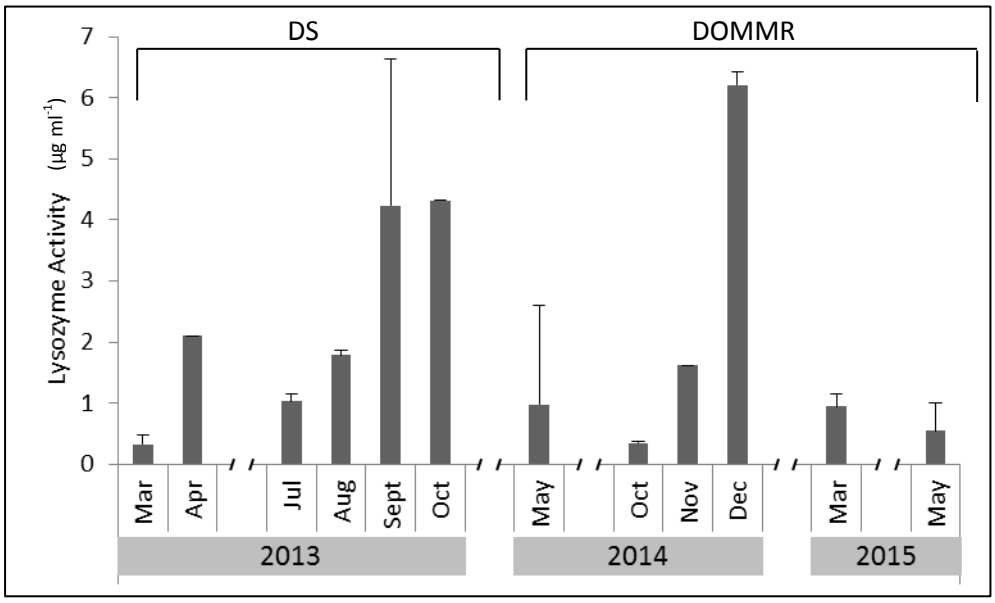


Figure 4: Mean lysozyme activity levels (µg ml^{-1}) of baseline (control) data from *P. lividus* individuals taken from past studies conducted in our lab since 2013. The // symbolizes missing data. Sea urchins were sourced from either Dunmanus Seafood Ltd (DS) or Daithi O'Murchu Marine Research Station (DOMMRS), both located in southwest Ireland.

Discussion

The aim of this study was to look at the impact of annual patterns of several immune parameters in the sea urchin, *P. lividus.* CV, NO, and lysozyme activity levels were observed to be higher in 2013 than in either 2014 or 2015. Since these data were compiled from baseline (control) data taken from different studies, it is important to note the differences in experimental design. Firstly, urchins were fed *L. digitata* throughout experiments in 2013 while they were not in 2014/2015. According to Cipriano *et al.* (*unpublished;* Chapter 3), *Laminaria digitata* (diet used in 2013 experiments) can have an increasing effect on immune parameters, especially on commencement of feeding. Secondly, urchins in 2013 were sourced from a different supplier than in 2014/2015.

From our results, the most obvious difference in the NO and CV data are with the different sites. While both locations are in southwest, Ireland, the main differences were primarily in the culture techniques of the suppliers (DOMMRS and DS). DOMMRS keeps their sea urchins ranched in offshore net cages where they are kept contained in their natural environment with access to large amounts food. DS, on the other hand, is primarily a hatchery that keeps the juvenile urchins in flow-through systems with fresh seawater, aeration, and food access dependent on collection availability. The larger juveniles are then released into offshore net cages until they are large enough for market harvesting. While both techniques are effective, they are different and are likely to affect immune parameters differently.

Furthermore, higher optical density (OD) levels were observed in CV during the summer months with lower levels in autumn/winter months. Coteur *et al.* (2004) pointed out that echinoderms are ectothermic; therefore, changes in water temperature will be reflected in the coelomic fluid and coelomocyte activity. The

changes in OD levels coincide with the reproductive cycle of *P. lividus*, which has an annual pattern with a gonad maturation period from January to August and a short period of regeneration and maturation of the gonads during the rest of the year (Sánchez- España *et al.,* 2004). Therefore, the levels observed during the summer could be linked to an overall increase in cellular activity and energy usage. In addition, Bayne (1990) noted further cellular activity, specifically phagocytosis, in invertebrates can be influenced by environmental changes. This was also confirmed by Feng and Feng (1974), who observed that phagocytosis increased when temperature was increased.

NO levels could not be compared for seasonality as months measured were different between the years. However, previous studies, such as Novas *et al.* (2007) found that basal NO production by haemocytes of *Mytilus galloprovincialis* showed seasonal variations, with summer values statistically higher than those of winter due to the fluctuation of two protein (IL-2 and TNF-α) levels produced by the organism throughout the year. Furthermore, Malanga *et al.* (2009) observed that the oxidative stress in sea urchins increased during spawning (late summer) and decreased during the winter months.

Lastly, lysozyme levels between 2013 and 2015 show a general pattern of lower levels in the summer months and higher levels in the autumn/winter months. This was also observed by Cronin *et al.* (2001) who found higher levels of lysozyme in Rossmore Oysters from Irish and Dutch waters in September (1999) than levels from June (1999) and they remained elevated until December of that year. Additionally, lower levels were observed during June (1999) in *O. edulis* and *Crassostrea gigas* (Cronin *et al.,* 2001). Low summer lysozyme levels were also observed in other studies on *C. virginica* (Feng *et al.,* 1970; Chu & La Peyre, 1989). Previous studies

on oysters have noted a seasonal change in lysozyme levels and a high variation between individuals (Cronin *et al.,* 2001). Furthermore, it has been suggested that haemolymph lysozyme levels in oysters may also relate to the reproductive system, which is controlled in part by external factors such as temperature and food abundance (Cronin *et al*., 2001). Wang *et al.* (2008) found that lysozyme levels were strongly affected by increasing temperatures in the sea cucumber, *Apostichopus japonica* indicating increased lysozyme activity at sub-optimal conditions. Therefore, animals that experience additional stressors during the winter months (e.g. decrease in water temperatures, light availability, food availability and access) are more likely to have elevated lysozyme levels (antimicrobial activity) in order to protect the host.

In conclusion, while there are many physiological and environmental variables (e.g. site, season, and reproductive status), which are all important variables to take into consideration during seasonality studies, the results presented in this study suggest that site location was the most influential variable. Therefore, it is suggested that another long-term repeatable seasonality study be conducted that standardizes site and culture techniques. This is turn would provide useful information that could be applied to helping make sea urchins reliable bio-indicators of their environments. Therefore, future studies using this sea urchin species should use individuals collected in the late autumn months with a test diameter size of 30 ± 5 mm when evaluating these parameters in order to get results that are more consistent. This would ensure a healthy mature individual not influenced by spawning or yet by winter temperatures.

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General discussion and conclusions

This thesis examined the immunostimulatory effect of several components of sea urchin culture. Several important discoveries were made: (1) PIT tagging was shown to be a successful way of identifying individual sea urchins with a high tag retention rate and good possibilities for field use (*Chapter 2*), (2) It was found that repeat sampling via syringe to measure host response of an individual caused stress which could mask potential results (*Chapter 2*), (3) Both *L. digitata* and *M. edulis,* as diet sources, were shown to influence measured immune parameters of DCC, NO, and lysozyme activity (*Chapter 3*), (4) PNZ and GTF are not suitable immunostimulant candidates for *P. lividus* under the presented experimental conditions, (5) Zymosan A was shown to be an effective immunostimulatory agent in *P. lividus* (*Chapter 4*), (6) Zymosan A suppressed the immune system's response to the tested stressor of storage (*Chapter 5*), (7) *Vibrio anguillarum* impacted immune parameters of *P. lividus* (*Chapter 6*), and (8) both seasonality and sourcing sites were found to influence immune parameters measurements.

Following a 4-month investigation, PIT tagging was shown to be a successful way of identifying individual sea urchins with a high tag retention rate and feasibility for field use (*Chapter 2*). Tagging is important for future culturing and ranching as it has applications in monitoring growth rate and survival of marked individuals in the laboratory and in the field, as well as denoting ownership, broodstock management, and tracking of animals in the market chain and in experiments. Although individual tagging has been used for many years in different species, the published results had varying success rates. Therefore, in this thesis, the specific PIT tag used was 4 mm

smaller than tags used in previous studies. It was observed that PIT tags were either lodged into the individual's tissue or remained "floating" around, which may provide insight into the mortalities observed in this study and warrants further investigation. Additionally, this was the first study, to the best of our knowledge, which looked at host response to tagging in *P. lividus* using immune parameters as indicators of host response. However, whether the initiation of the host response was due to the tags or to the sampling methodology is unclear and would need further verification. In the future, behavior studies should be developed to determine a less invasive way of measuring stress and host response because the methodology used in this study challenged all individuals including the controls. In the capture, monitor, and release study, there are two limitations to using PIT tags in the field: 1) sea urchins preferably hide in the crevices between rocks which limits accessibility and 2) the relatively short distance from which tags can be detected. Finding ways to address these limitations are necessary.

L. digitata and *M. edulis*, as diet sources, were shown to influence measured immune parameters (*Chapter 3*). *L. digitata* contains a derivative known as laminarin which has known immunostimulating benefits in other organisms, such as pigs. This is important when studying immune parameters as some diet ingredients could mask true results. To our knowledge, this is the first study to look at the effect of different feeding strategies on immune parameters in sea urchins. The algal and mussel diets affected the sea urchins in different ways over time and this could be observed by using the different immunoassays. Additionally, the potential for additional benefits from an algal-based diet, despite its lower calorific value, should be further investigated. Furthermore, this work demonstrated that starving a group of urchins

over a period of six weeks had little impact on the stability of immune parameters measured. In addition, it would be interesting to design a study that observes how often sea urchins consume different types of foodstuffs in order to properly determine how often mussels are actually consumed versus other items (e.g. kelp, bivalves, crustaceans).

PNZ and GTF are not suitable immunostimulant candidates for *P. lividus* under the presented experimental conditions; therefore, its use was discontinued. Zymosan A was shown to elicit an immunostimulatory effect on the immune response of *P. lividus,* especially at higher doses (*Chapter 4*). Upon ingestion of Zymosan A in an invertebrate, phagocytes engulf the particles and treat them as a possibly dangerous foreign agent; thereby, alerting the immune system to be on standby for future stressors. Sea urchin aquaculture is limited by stress and disease, often associated with transportation, handling, and over-crowding mortalities. Therefore, the next step was to test handling and storage stressors and a virulent strain of *V. anguillarum* on the immune function of *P. lividus* (*Chapters 5 & 6*). It was observed that Zymosan A, prior to storage, reduced the measured levels of some immune parameters (i.e. nitric oxide and lysozyme activity) relative to the control which may reduce the amount of stress the animals experience. This is crucial to sustained success in the live export market and warrants further investigation and eventual application. Future studies would need to test repeatability and market safety. In addition, a virulent strain of *V. anguillarum* was found to have an impact on the immune system of *P. lividus*; however, there was unfortunately not enough time to conclude experiments on the effects that Zymosan A might have had on infected animals. This

is important as finding an immunostimulant that can combat both handling/storage and vibriosis would be highly beneficial to the culturing practices of *P. lividus.*

Finally, both seasonality and site of origin were found to influence immune parameters in the sea urchin, *P. lividus* throughout this thesis research (*Chapter 7*). Two different sea urchin suppliers were used due to commercial availability. However, while both locations are in southwest, Ireland, the main differences were primarily in the culture techniques of the suppliers (offshore holding versus hatchery setting). In conclusion, while there are many physiological and environmental variables (e.g. site, season, and reproductive status), which are all important variables to take into consideration when designing experiments. It is therefore suggested that another long-term seasonality study be conducted that standardizes site and culture techniques. In addition, it would be interesting to conduct studies that would eventually lead to the use of sea urchins as bioindicators (i.e. through stable isotope analysis, fatty acid analysis) as they are both primary consumers and detritivores.

Thank you for taking the time to read my work. If you have any questions of comments please do not hesitate to contact me at ashlie.maack@gmail.com.

Appendix

Chapter 4:

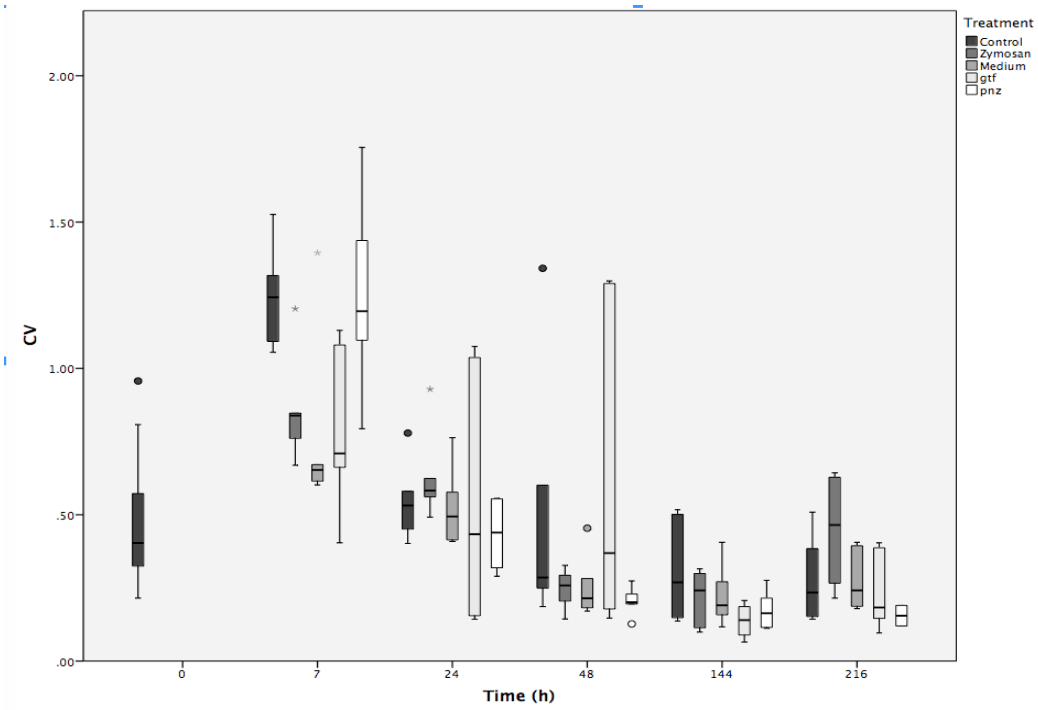


Figure 1: This represents data presented in Ch. 4, Figures 6-7, which represents the mean cell viability levels ± SE (OD_{540}) for *P. lividus* individuals treated with a 0.9 mg L^{-1} with Zymosan A, a control group, 10^9 CFU mL^{-1} of GTF and PNZ, and a 15% trehalose medium control group over a 9 day period.

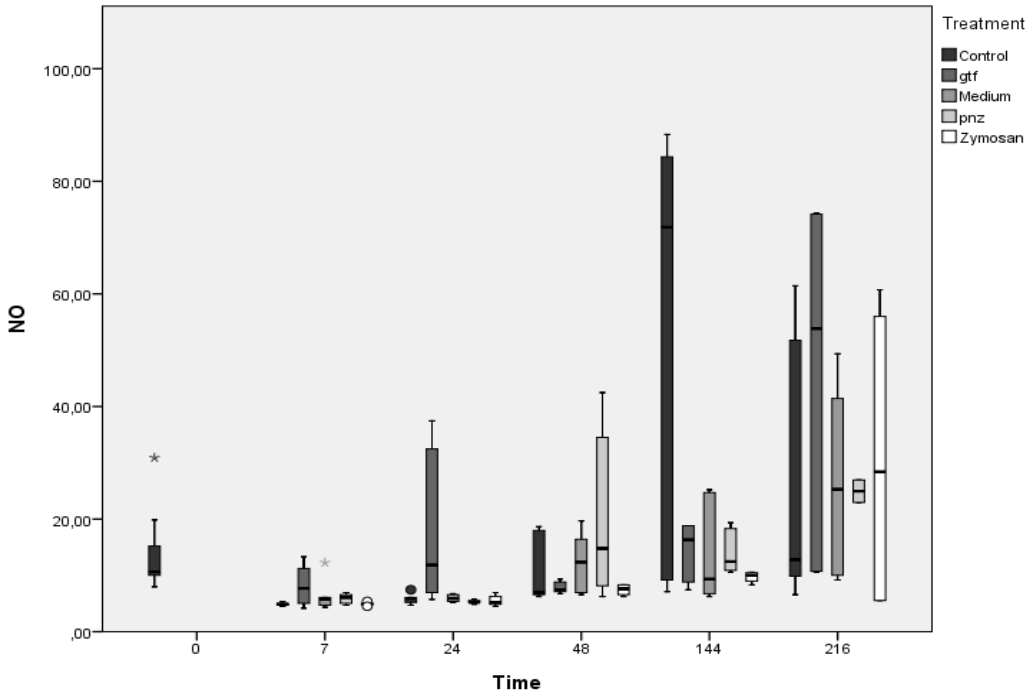


Figure 2: This represents data presented in Ch. 4, Figures 8-9, which represents the mean nitric oxide levels ± SE (µM) for *P. lividus* individuals treated with a 0.9 mg L^{-1} with Zymosan A, a control group, 10^9 CFU mL^{-1} of GTF and PNZ, and a 15% trehalose medium control group over a 9 day period.

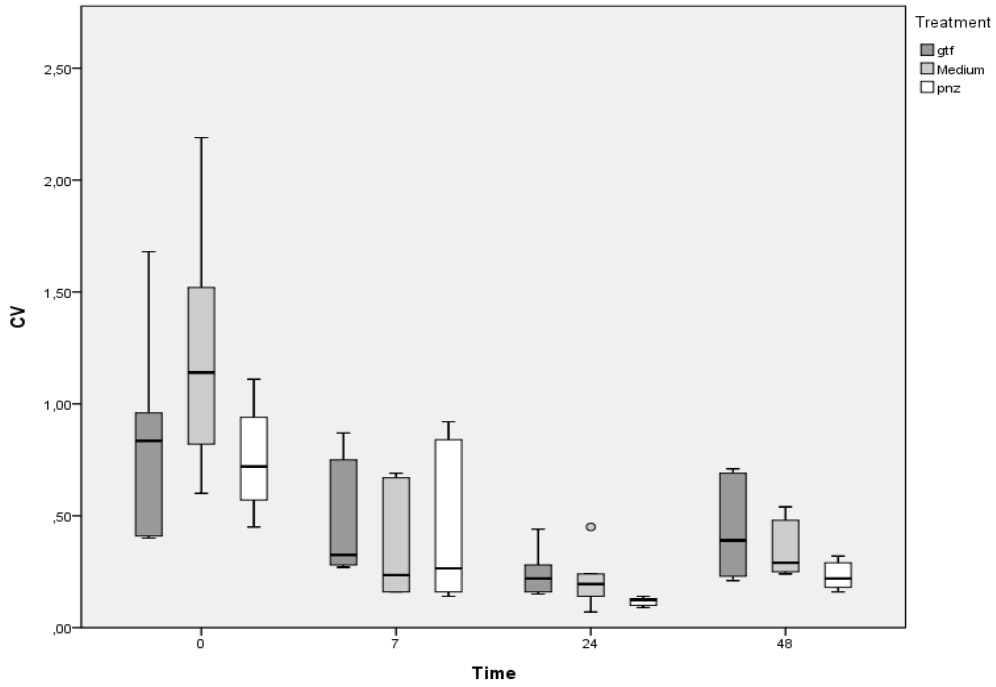


Figure 3: This represents data presented in Ch. 4, Figures 10, which represents the mean cell viability levels ± SE (OD_{540}) for *P. lividus* individuals (40 ± 5 mm) treated with a 10^7 CFU mL^{-1} of GTF and PNZ, compared with a 15% trehalose medium control group over 48 hours.

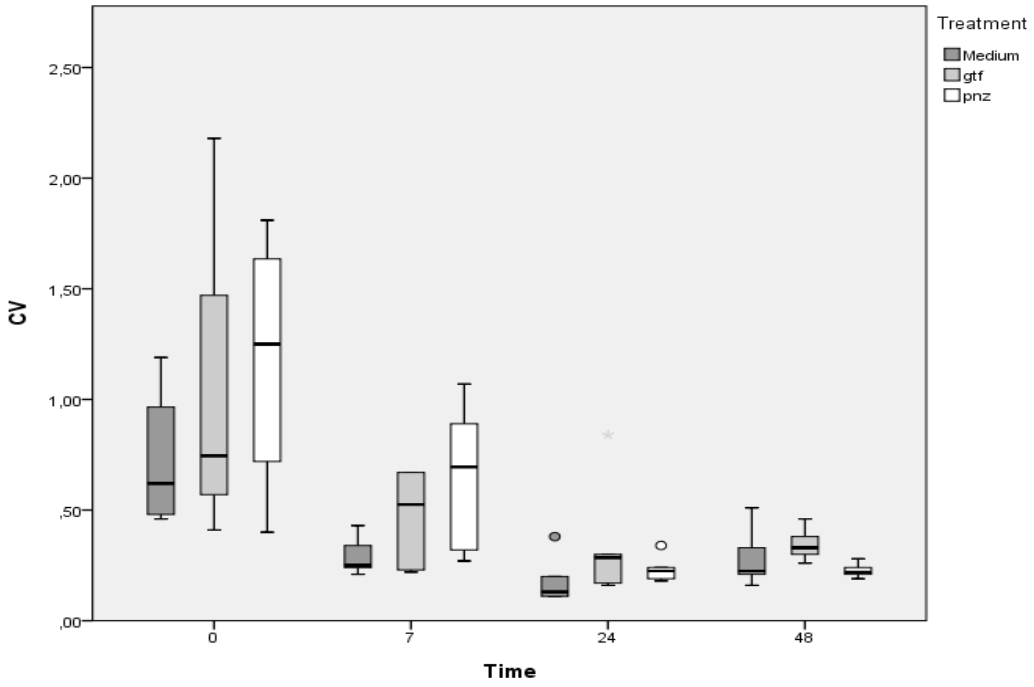


Figure 4: This represents data presented in Ch. 4, Figure 11 which represents the mean cell viability levels ± SE (OD_{540}) for *P. lividus* individuals (50 ± 5 mm) treated with a 10^7 CFU ml^{-1} of GTF and PNZ, compared with a 15% trehalose medium control group over 48 hours.

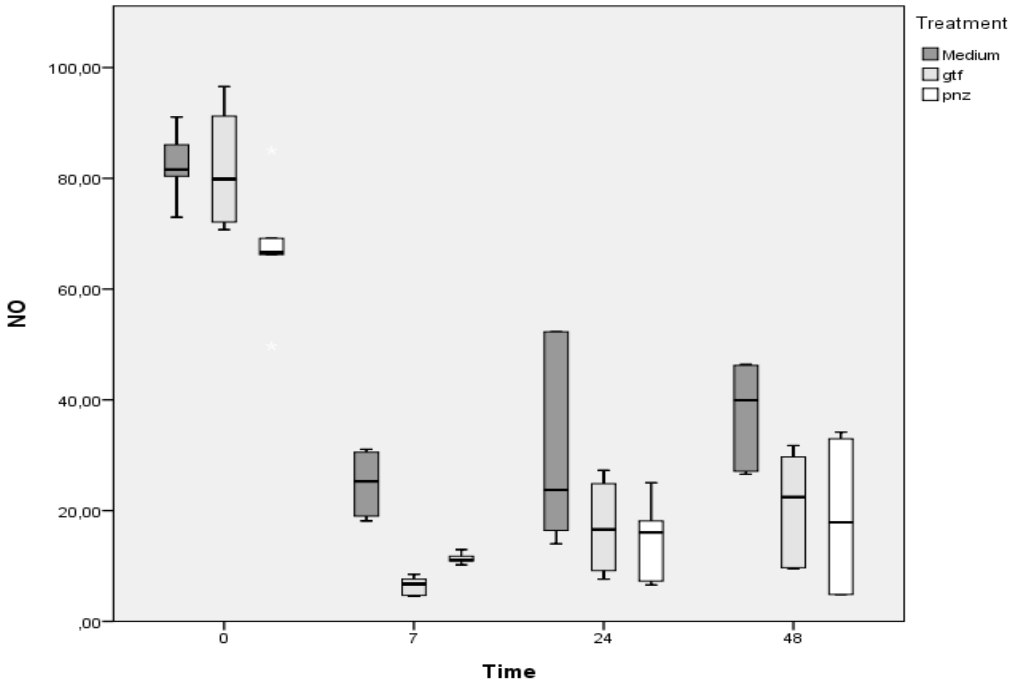


Figure 5: This represents data presented in Ch. 4, Figure 12 which represents the mean nitric oxide levels ± SE (µM) for *P. lividus* individuals (40 ± 5 mm) treated with a 10^7 CFU ml^{-1} of GTF and PNZ, compared with a 15% trehalose medium control group over 48 hours.

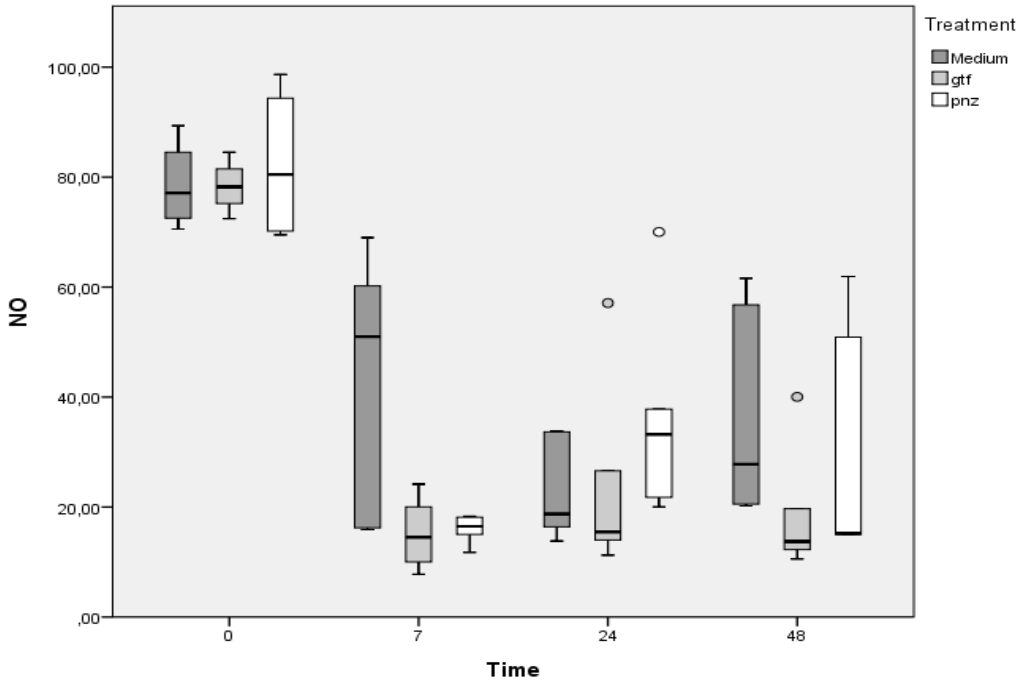


Figure 6: This represents data presented in Ch. 4, Figure 13 which represents the mean nitric oxide levels ± SE (µM) for *P. lividus* individuals (50 ± 5mm) treated with a 10^7 CFU ml^{-1} of GTF and PNZ, compared with a 15% trehalose medium control group over 48 hours.

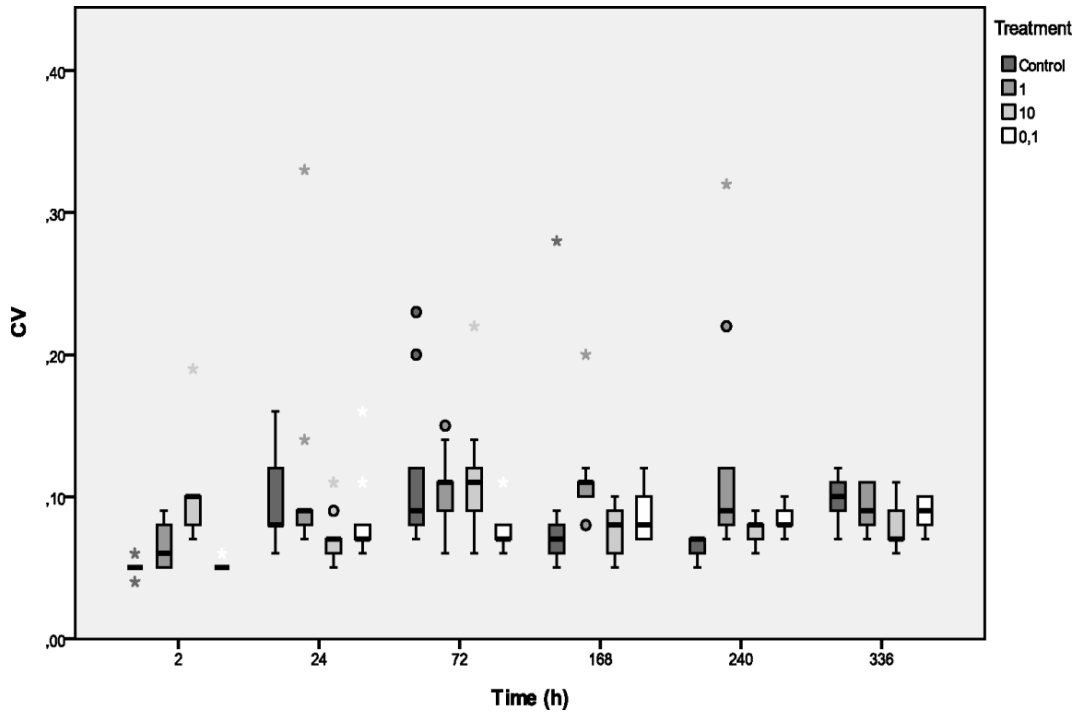


Figure 7: This represents data presented in Ch. 4, Figure 17 which represents the mean cell viability levels ± SE (OD_{540}) for *P. lividus* individuals (30 ± 5 mm) treated with different doses of Zymosan A (0.1 mg L^{-1}, 1 mg L^{-1}, and 10 mg L^{-1}) compared with a control group over 14 days.

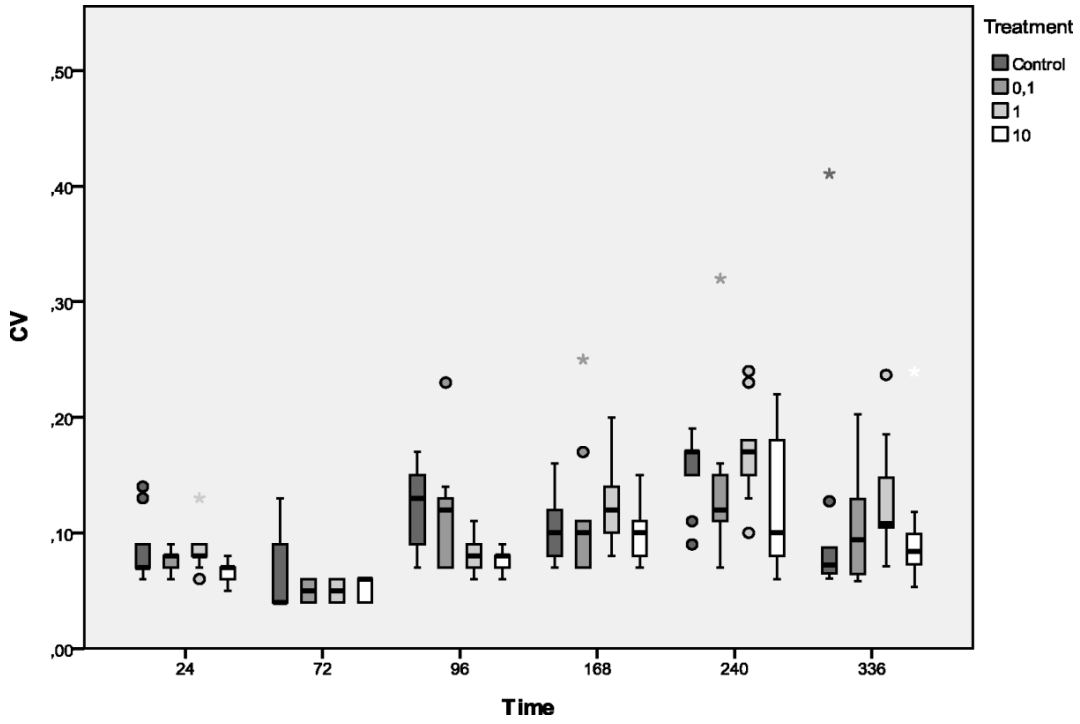


Figure 8: This represents data presented in Ch. 5, Figure 2 which represents the mean cell viability levels ± SE (OD_{540}) for stored *P. lividus* individuals (30 ± 5mm) treated with different concentrations of Zymosan A (0.1 mg L^{-1}, 1 mg L^{-1}, and 10 mg L^{-1}) compared with a control group over 14 days**.** First measurement was taken 24 hr. (1 day) after Zymosan A was initially added. SS = Simulated Storage.

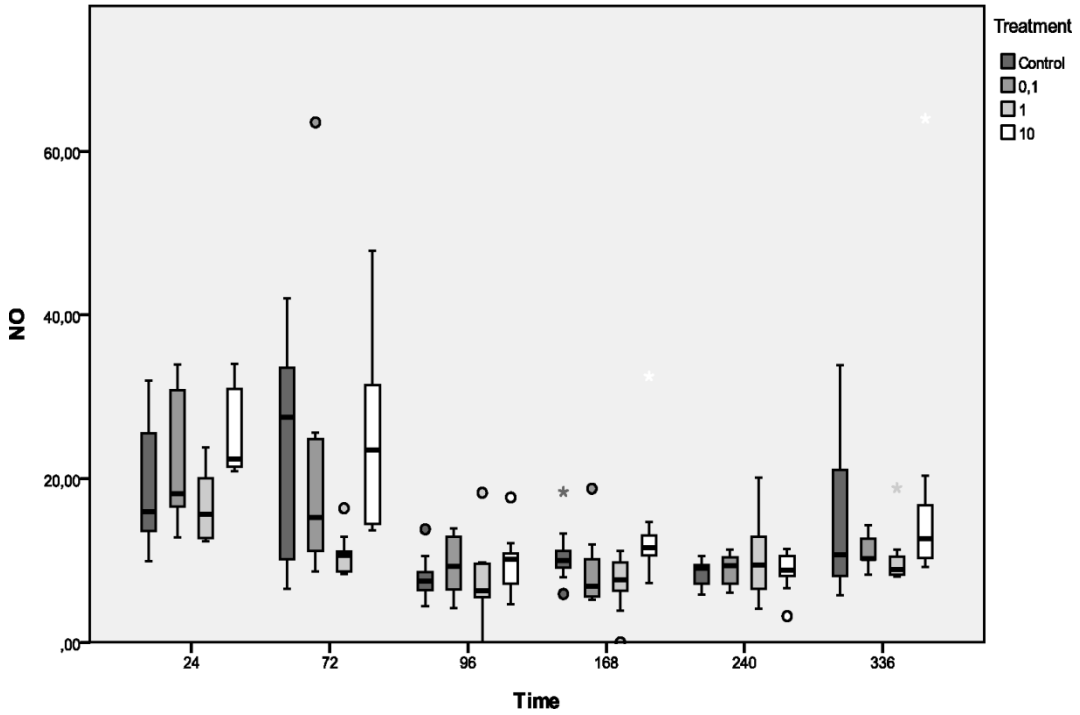


Figure 9: This represents data presented in Ch. 5, Figure 5 which represents the mean nitric oxide levels ± SE (µM) for stored *P. lividus* individuals (20 mm ± 5 mm) treated with different concentrations of Zymosan A (0.1 mg L^{-1}, 1 mg L^{-1}, and 10 mg L^{-1}) compared with a control group over 14 days. First measurement was taken 24 hr. (1 day) after Zymosan A was initially added. SS = Simulated Storage.

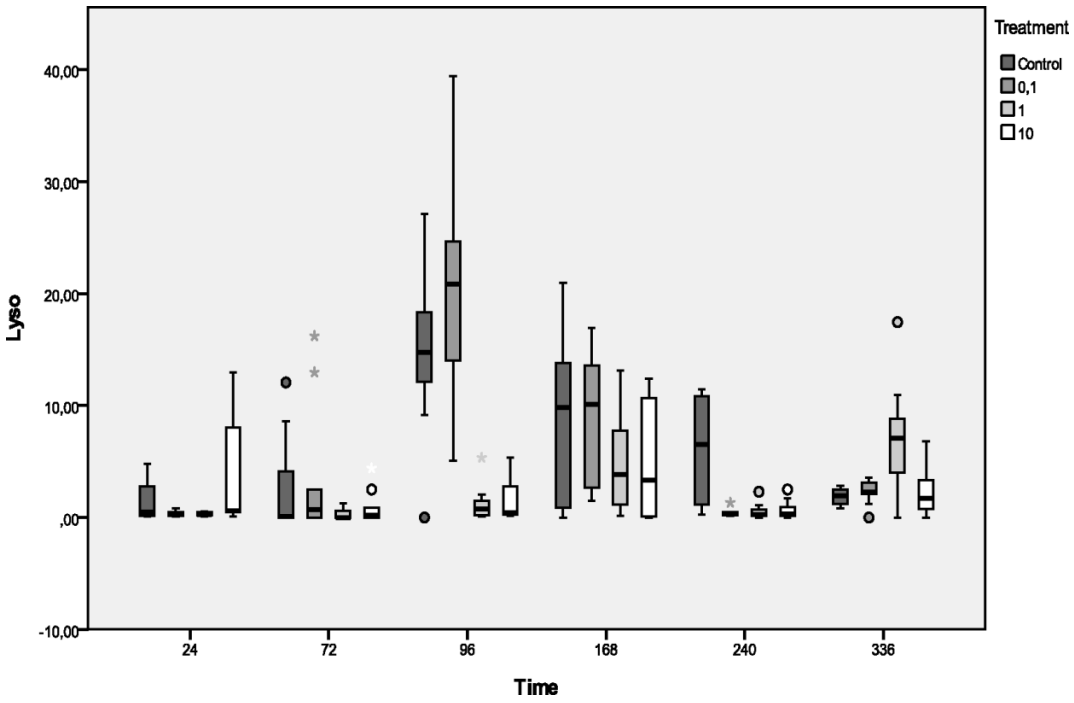


Figure 9: This represents data presented in Ch. 5, Figure 6 which represents the mean lysozyme activity levels ± SE (µg ml^{-1}) for stored *P. lividus* individuals (30 ± 5mm) treated with different concentrations of Zymosan A (0.1 mg L^{-1}, 1 mg L^{-1}, and 10 mg L^{-1} compared with a control group over 14 days. First measurement was taken 24 hr. (1 day) after Zymosan A was initially added. SS = Simulated Storage.

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Organizations to acknowledge:

Graduate research council (UCC), Thomas Crawford-Hayes Fellowship, Daithi O'Murchu Marine Research (DOMMR) Station in Bantry, Co. Cork, Ireland, and Dunmanus Seafoods Ltd. in Dunmanus Bay, Co. Cork, Ireland.

Curriculum vitae

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EDUCATION

University College Cork, Cork, Ireland ***Present***

- *PhD,* Marine Invertebrate Biologist
  Title: Immunostimulatory effects of different aspects of aquaculture on the host response in the purple sea urchin, *Paracentrotus lividus*

University of Kiel, IFM-GEOMAR: Leibniz Institute of Marine Sciences, Kiel, Germany ***July 2012***

- *Master of Science,* Biological Oceanography
- *Title:* Stable isotopes as a way to assess the trophic interactions of jellyfish, western Baltic Sea

American University, College of Arts and Sciences, Washington, D.C. ***May 2010***

- Bachelor of Science, Marine Science and Biology (Dual Degree)
- GPA: 3.24 (4.0 scale)

GRANTS, SCHOLARSHIPS, & FELLOWSHIPS

School of BEES, Graduate Studies Council, Travel Grant
To attend the Asian-Pacific Aquaculture 2013 conference, Vietnam (€1150) **August 2013**

Thomas Crawford Hayes Fellowship, PhD Fellowship **October 2012-September 2016**
Four years of full funding

DAAD, Scientific Exchange, Co-collaborator **October 2011-2013**
Supervisors: *Dr. Jamileh Javidpour (Post-doc at the Hemholtz Institute of Marine Sciences (GEOMAR), Department of Food Web Ecology in Kiel, Germany) and Dr. Mark Martindale (Director of the Kewalo Marine Laboratory, Professor of Organismal Biology, and Principle Investigator at the University of Hawai'i).*

ACADEMIC PUBLICATIONS

Cipriano AN, Wood, CT, Culloty SC, Burnell G. The effect of diet, *Laminaria digitata* and *Mytilus edulis*, on the immune response of the purple sea urchin, *Paracentrotus lividus. Manuscript submitted.*

Cipriano AN, Long SA, Culloty SC, Burnell, G. (2014). Evaluation of three tagging methods in marking sea urchin, *Paracentrotus lividus p*opulations under both laboratory and field conditions. *Journal of Aquaculture Research and Development*,*5*(6).

Javidpour J, **Cipriano AN,** Mittermayr A, Dierking J. Temporal dietary shift in jellyfish revealed by stable isotope analysis**.** *Accepted to Marine Biology March 2016.*

Cipriano AN, Moller LF, Javidpour J, Dierking J. *Experimental determination of discrimination factors and turnover rates of the ctenophore Mnemiopsis leidyi. Manuscript In Preparation.*

RESEARCH EXPERIENCE

Dunmannus Seafoods Hatchery, West Cork, Ireland **October 2012 - Present**

Harbour Porpoise Bycatch, *Iceland* **06-2011-2014**

Seven Lovén Centre for Marine Sciences, *Fiskebäckskil, Sweden* **02/2012-2013**
Stable Isotope Analysis Experiment: fractionation and turnover rates in M. leidyi

Development of Ctenophores, GEOMAR Aquarium, *Kiel, Germany* **10/2010-09/2011**
Intern

- Developed Ctenophores as a model organism for the aquarium
- Maintained breeding environment, while researching new breeding in captivity methods
- Studied the development of Ctenophore larvae
- Went on expeditions to collect organisms from the Baltic Sea and Kiel Fjord

Effects of Oceanic Acidification on Marine Organisms, *Kiel/Sylt, Germany* ***07/2010***

- Studied developmental response to different pCO_2 levels in Cephalopods
- Studied at the IFM-GEOMAR and Alfred-Wegener Institutes
- Cared for over 400 Cephalopod hatchlings, while maintaining their aquatic environments

Sea Urchin Development (National Institute of Health), *Maryland USA* ***12/2009- 05/2010***

- Designed and conducted different methodologies that look at specific genes that are involved in the signaling pathways between the ectoderm and primary mesenchyme cells (PMCs) of sea urchin larvae
- Studied the effects of different environmental cues on Sea Urchin development (i.e. food, predation)

Personal Care Product Effects on the Development of Zebrafish Larvae, American University, Washington, D.C., U.S.A. ***2008-2010***

- Designed and conducted experiments to determine the effects of sunscreen on development
- Designed and implemented all aspects of experiments (obtaining eggs/larvae, culture maintenance, data analysis)

Enrichment and Behavior in Cephalopods (Smithsonian National Zoo) ***2007-2008***

- Participated in Cuttlefish and Octopod behavioral studies
- Prepared enrichment sources for experiments
- Studied nervous systems involved with certain behaviors (recognition and movement)
- Prepared a paper summarizing evolutionary relationships and behavior

RELEVANT WORK EXPERIENCE

PetsPlus, Cobh, Ireland **October 2013 - 2015**
Shop Assistant

Sunbeam Animal Veterinary Hospital, Blackpool, Ireland **August 2013 - 2015**
Foster home and wildlife rehabilitation

Autism Assistance Dogs Ireland (AADI), Cork, Ireland **August 2013 - 2015**
Foster home and fundraiser for service dogs for autistic children

Animal Rescue, Ireland **July 2013 - 2015**
Foster home for dogs/cats and exotic animals

Harbour Porpoise Dissection Course, Husavik, Iceland **06/2012 & 06/2013**
Assistant lecturer

Department of Experimental Ecology, Kiel, Germany **09/2011-09/2012**
Hilfswissenschaftler (Laboratory Assistant)

- MesoAQUA, Conference. *Planning committee.*
- Weekly sampling on the Polarfuchs research vessel
- Narrator of published MesoAqua documentary film

Physiology Department, Kiel University, Kiel, Germany **10/2010-09/2011**
Hilfswissenschaftler (Laboratory Assistant)

- Observed developmental response to different pCO_2 levels in Cephalopods and Sea Urchins
- Studied at the IFM-GEOMAR and Physiology Department at Kiel University
- Cared for Cephalopod hatchlings and Sea Urchins, while maintaining their aquatic environments

General Education Faculty Assistance Program, American University ***01/2010 -05/2010***
Teaching Assistant, General Biology II

National Institute of Health ***12/2009-05/2010***
Intern

- Studied the effects of different environmental cues on Sea Urchin development (i.e. food, predation)
- Conducted experiments and developed laboratory skills (i.e. immunostaining, PCR, etc.)

ACADEMIC PRESENTATIONS, POSTERS, AND CONFERENCES

Asian-Pacific Aquaculture – Positioning for Profit Conference
Oral Presentation, EXTERNAL AND INTERNAL TAGGING METHODS FOR Paracentrotus lividus, December 2013 in Ho Chi Minh City, Vietnam

Asian-Pacific Aquaculture – Positioning for Profit Conference
Oral Presentation, EFFECTS OF IMMUNOSTIMULANTS ON THE PURPLE SEA URCHIN, Paracentrotus lividus, December 2013 in Ho Chi Minh City, Vietnam

Doctoral Showcase - University College Cork
Finalist in the Grand Plan Category of the UCC Doctoral Showcase 2013

ISOECOL - International Conference on Applications of Stable Isotope Techniques to Ecological Studies
Brest, France - August 2012

GEOMAR's mesocosm facilities and the last MESOAQUA PhD conference in mesocosm ecology
Narrator of published documentary film on mesoaqua.eu, December 2011

***Mnemiopsis leidyi* in European Waters: Where are they and what do we know?**
2nd Technical University of Denmark (DTU) Jelly Day, National Institute of Aquatic Resources, Kavalegåden 6, 2920 Chalottenlnd, Denmark, October 2011

Mineralization in Vertebrates
Oral Presentation, Mechanisms of Biomineralization, IFM-GEOMAR: Leibniz Institute of Marine Sciences, *presented Spring 2011.*

Chemical Compounds of Sunscreen
Poster Presentation, Robyn Rafferty Matthias Student Research Conference, College of Arts and Sciences, American University, Spring 2009. Co-presented with Kimberly Fitzgibbons.

The Effects of Sunscreen on Zebrafish Embryonic Development
Oral Presentation, Robyn Rafferty Matthias Student Research Conference, College of Arts and Sciences, American University, Spring 2009.

SEA TIME

2011. Culturing of *Mnemiopsis leidyi*. Sweden. IFM-Geomar. August 23-25.

2011. Marine Mammals in the Wild. Iceland. University of Iceland. July 1-10.

2011. Net Sampling. Cruise Ship Polarfuchs. Kiel Fjord. Weekly from February 2011-Present.

2010. Marine Biogeochemistry. Cruise ship RV Heincke. Kiel Fjord/Baltic Sea. December 1.

2010. Benthos Ecology. Cruise ship RV Heincke. Kiel Fjord/Baltic Sea. November 29.

RELEVANT SKILLS

- Skilled research images in Image J
- Skilled in Statistical Databases SPSS and the "R" program
- Skilled in laboratory skills (Immunological assays, histology, microscopy)
- Skilled in aquatic systems set-up and maintenance
- Skilled in Microsoft Office (Word, Excel, PowerPoint, Outlook)
- Scuba Certified (PADI)

RELEVANT COURSES

- BEES teaching and learning module

Published Papers



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Research Article Open Access

Evaluation of three tagging methods in marking sea urchin, *Paracentrotus lividus*, populations under both laboratory and field conditions

Cipriano A*, Long S, Culloty S, and Burnell G

Aquaculture and Fisheries Development Centre School of Biological, Earth and Environmental Sciences University College Cork Distillery Fields North Mall Campus Cork, Ireland

Abstract

The purple sea urchin, *Paracentrotus lividus* is an Atlanto-Mediterranean species that is of commercial interest for its gonads (or roe) in Europe and Pacific/Asian countries. Individual identification of sea urchins is difficult due to the presence of spines and the structure of the skeletal-like test. However, a successful tagging technique is important for monitoring growth rate and survival of marked individuals in the laboratory and in the field. In addition, tagging can denote ownership, help in brood stock management, and allow for the tracking of animals in the market chain and laboratory experiments. In this study, smaller than previously reported passive integrated.

Transponder (PIT) tags and two external methods (fingernail polish and beads glued to the spines) were tested on *P. lividus* individuals to assess tagging capability, survival, and host response (e.g. lysozyme activity, nitric oxide levels, and cell viability). Additionally, PIT tagged individuals were released in an intertidal rock pool and monitored in order to test field application. Of the three different tagging methodologies, PIT tags were found to be the most successful in both studies carried out in the laboratory in regards to survival and tag retention. In the field, PIT tagged individuals were released and recaptured successfully. Furthermore, host response to individual tagging showed that the individuals were challenged by the sampling methodology which caused increased mortality.

Keywords: Fisheries; *Paracentrotus lividus*; Lysozyme; Aquaculture industry

Introduction

Demand for high quality seafood is increasing; resulting in the rapid growth of the aquaculture industry, which in turn reduces pressure on capture fisheries. Maximizing the potential of the aquaculture industry though, requires innovation to refine existing techniques and apply new technologies [1]. Individual identification in holding conditions is important to monitor growth, behavior, genetics, and population dynamics [2]. The mechanism used to identify individuals must be easily distinguishable, be retained for long periods of time, and have minimal impact on growth and behavior if it is to have practical applications [3, 4]. Individual identification also has further applications in brood stock management, denoting ownership, tracking animals in the market chain, and ecological studies [5] by serving as a marker within the population. However, many commonly farmed aquatic organisms, such as shrimp, sea urchins, and other marine invertebrates, have proven difficult to tag despite recent advances in tagging technology [5-7].

The purple sea urchin, *Paracentrotus lividus*, is an Atlanto-Mediterranean species that is of commercial interest for its gonads (or roe) in Europe and Pacific/Asian countries [8, 9]. This commercial demand has placed pressure on wild sea urchin populations worldwide and has led to an increased need for aquaculture and hatcheries. In 2010, marine aquaculture produced an estimated 384,300 tons of echinoderms for consumption [10, 11] necessitating the establishment of more hatcheries. Individual tagging or identification of sea urchins is difficult due to the presence of spines and the structure of the skeletal-like test. Previous studies have focused on external markings to the spines and test [12-14], drilling holes in the test [15-18], fingernail polish plus dental adhesive [14] internal markings, such as tetracycline injections [19-22] and passive integrated transponders (PITs) [6, 23-26]. However, these techniques are often invasive and result in altered behavior and high mortality rates [27, 28].

The invasiveness of the tags challenges the individual and could lead to a compromised immune system and possible mortality. Factors affecting the immune system include diseases, condition, and diet [29]. Any factor that challenges an individual can elicit a host response. A tag, whether attached to the spine or test, or inserted into the coelomic cavity, may be treated by the sea urchins immune system as potential invaders. The sea urchins immune system is defined by its immune effectors 35 which have the capacity to respond to injuries, host invasion, and cytotoxic agents [29]. Using immune parameters, there are two means of evaluating host response: 1) humoral components such as nitric oxide and lysozyme activity assays and 2) cellular components such cell differentiation counts and cell viability assay. The humoral responses use antimicrobial compounds as a first response to invaders. Nitric oxide, a nitrogen radical produced from L-arginine during phagocytosis, serves as a mechanism of fighting off invasive pathogens [30]. Additionally, lysozyme levels demonstrate defense capabilities through the enzymatic break down of pathogenic cell membranes [31]. Cellular responses directly involve coelomocytes, circulating immune

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cells, Located within the coelomic cavity. Therefore, cell viability and immune cell differentiation are important immune parameters which allow insight into the effects of tagging on *P. lividus*.

In this study, two laboratory trials looked at internal implanted passive integrated transponder (PIT) tags and two external methods (fingernail polish and beads glued to the spines) when tested on *P. lividus* individuals over a two or four month time period (February–May 2013) in order to assess individual tagging viability and host response. Additionally, PIT tagged individuals were released and detected in the field in West Cork, Ireland using a portable universal microchip reader (RealTrace® RT100) in a water proof scuba bag. The overall aim of the study was to identify a tag that was the most suitable for identifying an individual based on (a) tag retention, (b) host response to tags, and (c) survival of *P.lividus* in the laboratory and in the field.

Materials and Methods

In this study, three trials were conducted on sea urchins. The first trial (Trial 1) looked at tag retention and survival for 8 weeks in the laboratory. The second trial (Trial 2) looked at tag retention and host response for 4 weeks in the laboratory. The final trial (Trial 3) assessed PIT tag viability in the field.

All *P. lividus* individuals were sourced from Dunmannus 68 Sea foods sea urchin hatchery in West Cork, Ireland. Both laboratory trials were carried out at ambient temperature (14.0 ± 1.0°C; maintained with PSA Aquaclim 10 reversible heat bpump/chiller) with continuous water circulation (1000 L sump filled with fresh sea water every 3 days) in four 400 L black plastic circular tanks. pH and oxygen saturation (DO) was monitored throughout the experiment to ensure water quality (pH: 8.0 ± 0.05 and DO: 8.0 mg/L ± 0.4 mg/L). Each tank (both trials) contained 4-5 plastic mesh baskets which each held 10 P. lividus individuals and underwent a different tag treatment. In order to acclimate the sea urchins, they were held for 7 days prior to trial commencement. No sea urchin mortalities were recorded during the acclimation period. Animals were fed *ab libitum* with *Laminaria* sp.

Trial 1: Assessment of different tag options in sea urchins

General set-up: In each of the tanks, 1 basket held controls, 1 held sea urchins tagged with fingernail polish (40 ± 5 mm individuals), 1 basket held specimens tagged with beads (40 ± 5 mm individuals), and 2 baskets each held a different specimen size class (20 ± 5 mm or 40 ± 5 mm individuals) tagged with PIT tags. The control consisted of 10 un-tagged sea urchins (40 ± 5 mm).

External tagging: Two external tags were used (Figure 1b-1c). The first tag type was fingernail polish (Boot's Natural Collection®) applied to the top of an individual sea urchin's spines (approx. 20 spines) after drying spines with cotton. The second tag type was 2mm craft beads glued to 5 spines per sea urchin with a BISON non-toxic, non-drip formula super glue gel.

Internal tagging: 1.4 mm×8 mm PIT tags (Trovan®) (Chips4Fish, Zoo Chip, UK) programmed with a unique 12-digit identification number [32] were inserted through the peristome membrane via syringe application [33] (Figure 1a).

Monitoring: Animals were monitored daily for 8 weeks (119 days). External tags (fingernail polish and beads) on each individual sea urchin were counted. Internal tags (PIT Tags) were scanned using a portable universal microchip reader (RealTrace® RT100). Any Dead Sea urchins or sea urchins that had lost their tags (expelled PIT tag or lost all beads/fingernail polish) were removed from the experimental system.



Figure 1: Physical representation of different tagging methodologies.

Trial 2: Host response from tagging of sea urchins in the laboratory

Tagging: Please refer to Trial 1 for general set-up, tagging, and monitoring with the exception that only smaller individuals (20 mm ± 5 mm) were used for this trial.

Host response measurements:

Coelomic fluid collection: Due to the small size of each individual urchin (20 ± 5 mm), samples were pooled from the 10 individuals per treatment per tank. An initial baseline sample was taken from 10 individuals prior to tagging. Sampling took place at 2 hrs (T2), 24 hrs (T48), 48 hrs (T48), and then occurred once a week for 4 weeks after tagging to allow for coelomic fluid levels to return to normal and for animals to de-stress between sampling episodes. Each week, 30 µl of coelomic fluid was taken from each individual and placed into a 2 ml eppendorf tube for pooling. Host response was monitored using lysozyme activity, nitric oxide measurements, and cell viability assays. In total, 40 individuals were analyzed per treatment.

Lysozyme activity assay: 200 µl of coelomic fluid, pooled from 10 sea urchins per treatment was immediately placed in a 2 ml eppendorf and placed on ice to prevent degradation of the samples. The samples were centrifuged at 3,000 rpm for 10 min to separate the cells from the serum. The supernatant was removed without disturbing the pellet formed at the bottom of the tube, placed into a clean 2ml eppendorf

and stored at -20°C until analysis. The corresponding Pellet was also frozen at -20°C. The lysozyme activity assay was carried out as described by Cronin et al. [31], according to Caraballal et al. [34], a modification of Shugar [35]. Lysozyme activity was measured using a 96 well plate reader at a wavelength (λ) of 450 nm which calculated the mean decrease in absorbance at T0, 1 min, 2 min, 3 min and 4 min. Duplicate lysozyme standard solutions (30 µl) made from hen egg white lysozyme (SIGMA) were serially diluted, were included on each plate and consisted of seven concentrations 5.0 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml etc. A corresponding number of blank wells, consisting of 200 µl phosphate buffer (0.1M; pH 7.5), were included on each plate. 30 µl of the supernatant of each sea urchin sample (in triplicate) was added to the wells of each plate. 170 µl of *M. lysodeikticus* suspension (pH 6.4) was added to the wells containing the standard solutions and the sample solutions on each plate to make up to a total volume of 200 µl per well.

Nitric Oxide production, Griess reaction: 100 µl of coelomic fluid, pooled from 10 sea urchins per treatment, was incubated in a 96 well-plate for 30 min at room temperature (in triplicate). The same volume of filtered sea water (FSW) was added to the controls and left to incubate for 2 hours. 50 µl of supernatant was removed and transferred to a new plate. 50 µl of each of the Sodium Nitrite standards: 0.1, 0.5, 1, 5, 10, 50, 100 µM, was added to new wells. 100 µl of Solution A (1% sulphanil amide in 2.5% phosphoric acid) then 100 µl of Solution B (0.1% N157 naphthyl-ethylenediamine in 2.5% phosphoric acid) was then added to all wells (samples, standards and blank) and incubated for 5 min at room temperature. The 96 well plates were placed in a spectrophotometer reader (Elx808 Ultra Microplate Reader, BIO-TEK instruments, INC.) and read at 540 nm.

Cell viability: 100 µl of coelomic fluid, pooled from 10 sea urchins per treatment, was incubated in a 96 well-plate for 30 min at room temperature (in triplicate). The supernatant was removed by overturning the plate. 100 µl of working neutral red solution (1/50 of stock solution: 0.02 g in 5 ml of filtered sea water (FSW), filter and maintain in dark) was added to each well and incubated for 2 hours. The supernant was discarded by overturning. Samples were washed with FSW and discarded again. 100 µl Lysis169 Solution (1% Acetic acid and 50% Ethanol in distilled H_2O) was then added to each well. The 96 well plates were placed in a spectrophotometer reader (540 nm) after being shaken for 1 min.

Trial 3: Individual tagging of sea urchins in the field

The remaining 44 PIT tagged sea urchins from Trial 1 and 2 (sizes ranging from 20 ± 5 mm to 40 ± 5 mm) were ranched in shallow rocky tide pools near the Dunmannus Sea foods sea urchin hatchery in West Cork, Ireland from August to October 2013 for a total of 6 weeks. The urchins were simply released into the rock pool and monitored fortnightly at spring tides using a portable universal microchip reader (Real Trace® RT100).

Data analysis

For both laboratory experiments (Trial 1 and 2), a chi-squared (χ^2) 183 test was used to indicate the significance of a particular chosen tag type on sea urchin mortality. In Trial 2, post hoc analyses were conducted given the statistically significant ANOVA ($p<0.05$) for the cell viability and nitric oxide assay results on day 14 (last day where all treatments were still measured). Specifically, Tukey HSD tests were conducted on all possible pair wise contrasts. For the lysozyme activity assay results, individual Kruskal Wallis tests were used to test for significance on day 14 due to the missing data from later sampling points (Table 1).

Results

Trial 1: Assessment of different tag options in sea urchins

The controls (urchin size: 40 mm ± 5 mm) had a 25% mortality rate over the four month study period. Within 24hrs, the individuals with fingernail polish painted on their spines (urchin size: 40 mm ± 5 mm) started to lift the entire epidermal layer off their tests and drop their spines. By day 29, 100% mortality was observed in this group. Although the bead methodology had 100% survival, it was only successful in the short-term as sea urchins survived with the beads up to 29 days before all the beads fell off or the sea urchins dropped the spines holding the beads. Lastly, two size classes of sea urchins contained the PIT tags; small (20 ± 5 mm) and large (40 ± 5 mm). The large sea 203 urchins showed a 52.5% mortality rate and the small urchins had a 22.5% mortality rate over the 8 week study period. All PIT tags remained operational throughout the experiment. A chi-square test indicated that the Choice of a particular tag type was associated with the survival of the sea urchin (χ^2; $p<6x10^{-5}$).

Trial 2: Host response from tagging of sea urchins in the laboratory

For *Trial 2*, only 20 mm (± 5 mm) individuals were used. The controls showed a 20% mortality rate. Within the first week, the individuals with fingernail polish started to lift the entire epidermal layer off their tests and dropped their spines with 90% mortality. By day 21,100% mortality was observed. As in *Trial 1*, the bead methodology was more successful, but still observed a 90% loss of tags by the end of the 28 day experiment. Lastly, urchins that contained the PIT tags had 60% mortality. The individuals that retained their tags survived and were healthy until the end of the experiment. A chi-square test indicated that there was a significant difference between the tag retention by the sea urchins based on the tagging option employed (χ^2; $p < 5.2 \times 10^{-3}$). Cell viability, nitric oxide levels, and lysozyme activity were measured to evaluate host response to tagging. The tag types were statistically compared on day 14 as it was the last sampling point when all tag types were still viable.

The cell viability measurements (Figure 2) indicated an overall decrease for all treatments and the control. Two hours after tagging, 0.36 OD_{540} and stabilized at 0.12 OD_{540} throughout the remainder of the trial. The different tags types followed a similar pattern. PIT tagged individuals after hours measured 0.36 OD_{540} and stabilized at 0.12 OD_{540}, while beaded individuals, after two hours, were 0.58 OD_{540} and stabilized at 0.10 OD_{540}. Fingernail polished individuals after two hours was measured at 0.32 OD_{540} and stabilized at 0.11 OD_{540}. The fingernail polish tag type was significantly different ($p<0.05$) from other tag types on day 14. There was not enough sea urchin coelomocyte left over to sample until day 28 due to mortality. Nitric oxide

Tag Option	Trial 1.1		Trial 1.2 (Mortality)
	Mortality	Tag Loss	
Control	25%		20%
Bead		100%	90%
PIT Tag			
20 mm Individuals	22.5%		60%
40 mm Individuals		52.5%	
Nail Polish	100%		100%

Table 1: Tag retention and survival in *P. lividus*.

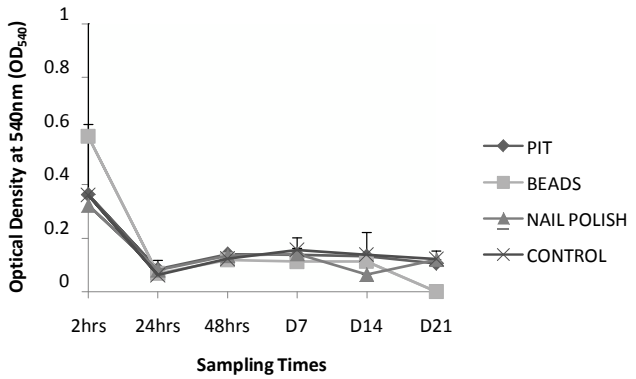


Figure 2: Cell viability, measured in optical density, for pooled *P. lividus* individuals tagged with different tagging options over a 28 day period.

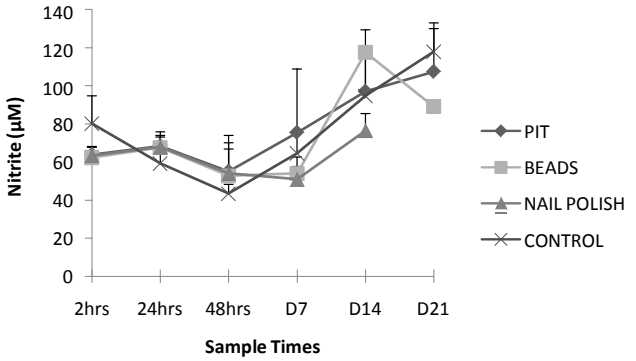


Figure 3: Nitric oxide levels for pooled *P. lividus* individuals tagged with different tagging options over a 28 day period.

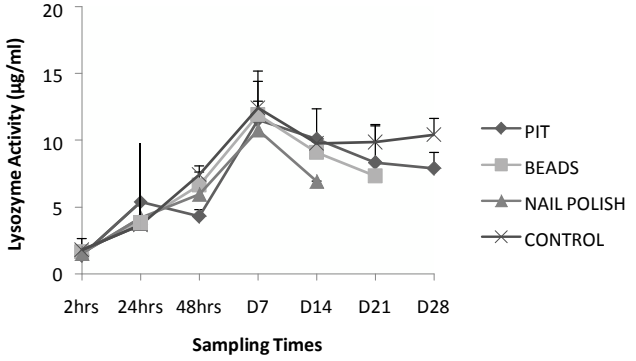


Figure 4: Lysozyme activity data pooled *P. lividus* individuals tagged with different tagging options over a 28 day period.

levels (Figure 3) showed an initial 48hr decrease after tagging followed by general increases in all tag types and controls until the end of the experiment. The controls at T2 measured 80.04 µM which decreased to 43.3 µM237 at T48 and then increased to 117.78 µM on day 21. The different tag types followed a similar pattern. PIT tagged individuals at T2 measured 63.8 µM which decreased to 55.01 µM at T48 and then increased to 107.28 µM on day 21. Beaded individuals at T2 were 62.35 µM which decreased to 52.7 µM at T48 and then increased to 107.28 µM at T21. Fingernail polished individuals at T2 were 63.26 µM which decreased to 54 µM at T48 and then increased until they died off on day 14. The bead tag type was significantly different ($p<0.001$) from other tag types on day 14. There was not enough sea urchin coelomocyte left over to sample until day 28 due to mortality. Lysozyme activity (Figure 4) showed a general increase in all tag types and the control until day 7. The controls at T2 measured 1.79 µg/ml, peaked at 12.42 µg/ml and measured 10.41 µg/ml at TDay28. The different tags types followed a similar pattern. PIT tagged individuals at T2 measured 1.37 µg/ml, peaked at 11.51 µg/ml and measured 7.89 µg/ml at TDay28, beaded individuals at T2 were 1.63 µg/ml peaked at 11.92 µg/ml and wasn't viable at TDay28, and fingernail polished individuals at T2 were 1.52 µg/ml, peaked at 10.77 µg/ml and wasn't viable at TDay28. The fingernail polish tag type was significantly different ($p<0.05$) from other tag types on day 14 [36].

Trial 3: Field detection of PIT tags

In the field, individuals (n=44; 20 ± 5 mm and 40 ± 5 mm) were monitored four times over a two month period (Table 2). Tag identification numbers were recorded in order to observe individual occurrences and tag feasibility. One week after releasing the PIT tagged individuals into the rock pool, 2.3% of individuals were recaptured and identified. However, 3 weeks after the animals were replaced, 13.6% of individuals were recaptured. 11.4%, 20.5%, and 4.5% individuals were caught and recaptured on weeks 4, 5, and 6, respectively. Altogether 12 individuals (27%) were identified and captured over the 6 week period [37, 38].

Discussion

Previous studies using PIT tagged aquatic invertebrates claim that PIT tagging does not adversely affect survival [6, 39-42]. Therefore, the higher mortality from this study could be due to sensitivity of P. lividus, condition of the individuals at the time of the experiment or the host response to the tags. Three tag types were used 271 in this study: glued craft beads, nail polish and PIT tags. The glued crafts beads could be viable for short term experiments that last less than two weeks and don't involve host response measurements. It was observed that the sea urchins dropped the spines with the beads, possibly as a defense mechanism. The fingernail polish resulted in 100% mortality. This was could be due to the fact that a) the epithelial layer with the fingernail polish became detached within 24 hrs of application layer making the urchin susceptible to infection and b) some urchins were observed to have a swollen peristomal membrane possibly indicating the fingernail polish, when applied, covered the anus preventing waste expulsion (*personal observation*). The specific PIT tag used in this study was 4 mm smaller than tags used in previous studies [6,36-39]. In our initial tag retention trial, the mortality rate observed was lower in the smaller individuals than in the larger individuals possibly due to the life history and previous holding conditions of the urchins. The most successful marker in this study was the PIT tag with applications in denoting ownership and tracking individuals.

Trial 1 showed that survival in the smaller sized urchins with PIT tags was the same as in the control group. The poorer survival in the larger PIT tagged individuals may have been due to their previous life history in the hatchery. Upon dissection of PIT tagged individuals, it was observed that the tag was lodged in the membrane covering the inside of the test (personal observation). In a study by Parker and Ranken [39] on PIT tagging in Black Rockfish, it was suggested that the movement of tags could be the cause of observed mortalities. Christy [26] observed PIT tags lodged in the outer peritoneum of two frogs (*Limnodynastes peronii*). This may have contributed to the mortalities observed in PIT tagged urchins in this study as the PIT tag was inserted

	Weeks post releasing to the field				
Individual PIT Codes	**Week 1 Aug. 23 2013**	**Week 3 Sept. 04 2013**	**Week 4 Sept. 12 2013**	**Week 5 Sept. 17 2013**	**Week 6 Oct. 21 2013**
345832 1 (15.4	✓				
348073 9 (41.2		✓	✓	✓	
344016 3 (12.1		✓		✓	
348389 3 (6.6 g)		✓		✓	
347416 4 (8.4 g)		✓	✓	✓	
348370 6 (38.6		✓		✓	
347523 1 (26.5		✓	✓	✓	
346660 2 (25.8			✓	✓	
346737 6 (30.2				✓	
344780 0 (12.6				✓	
347688 2 (33.5					✓
378486 0 (10. 4					✓
% of individuals recaptured	2.3	13.6	11.4	20.5	4.5

Table 2: Recapture rate for PIT tagged urchins in the rock pool over a 6 week period.

into the coelomic cavity where it could move around, conceivably causing internal damage. Additionally, upon the completion of the experiment, eight of the surviving PIT tagged animals were dissected in order to locate the PIT tag. It was observed that the tag was starting to be encased in the tissue lining of the test. This observation has not been reported before and warrants further investigation. Although individual tagging has been used for many years in different species (mainly vertebrates) [43], other studies using PIT tags on aquatic invertebrates include prawns, *Macrobrachium rosenbergii* [44], freshwater signal crawfish, *Pacifastacus leniusculus* [38], pot-bellied seahorses, *Hippocampus abdominalis*[41], green sea urchins, *Strongylocentrotus droebachiensis* [6,45], the kina sea urchin, Evechinuschloroticus, sea cucumbers, *Holothuria whitmaei* and *Actinopyga miliaris* [4], and freshwater pearl mussels, *Margaritifera margaritifera* [46], eastern lamp mussels, *Lampsilis diataradiata* [42] 305 with varying success as viable markers. Other studies using similar tagging techniques to this study, such as Agatsuma et al. [47] reported successful tagging of *Strongylocentrotus nudus* spines with different colors of fingernail polish and covering the polish with a quick drying dental adhesive but only for trials lasting for shorter than three days. No studies to our knowledge have used craft beads glued to the spines.

There have been few studies on the effects of tagging on the host, especially in invertebrates [46]. This is the first study looking at host response to tagging in P. lividus using immune parameters as indicators of host response. Trial *1* was designed to assess tagging viability and mortality, while Trial 2 was designed to test immune parameters of the tagged host. All animals in *Trial 2* showed host response, and higher mortality when compared to *Trial 1*, including the controls, due to handling and sampling of the coelomic fluid via insertion of a syringe into the peristomal membrane. The overall decrease in cell viability within the treatment groups, as well as the controls, may be due to tag effects. With less viable cells, phagocytosis decreases as well; therefore decreasing capability of an immune response. Upon introduction of a stressor, the host will liberate oxygen and free radicals, such as nitric oxide which is a potent bactericidal [48-51]. Our nitric oxide (NO) measurements indicate that the sea urchin coelomocytes produced NO with increased production at 48hrs; however, whether it was due to the tags or to the sampling is unclear and would need further verification. Lysozyme is an enzyme that can hydrolyse components of bacterial walls; therefore, aiding in immune defense and digestion [52]. Lysozyme results from this study indicate an increase in host response until day seven followed by a decrease in lysozyme activity in all treatments excluding the controls. Again, the initiation of the host response, whether it was due to the tags or to the sampling methodology, is unclear and would need further verification. Behavior studies should be developed to determine a less invasive ways of measuring stress and host response because the methodology used in this study challenged all individuals including the controls. Therefore, it is necessary to develop an alternative way of measuring host response (i.e. the activity of the podia and the configuration of the spines). In the capture, monitor, and release study, surviving tagged individuals from the tag retention experiment were ranched in natural rock pools on the west coast of Ireland. The recaptured urchins had retained their PIT tags for at least five months (laboratory and field) and were easily scanned with a portable universal microchip reader (RealTrace® RT100) (standard in veterinary practices); however, there are two limitations 339 to using PIT tags in the field: 1) sea urchins preferably hide in the crevasses between rocks which limits accessibility and 2) the relatively short distance from which tags can be detected (also reported in Bubb et al. [38]). One way to address these limitations is to apply a technique suggested by Bubb et al. [38] and Roussel et al. [53], which calls for the use of a coil antenna or and 'open coil' antenna mounted on a pole to facilitate searching for tagged individuals in aquatic environments full of rocky crevasses. PIT tagging permits repeated non-destructive sampling of individuals. The claim that this technique has a theoretically indefinite life span, negligible tagging mortality, high

tag retention, and no apparent long term effects on growth and survival of tagged individuals [38], needs further verification in *P. lividus*. The PIT tags used in this study was a useful mechanism for individual sea urchin identification in the laboratory and in the subsequent field study. Additionally, this method provided tagged individuals are held in captivity for three months to test for tag retention, allows for a large number of animals to be marked and has the potential to address numerous questions relating to the behavior, mobility, habitat use, brood stock management, and denotes ownership within the laboratory and in the field.

Fingernail polish was the least successful tagging technique and caused 100% mortality. The bead technique is a temporary tagging solution but is highly stressful when compared to the control and PIT tagged individuals. Urchins released into rock pools were detected up to 6 weeks after release indicating that the use of these smaller PIT tags are a viable option in sea urchin culture.

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