SEA STAR, LUIDIA CLATHRATA, RESPONSES TO PHYSICAL AND THERMAL STRESS
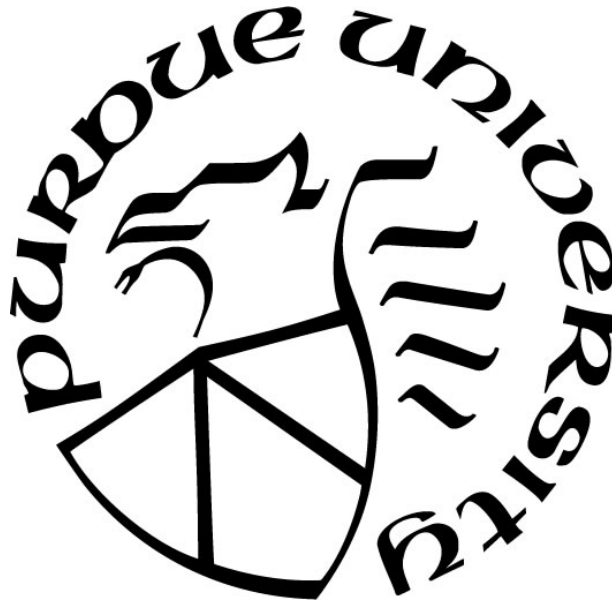
by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Biological Sciences at Purdue Fort Wayne

Fort Wayne, Indiana

August 2023

THE PURDUE UNIVERSITY GRADUATE SCHOOL
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This Dissertation is dedicated to my beloved grandfather Lok Bahadur Parajuli who was a man of wisdom and a constant source of inspiration to my family.
I am honored to fulfill his dream and continue my father's legacy by pursuing higher education.

ACKNOWLEDGMENTS

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ABSTRACT

Human actions and the resultant global warming are leading to considerable environmental changes that are negatively impacting marine ecosystems and their biodiversity. *Luidia clathrata*, a starfish species, is essential to the marine ecosystem, and understanding its sensitivity to stressors can help predict its future adaptations and role in the reef ecosystem. The study involved subjecting *L. clathrata* to thermal stress by incrementally raising the temperature by 1°C each day for a period of seven days. Physiological responses were evaluated on two separate occasions: day 1, which corresponded to the acute stress response, and day 7, which corresponded to the chronic stress response. The results showed a minor increase in phagocytic activity during acute thermal stress, but a significant decrease during chronic exposure. Although there was a slight decrease in total coelomic plasma protein during acute thermal stress, it significantly increased during post-chronic exposure. The amputated starfish avoided using the injured arm when righting themselves, indicating the development of neurosensory potential. Total cell count increased slightly in all stressed groups during acute stress but decreased after prolonged exposure to stressors. The mortality rate of the temperature-stressed groups was 33%, indicating that prolonged exposure to temperatures exceeding expected future temperatures could be harmful to *L. clathrata*. To support the hypothesis at the molecular level, RNA/DNA ratios and Heat shock protein gene 90, a molecular marker for cellular stress, were studied. Although no significant differences were observed in transcriptomic level, the temperature-stressed group showed slightly upregulated hsp90 gene expression. The findings indicate that *L. clathrata* responds to stress similarly to vertebrates, highlighting the potential impact of climate change on marine ecosystems. This study provides a baseline for comprehending the stress response of starfish, and further research is recommended with a larger sample size and over a more extended period. It is interesting to note that the gonad and body wall extracts of starfish exhibit significant inhibitory activity against various tested pathogens. The findings suggest that starfish extracts may have potential medicinal uses as antimicrobial agents. However further research is needed to understand the mechanisms of action behind these inhibitory activities and to identify the specific compounds responsible for them.

CHAPTER 1.   GENERAL INTRODUCTION

1.1   Introduction

The present state of global environmental change is unprecedented and more severe than any previously recorded. It is causing widespread displacement of organisms due to its local impacts. Both scientists and the general public acknowledge that climate change, largely caused by human activities, is causing a rise in global temperatures (Bose, 2010). The current global temperature has risen by 1°C (1.8 degrees Fahrenheit) since the late 19th century, and the rate of warming has accelerated in recent decades. Global warming is causing the ocean to warm and acidify, which is having a profound impact on marine ecosystems. Deterioration of local environments, combined with the local effects of global climate change, can lead to the depletion of crucial resources and ecosystem services (Bose, 2010; Poloczanska et al., 2013). The devastation caused by anthropogenic activities highlights the urgency in developing the necessary tools to tackle the growing challenges posed by climate change, environmental degradation, increasing population density, and natural disasters that lead to population displacement, resettlement, and mass extinction (Belkin, 2009; Bose, 2010; Mckinney et al., 2015; Poloczanska et al., 2013).

The marine environment is an incredibly diverse and complex ecosystem that supports a wide range of species. The movement of ocean currents helps to distribute nutrients and other essential elements throughout the marine environment, creating habitats that support a rich diversity of plant and animal life (Zhukova, 2022). In addition, the different types of undersea landscapes, such as coral reefs, kelp forests, and deep-sea trenches, provide unique habitats that allow different species to thrive. The ocean also plays a critical role in regulating the earth's climate and supporting global food security through the provision of important food resources, such as fish and shellfish (Zhukova, 2022). Unfortunately, the ocean and its marine life are facing numerous challenges, including the impacts of global warming and anthropogenic (human-caused) exploitation (Bose, 2010; Mckinney et al., 2015; Poloczanska et al., 2013). As the ocean warms, many species are moving to cooler waters in search of suitable habitats, and some are even going extinct (Bose, 2010; Mckinney et al., 2015; Poloczanska et al., 2013). Additionally, ocean acidification is making it more difficult for species such as corals, mollusks, echinoderms, and

certain types of phytoplankton to build and maintain their calcium carbonate skeletons, which are critical to their survival (Mckinney et al., 2015; Poloczanska et al., 2013).

Human activities, such as overfishing and recreation, pollution, and habitat destruction, are particularly having a significant impact on marine ecosystems. Specifically, overfishing is reducing the populations of many economically important aquatic species, and some are even on the verge of extinction. Pollution from sources such as oil spills, sewage, and plastic waste is harming marine life and habitats and destroying the biodiversity of the ocean (Belkin, 2009). Destruction of coastal habitats (wetlands and mangroves) due to recreational activities, is also affecting marine species by destroying their critical breeding and nursery ground, and destroying the biodiversity of the ocean (Belkin, 2009; Mckinney et al., 2015; Poloczanska et al., 2013).

Echinoderms are a diverse group of marine creatures known for their spiny skin and radial symmetry, which include well-known animals such as starfish, sea urchins, sand dollars, and sea cucumbers (Zhukova, 2022). These unique marine invertebrates with their bizarre appearances play essential ecological roles in marine ecosystems (Zhukova, 2022). For instance, sand dollars and sea cucumbers contribute to higher oxygen levels in deep-sea sediments, while starfish play a critical role in limiting the growth of algae on coral reefs (Zhukova, 2022). Additionally, they are gaining intense popularity because of their food values (delicacies), therapeutic potential, and agricultural benefits. Sea cucumbers, for instance, are quite famous in southeastern Asia as delicacies (Gomes et al., 2014; Micael et al., 2016; Zhukova, 2022). Specifically, in the Chinese continent, sea cucumbers are used as a basis for gelatinous soups and stews. Sea urchin gonads are consumed mostly in Japan, France, Spain, and Peru and are liked by many people for their unique taste described as the mixture of fruit and seafood (Gomes et al., 2014; Micael et al., 2016; Zhukova, 2022). Starfish, on the other hand, has not been fully explored for its food values however it has been documented as a famous street food cuisine in some parts of China, Indonesia, and Thailand (Gomes et al., 2014; Micael et al., 2016; Zhukova, 2022). Echinoderms have important uses in both medicine and scientific research. Sea cucumbers, for example, contain compounds that have shown promise in slowing the growth of cancer cells (Gomes et al., 2014; Micael et al., 2016; Zhukova, 2022). Sea urchins and sea stars are important model organisms in developmental biology research, as they undergo a variety of key physiological processes during early development that are relevant to many different areas of biological study (Gomes et al., 2014; Micael et al., 2016; Zhukova, 2022). In addition, the molecular mechanisms underlying these

developmental processes have been studied extensively in echinoderms, making them a valuable tool in many areas of research (Gomes et al., 2014; Micael et al., 2016; Zhukova, 2022). Interestingly, the hard skeletons of some echinoderms, such as sea urchins and certain types of starfish, can be a valuable source of lime for farmers in areas where limestone is scarce (Zhukova, 2022). The echinoderm skeletons are ground up and added to soil to improve its nutrient content, which in turn can promote better plant growth (Zhukova, 2022). This practice is particularly common in some parts of Asia, where echinoderms are harvested and processed for this purpose on a large scale (Zhukova, 2022). It is estimated that around 4,000 tons of echinoderms are used each year for agricultural purposes in this way (Zhukova, 2022).

Despite its wide range of ecological and medicinal values, echinoderms, specifically, starfish, are not fully explored (McClintock and Lawrence, 1985). *Luidia clathrata* commonly known as Slender arm starfish or gray sea stars are commonly found in the shallow waters of estuaries on the west coast of Florida (McClintock and Lawrence, 1985). Since these environments often experience fluctuations in water parameters such as temperature, salinity, and pH, starfish living in these areas can be easily affected by even slight changes in their environment (McClintock and Lawrence, 1985). As a result, we wanted to investigate how *L. clathrata* adapts to thermal and physical stress and if they likely to have unique adaptive mechanism at both physiological and molecular levels. Additionally, as *L. clathrata* is one of the least studied species, we also wanted to explore if they have any therapeutic potential by testing their antibacterial and hemolytic potential against pathogenic bacteria in vitro. We believe that a fine-grained understanding of stress response in terms of physiological and molecular parameters provides baseline information on adaptation mechanisms and internal functioning. Additionally, this information gives insight to conservation practitioners to preserve those species in the upcoming future as they are the driver of the marine ecosystem.

Our specific objectives included

1. To explore the physiological response of Sea Star (*L. clathrata),* exposed to thermal and physical stress.
2. To explore the antibacterial and activity of Sea Star (*L. clathrata*), tissue extract against selected pathogenic bacteria.
3. To explore the molecular response of Sea Star *(L. clathrata),* exposed to thermal and physical stress.

1.2   References

Belkin, I. M. (2009). Rapid warming of large marine ecosystems. *Progress in Oceanography*, *81*(1-4), 207-213. 10.1016/j.proenv.2011.03.030

Bose, B. K. (2010). Global warming: Energy, environmental pollution, and the impact of power electronics. *IEEE Industrial Electronics Magazine*, *4*(1), 6-17. 10.1109/MIE.2010.935860

Gomes, A. R., Freitas, A. C., Rocha-Santos, T. A., & Duarte, A. C. (2014). Bioactive compounds derived from echinoderms. *Rsc Advances*, *4*(56), 29365-29382. 10.1039/C4RA03352C

McClintock, J. B., & Lawrence, J. M. (1985). Characteristics of foraging in the soft-bottom benthic starfish *Luidia clathrata* (Echinodermata: Asteroidea): prey selectivity, switching behavior, functional responses and movement patterns. *Oecologia*, *66*, 291-298. https://doi.org/10.1007/BF00379867

McKinney, M.A., Pedro, S., Dietz, R., Sonne, C., Fisk, A.T., Roy, D., Jenssen, B.M. and Letcher, R.J. (2015). A review of ecological impacts of global climate change on persistent organic pollutant and mercury pathways and exposures in arctic marine ecosystems. *Current Zoology*, *61*(4), 617-628. https://doi.org/10.1093/czoolo/61.4.617

Micael, J., Alves, M. J., Costa, A. C., & Jones, M. B. (2016). Exploitation and conservation of echinoderms. In *Oceanography and Marine Biology* (pp. 203-220). 10.1201/9781420094220-7

Poloczanska, E.S., Brown, C.J., Sydeman, W.J., Kiessling, W., Schoeman, D.S., Moore, P.J., Brander, K., Bruno, J.F., Buckley, L.B., Burrows, M.T. and Duarte, C.M. (2013). Global imprint of climate change on marine life. *Nature Climate Change*, *3*(10), 919-925. https://doi.org/10.1038/nclimate1958

Zhukova, N. V. (2022). Fatty Acids of Echinoderms: Diversity, Current Applications and Future Opportunities. *Marine Drugs*, *21*(1), 21. 10.3390/md21010021

CHAPTER 2.   PHYSIOLOGICAL AND BEHAVIORAL RESPONSES OF SEA STAR, *LUIDIA CLATHRATA*, EXPOSED TO THERMAL AND PHYSICAL STRESS

2.1   Abstract

Human activities such as climate change, pollution, habitat destruction, and overfishing are causing significant environmental changes that are having a severe impact on marine ecosystems and biodiversity. Echinoderms, including the starfish species, *Luidia clathrata*, are important in marine ecosystems, and understanding their sensitivity to stressors can help predict their future adaptations and their role in the reef ecosystem. To investigate this, we exposed *L. clathrata* to thermal stress by gradually increasing the temperature by 1°C each day for 7 days and physical stress by amputating one of their arms. We measured various physiological responses on day 1 (post-acute exposure) and day 7 (post-chronic exposure). Our results showed a slight increase in phagocytic activity during acute thermal stress, but a significant ($p<0.001$) drop during chronic exposure. Total coelomic plasma protein was reduced slightly during acute thermal stress but significantly ($p<0.001$) increased during post-chronic exposure. We also observed some behavioral change such as righting activity and righting mode which is neurosensory mechanism. Although we did not observe significant changes in righting timing among the different stressed groups, we did notice that arm-amputated starfish tended to avoid using the injured arm when righting themselves, which suggests that they have developed neurosensory potential. We observed a slight increase in total cell count in all stressed groups during acute stress, but this count decreased after prolonged exposure to stressors. The temperature stressed groups had a mortality rate of 33%, indicating that prolonged exposure to temperatures that are expected to be exceeded in the future could be harmful to *L. clathrata*. Our results suggest that *L. clathrata* responds to stress in ways similar to vertebrates, highlighting the potential impact of climate change on marine ecosystems.

2.2   Introduction

The stress response in animals is primarily coordinated by the release of neuroendocrine factors, which induce physiological changes that are mostly adaptive (Adamo, 2012; Tort et al., 2020). However, stress-induced changes in immune function are largely immunosuppressive, which raises questions about their fitness advantage. The fact that receptors of stress hormones and their reciprocal signaling pathways are present in immune cells indicates that the impact of stress response on the immune system is likely to be beneficial. (Adamo, 2012; Tort et al., 2020). Due to the intricate nature of vertebrate systems, it presents challenges in testing for adaptive functions. However, studies conducted on both vertebrates and invertebrates indicate that certain neuroendocrine/immune connections are shared across different animal groups, as evidenced by research conducted by Adamo (2012), Smith et al. (2018), and Tort et al. (2020). This conservation implies that investigating the neuroendocrine/immune relationships in organisms with simpler physiological structures may yield outcomes that can be broadly applied (Tort et al., 2020; Smith et al., 2018). Invertebrate immune systems use three main mechanisms to defend against pathogens: physical barriers, humoral mechanisms, and cellular defenses (Smith et al., 2018; Tort et al., 2020). These mechanisms are similar to those found in vertebrates' innate immune systems (Adamo, 2012; Ader and Cohen, 1993; Hatanaka et al., 2009). Invertebrates have molecules in their hemolymph/hemocyanin that can recognize pathogen-associated molecular patterns (PAMPs) and induce an immune response (Adamo, 2012; Ader and Cohen, 1993). This response includes the production of cytotoxic compounds and activation of cell-mediated responses (Adamo, 2012; Ader and Cohen, 1993). Hemocytes, which are similar to macrophages, are important for invertebrate immunity and can perform functions such as phagocytosis. Invertebrates lack the acquired immune system found in vertebrates but have evolved mechanisms to produce a diverse range of proteins that can recognize various pathogens (Pinsino et al., 2007; Smith et al., 2018).

Invertebrates also utilize neuroendocrine system to optimize their physiological reaction for hyperarousal behaviour such as fight-or-flight response, as suggested by studies conducted by Adamo (2012) and Roeder (1999). While there is greater diversity in neuroendocrine systems among invertebrate groups compared to vertebrates, certain molecules play a role in mediating aspects of the acute stress response across different animal phyla (Adamo, 2012; Roeder, 1999). In invertebrates, the acute stress response initiates with the rapid release of a biogenic amine, followed by the release of a peptide or protein that mobilizes energy reserves, as noted by Elis et

al. (2011). The adaptation of an animal's physiology to prepare for fight-or-flight may represent an ancient and well-preserved function of these substances. Cortisol is a steroid hormone that is often associated with stress, and the detection of cortisol in non-chordate groups such as echinoderms, bivalves, and holothuroids suggests that these organisms may also experience stress responses similar to those observed in chordates (Hamel et al., 2021; Pei et al., 2012). This finding has important implications for our understanding of stress and stress responses across the animal kingdom, and highlights the importance of considering the well-being of all animals, not just those traditionally considered to be "higher" animals such as mammals. Chronic stressors for invertebrates also include predation attempts, adverse environmental conditions, and parasitism (Adamo, 2012). Chronic stress often leads to elevated stress hormone levels in both vertebrates and invertebrates, which can result in negative effects such as a decline in immune function, weight loss, reduced growth, reproduction, and survival (Adamo, 2012; Smith et al., 2018). However, chronic stress can also have positive effects on some invertebrates, such as improving sustained flight and anti-predator behavior. Although it is not confirmed, the idea that stress hormones play a role in mediating these effects is possible. It is conceivable that elevated stress hormone levels at baseline are a typical response among animals from various phyla to sub-optimal environments (Adamo, 2012; Smith et al., 2018). In their study, Hamel et al. (2021) discovered that exposing *Cucumaria frondosa* to physical stressors caused a significant increase in certain markers, such as cortisol levels spiking within half an hour and cell counts in the hydrovascular fluid increasing by up to 700-770% over three hours. Long-term exposure to stress without physical contact led to a reduction in immune markers, suggesting habituation. These findings suggest that echinoderms may have adaptive stress responses similar to those observed in other vertebrates, as they belong to the phylum that is closest to vertebrates (Hamel et al., 2021).

Echinoderms, which are ancient deuterostomes, do not have a complex, adaptive immune system and rely primarily on innate immunity (Smith et al., 2018). Coelomocytes (collection of cellular morphotypes) are freely suspended in the coelomic fluid of the starfish. Coelomocytes have been well documented for its diverse set of immunological functions including but are not limited to formation of cellular clots, phagocytosis, encapsulation, clearance of the bacteria and other foreign pathogens, inflammatory reaction, oxygen transport and regeneration of lost tissue (Ballarin et al., 2021; Cooper, 2018; Pinsino et al., 2007).

Phagocytosis is an essential biological mechanism that safeguards the host organism against detrimental extrinsic particles, whether they are infectious or not, and eliminates unwanted or impaired host cells to sustain tissue homeostasis. In general, phagocytosis is a fundamental process that serves to maintain an organism's well-being and wholeness. Coelomocytes of echinoderms are known to pose impeccable phagocytic capacity and thus an important component of the immune response (Cooper, 2018; Pinsino et al., 2007). In addition to their role in immune defense and tissue regeneration, coelomocytes have also been studied for their potential as biomarkers of the environmental stress and pollution in echinoderm. Studies have shown that exposure to pollutants can cause changes in coelomocyte morphology, number, and activity such as encapsulation and phagocytosis (Ballarin et al., 2021; Ben et al., 2015; Cooper, 2018; Pinsino et al., 2007). Furthermore, the number of circulating coelomocytes and the coelomocyte free plasma protein in some holothurian and asteroid rapidly increases in the first hours after arm tip amputation or an immune challenge, indicating their importance in the early post-traumatic period (Lawrence et al., 2010; Pinsino et al., 2007).

Starfish which are highly sensitive to seawater temperature, and increased ocean temperatures due to climate change have negative effects on their development, growth, metabolism, immunity, behavior, and gene expression profiles (Lawrence et al., 2010; Leclerc and Bajelan, 1992; Pinsino et al., 2007). Various studies have demonstrated how higher temperatures can compromise early development in sea urchins, reduce growth rate and unsaturated fatty acid content in juvenile sea cucumbers, and alter immune response variations in different sea urchin species (Kohl et al., 2016; Shimizu et al., 1999). Sea stars have been observed to experience other sources of stress besides the ones mentioned earlier, such as salinity, ocean acidification, hypoxia, and predation. These stressors have also been found to impact the sea stars' ability to flip themselves over if they become overturned which is called righting ability. Righting time is a useful proxy for overall performance in response to temperature stress in echinoderms, as it measures neuromuscular coordination and the ability to move in response to environmental conditions (Ardor, 2019; Stickle and Diehl, 1987; Watts and Lawrence, 1990).

Human activities and global warming have been causing adverse effects on marine species, particularly shallow water echinoderms like starfish (McClintock and Lawrence, 1985; Zhukova, 2022). These disturbances have had a significant impact on the sand sifting sea star (*L. clathrata*), which typically inhabits estuaries with fluctuating environmental factors (McClintock and

Lawrence, 1985; Zhukova, 2022). Therefore, it is crucial to investigate how this species responds to changes in temperature and physical stress to understand its potential role in reef ecosystems. This study aims to determine post-acute (1 day) and post-chronic (7 days) physiological effects of elevated temperature (as a proxy for global warming) and arm amputation (as a representation of autotomy) on *L. clathrata*, using cellular, immunological, and behavioral markers.

2.3   Materials and methods

2.3.1   Animal acquisition and maintenance

Healthy adult *L. clathrata* (24.41±1.50gm body weight, 7.37±0.67cm arm length, n=24) were purchased from a certified animal vendor (Gulf Specimen Marine Lab, Panacea, Florida, USA) in May 2021. Individuals were transported to the invertebrate laboratory in Life Science Resource Center (LSRC), Purdue University Fort Wayne. To acclimate the starfish to the new environment and avoid any shock, they were carefully transferred to separate aquaria (Ten-gallon capacity) with three individuals. The process began by floating the bag containing the starfish in the aquarium for 15 to 20 minutes to ensure that the temperature inside the bag was the same as the tank's temperature. Next, a few small holes were made in the bag to allow for a gradual release of water into the tank. Once half of the water had been released, the starfish were finally introduced into the aquariums. Organisms were left for 24 hours for the acclimation in optimal water conditions (temperature: 68-70ºF, salinity: 28±1 ppt, ammonia: 0-0.25mg/L, pH:7.8-8.0) with 12:12 light-dark cycle in the lab.

2.3.2   Experimental design

Organisms were divided into four experimental groups and each experimental group had two technical replicates with three individuals as shown in figure 2.1. After 24 hours of acclimation, organisms in the stress groups were exposed to the respective stressor, including thermal, physical stress, and a combination of both (thermal + arm amputation). On the basis of the Temperature Coefficient (Q10) law, a 1°C increase in temperature per day for seven days was applied to the temperature-stressed group (Mundim et al., 2020). To bring the physical stress, the arm amputation group was stressed by cutting off one of the arm tips (1-1.5 cm) with the scalpel aseptically (Fan, 2011). Starfish were carefully returned to the aquarium after the arm excision and allowed to bury

themselves in the sand. For the combined stressed group, the arm tip was excised on day 1 and exposed to the gradual temperature rise for seven days. All the water quality parameters were monitored and assessed daily with API Saltwater Aquarium Master Kit and maintained throughout the experimental period. The temperature-stressed groups received a sufficient amount of water additions to keep the salinity and water volume constant as a result of the high temperatures causing water evaporation. Organisms were fed ad-libitum with the chopped frozen shrimp (10mm size) daily. The food remnants were removed as soon as the feeding stopped.
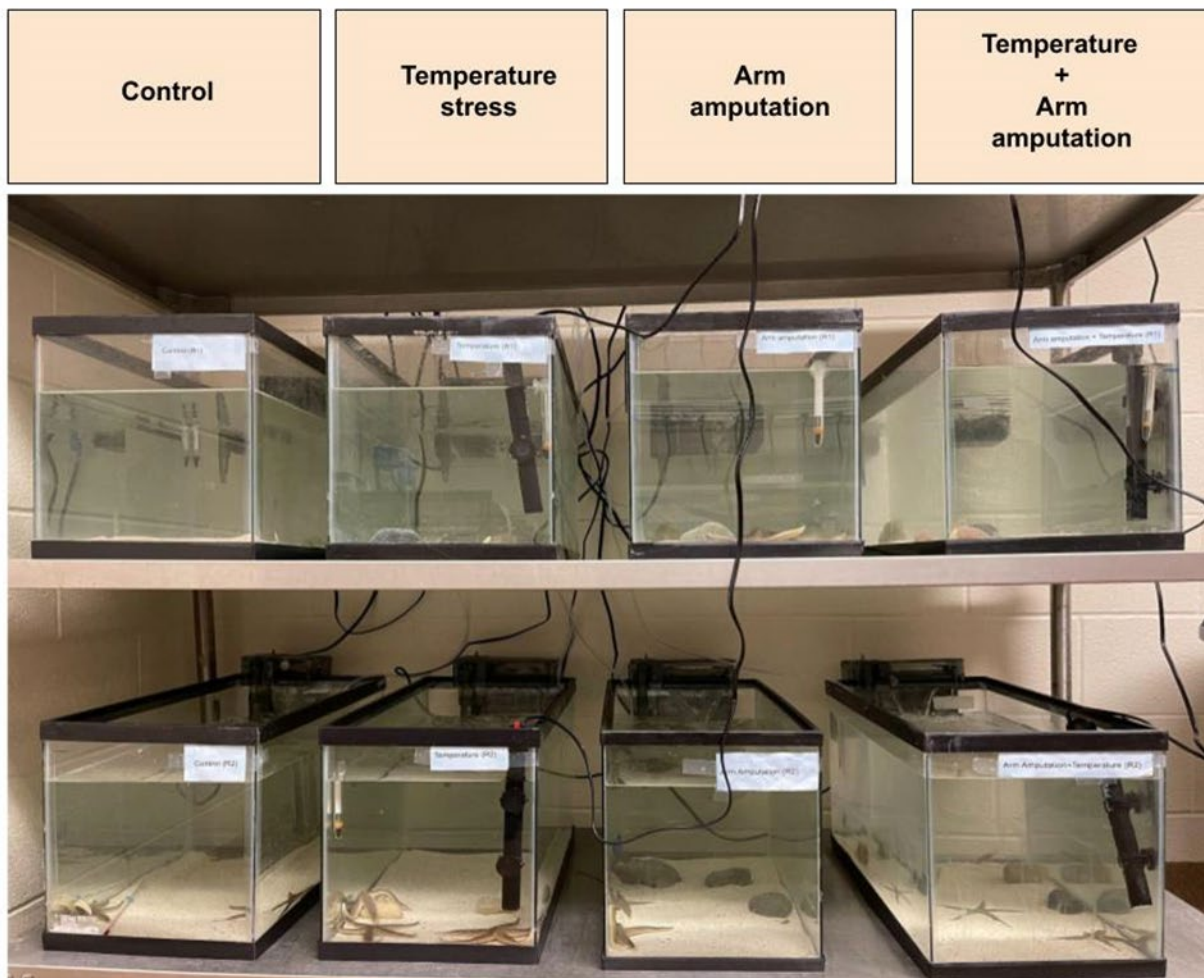
Figure 2.1 Experimental design for the stress study of starfish exposed to the designated stressor (Temperature, Arm amputation, and combination of both). The upper and lower row of the tanks represents technical replicates 1 and 2 respectively. Each tank housed three starfish.

2.3.3 Preparation of the coelomic fluid

To harvest Coelomic fluid (CF), an arm tip of the starfish was punctured by a 26 mm gauge needle attached to a 1 ml syringe (Dickinson and Beacon). Not more than 250μL of coelomic fluid was drawn from each individual to avoid sacrificing the starfish. Coelomic fluid was drawn carefully and transferred into the Eppendorf tube and kept in an ice bucket for the subsequent investigations.

2.3.4 Total coelomocyte count

A live coelomocyte count was performed with the freshly acquired CF. To begin with, a glass hemocytometer (Bright-Line, Hausser Scientific, USA) was taken and wiped out with absolute methanol to make it sterile. The coverslip was moistened with water and affixed to the hemocytometer. 10μL of the coelomic fluid was carefully loaded to both chambers located under the coverslip, allowing the suspension to be pulled into the chambers by capillary action. The CF-loaded hemocytometer was then observed under the microscope. Different cellular morphotypes were counted using the hand tally counter separately and summed up to get the total coelomocyte count. Total coelomocytes in an ml were counted by using the following formula (Guillard and Sieracki, 2005).

Total coelomocyte count (TTC) = Average number of coelomocytes from each 16 square$\times 10^4$

2.3.5 Total coelomic protein

The concentration of protein in the blood plasma is a valuable marker of an organism's overall health and stress levels. In response to stress, the body produces specific proteins that act as molecular chaperones to help repair damaged cells. To measure the total protein in CF, a protein refractometer (VEE GEE, CLX-1, USA) was used, which calculates the refractive index of all the solid substances dissolved in the plasma. Prior to usage, the refractometer was calibrated using a solution of phosphate buffer sodium (PBS). The cell-free coelomic fluid was prepared by hematocrit centrifugation (MB Microhematocrit Centrifuge, International Equipment Co.) at 1000 rpm for 10 minutes. To obtain a measurement, a small amount of cell-free CF was placed on the prism in such a way that it covers the entire surface, and the corresponding value was then noted.

2.3.6 Phagocytic capacity

The phagocytic capacity of *L. claharata's* coelomocytes was measured by following the protocol by Fast et al., (2002). To begin with, 50µL of CF was taken in an Eppendorf tube and 50µL anticoagulant was added. Cells were adequately mixed using a vortex and dispensed in 50µL volumes into each etched circle (10mm) on the Fluoro slides (Esco, Selected Microscopic Slides, Erie Scientific). The slides were incubated for 1 hour at room temperature. 50µL of *Bacillus megaterium* was then added and left for another hour. The slides were washed carefully with PBS and fixed in alcohol for a minute followed by staining with the Wright Giemsa stain for 20 seconds. Slides were then observed under the light microscope (100X) in oil immersion. At least a hundred cells were counted to determine the percentage of positive phagocyte cells. Cells with 5 or more bacteria inside them were counted as positive phagocyte cells.

$$\% \, Positive \, phagocytes = \frac{Number \, of \, positive \, cells}{Total \, number \, of \, counted \, cells} X100\%$$

2.3.7 Righting behavior

Since the righting response correlates with the functioning level of the neuromuscular system which involves locomotion and predatory activities, it was used as a behavioral stress marker in this study. Righting Activity Coefficient (RAC) is calculated by the following formula recommended by Stickle and Diehl (1987). To observe the righting activity, the starfish were inverted gently with the help of blunt forceps and timed up until it was completely right back to the normal position. Video of each starfish's righting activity was recorded with the help of iPhone 12 pro max (Apple Inc, California, USA) for later analysis. The individual which could not invert back itself within the 10 min was assigned a value of 0. Righting activity for all the individuals was observed with great care after they recovered from the trauma of syringe aspiration.

$$Righting \, Activity \, Coefficient \, (RAC) = \frac{1000}{Time \, taken \, to \, right \, back}$$

2.3.8 Statistical analysis

The data analysis was performed using SigmaPlot 14.0, Systat Software INC. Highly skewed data were log-transformed to meet the normality assumption. The statistical significance ($p<0.05$) of the means was determined using a one-way analysis of variance (ANOVA). To assess the significance between the three groups, a Tukey test was conducted. Data are presented as means ± standard error of means (SEM).

2.4 Results

2.4.1 Total coelomocyte count

Figure. 2.2 shows a typical profile for total cell count for all experimental groups during the post-acute and post-chronic exposure period of the present experiment. We did not find any significant changes in the total cell count in any of the experimental groups when compared to the control during both acute and chronic studies. However, we can see from the general patterns (Figure 2.2) that the acute stress has induced a slight increase in the total coenocytes in the stressed *L. clathrata* which later decreased in post chronic exposure.
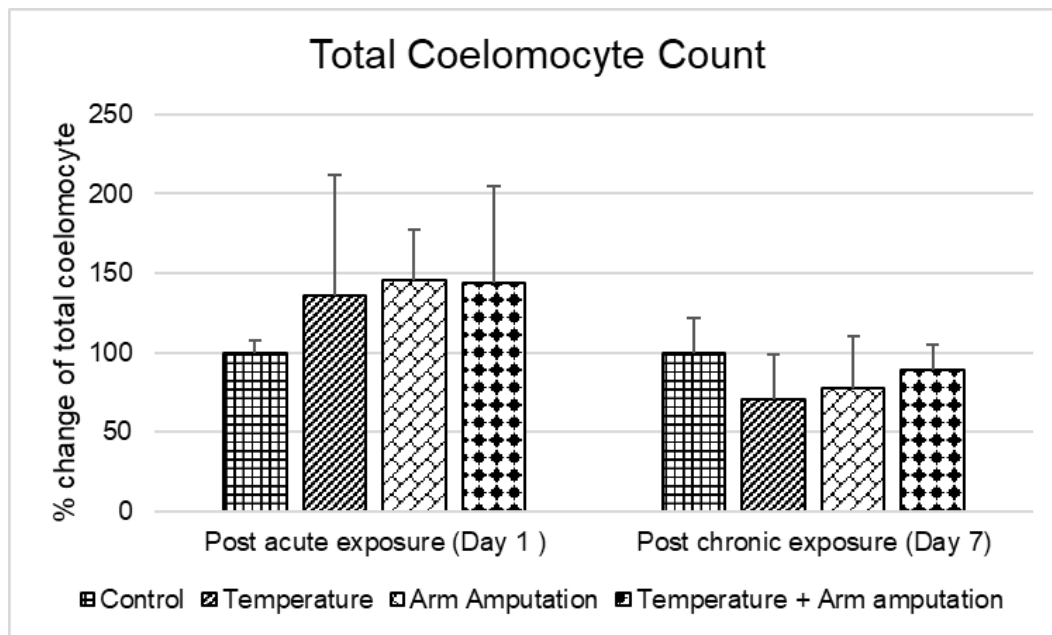
Figure 2.2 Total coelomocyte count of *L. clathrata* exposed to various stressors. Data are presented as means ± SEM ($P<0.05$, n=3).

2.4.2 Total Coelomic protein

We did not notice any changes in the total CF protein concentration during the acute exposure (Figure 2.3). We noticed the temperature-only stressed group exhibited a significantly ($p<0.001$) higher amount of total CF protein compared to the control during the chronic exposure (Figure 2.3). However, we did not notice any change in the total CF concentration in the group stressed by arm amputation when compared with the control. The combination group also demonstrates a significantly ($p<0.001$) higher level of CF protein production than the control. Our results indicate that arm amputation did not have any significant effect on the total CF protein production since the only difference in CF protein concentration was observed in the groups that received thermal stress. Thus, we inferred that the observed changes in CF protein concentration are due to thermal stress and not arm amputation.
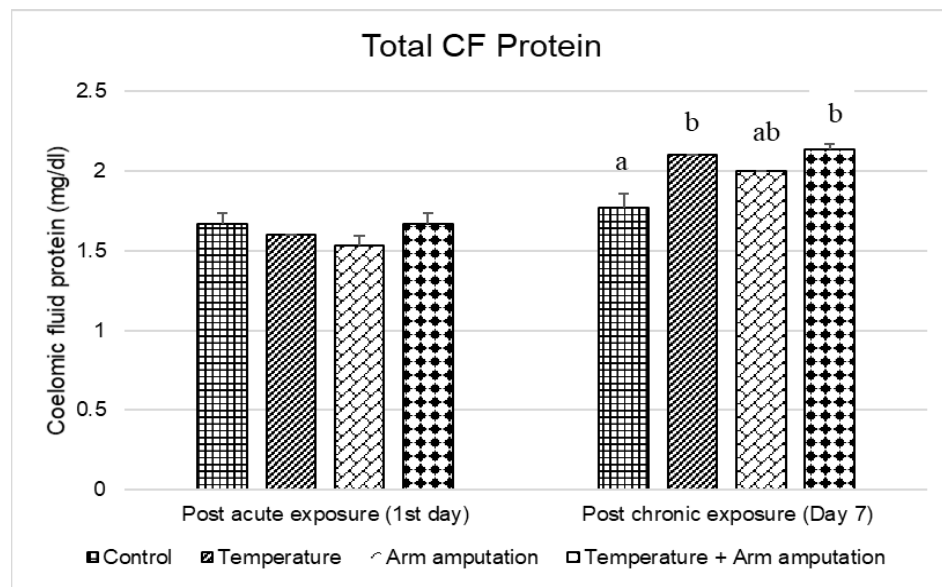
Figure 2.3 Total coelomic fluid protein (mg/dl) of *L. clathrata* exposed to various stressors. Data are presented as means ± SEM. Different Alphabet represents the significant difference in the experimental group compared to the control within post-acute and post-chronic exposure ($p<0.001$, n=3).

2.4.3   Phagocytic capacity

We did not notice any significant change in the phagocytic activity in any of the experimental groups after first day of exposure period. However, looking at the pattern of the result (Figure 2.4), we can see that the temperature-stressed and arm amputation group had relatively higher phagocytic activity compared to the control. During post-chronic exposure (after 7 days), we noticed a significant ($p < 0.001$) decrease in the phagocytic activity in the temperature-stressed and the combination group. We did not notice any changes in the phagocytic capacity for the arm amputation group indicating that autotomy may be the normal physiological activity for the starfish. The compromised phagocytic activity observed in both temperature-stressed groups indicates that an increase in temperature by 7°C is sufficient to cause an imbalance in their cellular homeostasis.
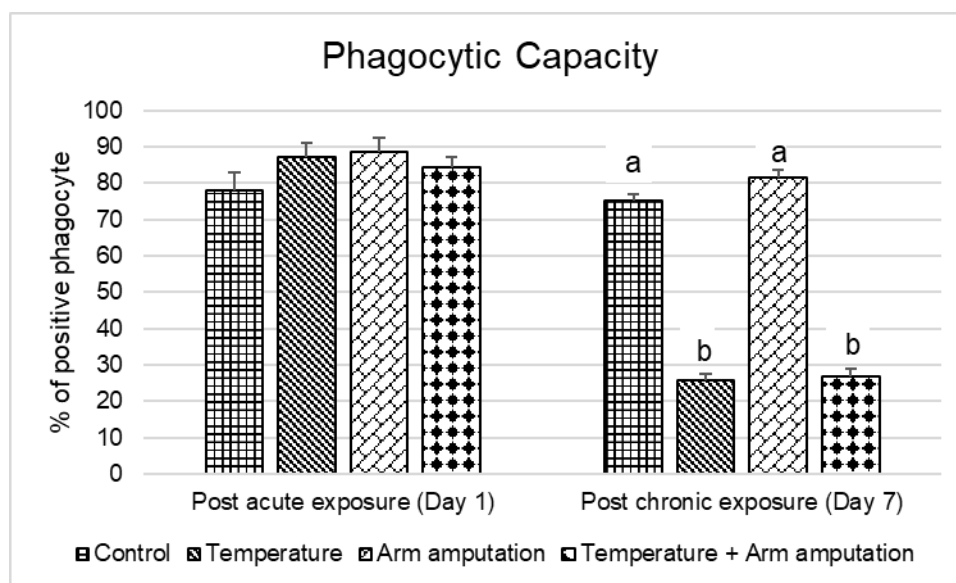
Figure 2.4 Phagocytic capacity of coelomocytes from *L. clathrata* exposed to various stressors. Data are presented as means ± SEM. Different Alphabet represents the significant difference in the experimental group compared to the control within post-acute and post chronic exposure ($p < 0.05$, n=3).

2.4.4   Righting behavior

We found no significant impact of any of the stressors (temperature, arm amputation, and combination of both) in righting time of *L. clathrata* (Figure 2.5). The results showed no significant difference in the time taken for the experimental group to right back compared to the control group during acute exposure, but while observing general behavioral patterns, we noticed that starfish from the arm amputation group were slightly lethargic and took longer time to initiate

the righting. During chronic exposure, the arm amputated group tended to right back relatively faster than control. Even though the results were inconclusive regarding the effects of stress on righting time in the present experiment, the experimental groups showed different righting modes. Notably, starfish with amputated arm tips avoided the injured arm while righting, whereas those in the other experimental groups somersaulted to right back using all five arms. They raised the central disk while attaching to the substratum with the other four arms, and one arm took the lead to tip their body sidewise and inverted. In contrast, the starfish with an amputated arm twisted and curled all of their arms to touch the substratum, allowing them to right themselves gently to regain the oral surface on the substratum.
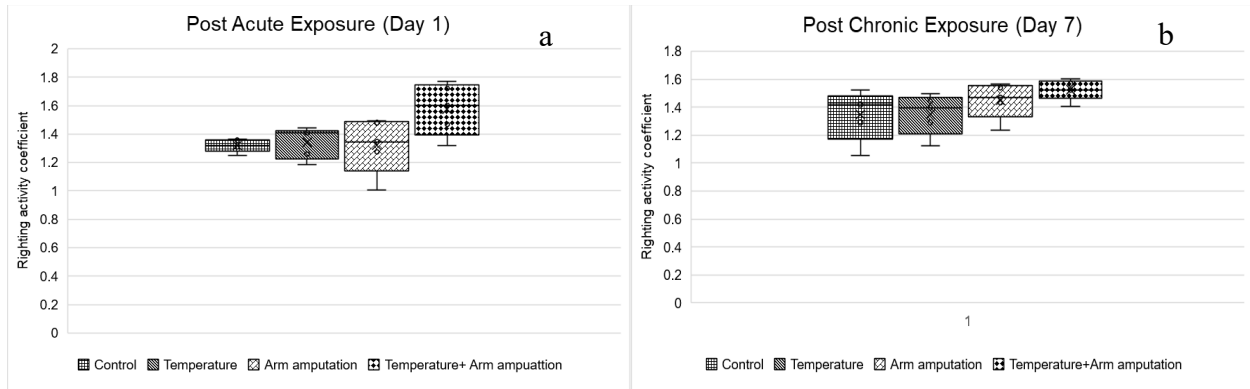
Figure 2.5 Day 1 (a) and day 7 (b) righting activity of *L. clathrata* exposed to various stressors. Data are presented as means ± SEM (p>0.05, n=3).

2.4.5   Survival rate

Figure. 2.6 illustrates the percentage of survivability among various groups in the post-acute (after 1 day) and post-chronic (after 7 days) exposure periods. No mortality was reported until the 7th day. The group that was exposed to thermal stress had a mortality rate of 16.66%, while the combination group had a mortality rate of 33.33%. There were no deaths in the groups with armed amputation. The overall survival rate for the thermal stress group during the chronic exposure period was 66.66%.
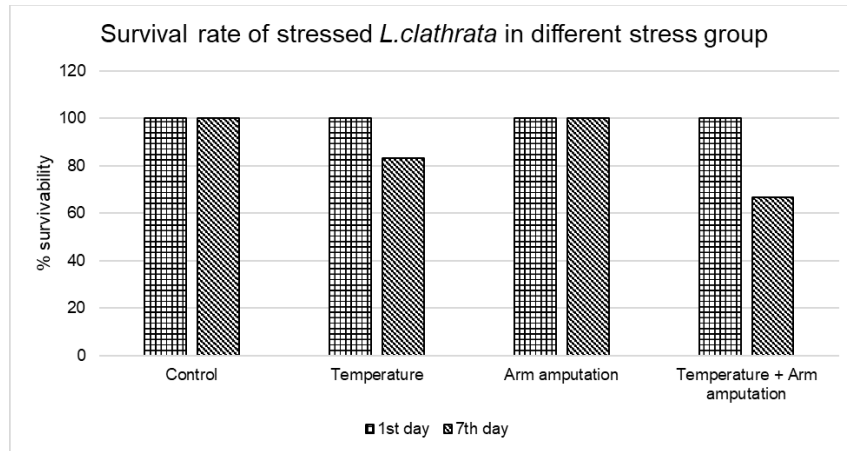
Figure 2.6 Survival rate of *L. clathrata* exposed to various stressors.

2.5   Discussion

L. clathrata is typically found in shallow waters in estuaries, where environmental factors fluctuate significantly with seasonal variation. However, recent global warming and human activities causing physical disturbances may have impacted the organismal activity of this species (Poloczanska et al., 2013). Therefore, it is essential to investigate the sensitivity of *L. clathrata* to physical stress and changes in temperature to understand the potential impact of future alterations in environmental conditions on this species and its role in the reef ecosystem. In this study, we aimed to determine how an elevated temperature (anticipatory global warming) and arm amputation (representing autotomy) affect the overall physiology of the asteroid, *L. clathrata,* by leveraging cellular, immunological and behavioral markers.

Coelomocytes have been investigated as potential biomarkers for environmental stress and pollution in invertebrates very well. The present study provides a breakthrough in understanding the acute vs chronic stress response of the *L. clathrata* coelomocytes when stressed with thermal and physical stress. Similar to sea urchins and other starfish, our study found that the majority of the cells are phagocytes, which constitute almost 95% of the coelomocyte population in starfish, with the remaining 5% consisting of other cell types (Pinsino et al., 2007; Smith et al., 2018). Although we did not observe significant changes in coelomocyte population in different stressed groups during acute and chronic exposure, we did observe a slight increase in total coelomocyte count in the stressed groups compared to the control during acute stress. However, we noticed a pattern of slight decrease in total coelomocyte count, consistent with changes in phagocytic

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capacity in different stressed groups. Findings of our study are consistent with those of Pinsino et al (2007), who observed that the levels of circulating coelomocytes were influenced by time. The researchers speculated that this effect could be due to either the fast multiplication of circulating stem cells or the mobilization of coelomocytes from other parts of the body. Our study also observed a slight increase in coelomocytes, which could be attributed to the body's preparation for the fight or flight response (Adamo, 2012; Smith et al., 2018).

The total CF protein production in our study is different from what has been studied previously. The total coelomic fluid protein production went down as soon as the organism was exposed to the stressor and increased significantly during chronic exposure. However, most of the vertebrate plasma protein went high during acute stress followed by the decrease during the long term exposure (Hamel et al., 2012; Holm et al., 2008; Lang et al., 2022). Several studies have shown an increase in protein production during the first hour of exposure, but no significant increase was observed in plasma free protein production. Different species exhibit varied responses in terms of protein production, with some showing an increase during acute stress followed by decreases during prolonged exposure, while others display an increase in protein production in chronically stressed species. It is not unexpected to observe discrepancies in our results when compared to previous studies, such as Pinsino et al., (2007) research on *Asterias rubens*. Their study reported a time-dependent modulation of proteins in which the level of hsp 70 peaked six hours after physical stress and decreased after 24 hours, but still remained higher than the control group. Furthermore, in a study by Zhan et al (2019), the sea urchin, *Strongylocentrotus intermedius*, showed an upregulation of heat shock protein 70 and heat shock protein 90 after 28 days of exposure to thermal stress. It is not clear why *L. clathrata* showed a decrease in total coelomic plasma protein after one day of exposure, followed by a significant increase after seven days. It is reasonable to consider that a slight increase in temperature, just one degree higher than normal, may not be significant enough to induce a change in the proteomic level of *L. clathrata*. As observed in the study, it was only after seven days of exposure to a temperature of 7 degrees higher than normal that a change in protein levels was detected. Therefore, it is possible that the stressor was not strong enough to cause an immediate impact within 24 hours.The differences between our findings and those of previous studies may be attributed to the fact that we measured the total protein level instead of specific proteins.

We observed no significant change in the phagocytic potential during acute stress but evidenced a significant reduction in phagocytic activity in the thermally stressed group during post-chronic exposure. Based on our results, a slight increase in the ability of coelomocytes to engulf foreign substances after 24 hours of exposure to a stressor suggests that the organism is activating its immune system to adapt to the stress and maintain a balanced internal environment. This result aligns with the well established vertebrate immune response where stress hormones secreted due to acute stress allows the body to re-allocate the resources to maximize the immune function during flight and fight response (Adamo, 2012; Smith et al., 2018; Tort et al., 2020). Our result is consistent with previous research on invertebrates, which has shown that some species, such as holothurians, undergo a "flight or fight " response when subjected to acute stress, which prepares them immunologically (Lavine and Strand, 2002). In certain mollusks and echinoderms, the acute stress response triggers the release of stress hormones like epinephrine, which prompts a series of biochemical changes that help reconfigure the molecular, cellular, and physiological networks in order to maintain optimal immunity in the face of changing internal conditions. This may allow them to more effectively deal with potential future adversities and attacks (Adamo, 2012; Hamel et al., 2021). Furthermore, a significant decrease in coelomocyte phagocytosis during post chronic period indicates that prolonged stress can lead to immunocompromisation. Interestingly, only the groups subjected to thermal stress showed a significant decline in phagocytic activity, indicating that natural predation attempts did not have an effect during chronic exposure. This may be because organisms with amputated limbs were able to redirect energy towards wound healing and regeneration during acute stress, which is a natural adaptive process known as autotomy (Pinsino et al., 2007). We did not observe any changes in the rate of wound healing in the group with amputated limbs due to temperature stress. Our findings are consistent with previous studies, which have shown that chronic temperature stress can compromise immunity in starfish such as *Asterias rubens* (Coteur, 2003).

Research has demonstrated that changes in temperature can have a substantial impact on the righting time and movement rate of echinoderms . Some species may not be affected by a small increase in temperature of 2°C, while others may experience significant declines in performance with just a 1°C increase (Ardor and Smith 2019; Watts and Lawrence, 1989). According to a study conducted by Watts and Lawrence (1989) exposure to low temperatures (17°C) for 30 days led to a decline in both the righting ability and feeding rates of *L. clathrata*. Furthermore, the study found

that there was little to no acclimation to the low temperature conditions. When exposed to combinations of high or low temperatures and low salinity levels, there was a negative impact on the organism's righting. These performance declines can have adverse effects on echinoderms' ability to find food and evade predators, ultimately limiting their capacity to cope with the higher energy demands of a warmer ocean. Although our study did not reveal any significant changes in the righting ability of starfish, we did observe a shift in the mode of righting in the arm amputated starfish. This change in behavior suggests an advanced neurosensory ability, as the starfish tried to avoid the injured arm (Pinsinno et al., 2007) . This finding is consistent with Pinsino et al (2007) research on arm amputated *Asterias rubens*, where the starfish raised the wounded tip upward to prevent the loss of coelomic fluid. Similarly, *Acanthaster solaris* exposed to elevated temperatures for over 60 days, showed no changes in their righting ability. However, they did exhibit different modes of righting and faster rates of movement, which allowed them to travel further to reach the edge of the aquaria.

Our study found that chronic exposure to a 7°C temperature increase resulted in a decreased chance of survival for *L. Clathara*, with mortality rates of 16% and 33.33% in the temperature-only stressed groups and combination groups, respectively. However, we did not observe any mortality in the arm amputated group, suggesting that autotomy may be an adaptive response in *L. clathrata*. This adaptation can occur within 24 hours. Our findings are consistent with the thermal stress response observed in *Acanthaster solaris*, where exposure to 32°C temperature had a significant effect on the probability of survival (Lang et al., 2022).

Invertebrates have less complex immune and endocrine systems than vertebrates, but still experience stress-induced changes in immune function, which may be adaptive and depend on various factors such as context, stressor, time, and concentration of stress hormones (Adamo, 2012; Smith et al., 2018; Tort et al., 2020). The presence of elevated levels of cortisol in echinoderms, bivalves, and holothuroids indicates that stress responses are conserved across different phyla (Hamel et al., 2021; Pei et al.,2012). This discovery holds significant implications for comprehending stress and stress responses throughout the animal kingdom. Stress hormones in organism are believed to play a role in redistributing resources during fight-or-flight responses by triggering molecular, cellular, and physiological changes that enable the animal to maintain optimal immunity as its internal environment shifts (Adamo, 2012; Smith et al., 2018; Tort et al., 2020). This reconfiguration results in the enhancement of certain immune functions while

suppressing others. Our findings are consistent with this idea, as we observed an increase in phagocytic capacity during acute exposure followed by a decrease in chronic exposure, as well as a slight decrease in total plasma protein during post-acute exposure.

2.6   Conclusion

The stress response is a necessary mechanism for survival, but prolonged exposure to stress can have negative effects on the body, leading to exhaustion and compromising the immune system. Present study investigated the response of *L. clathrata*, a species of starfish, to thermal and physical stress. The results showed that the species responded to stress in ways similar to vertebrates, including changes in phagocytic activity, coelomic plasma protein levels, and total cell count. Arm amputated starfish showed potential neurosensory development by avoiding using the injured arm when righting themselves. The study found that prolonged exposure to thermal stress could be harmful to *L. clathrata*, as evidenced by the mortality rate of 33% in the temperature-stressed groups. These findings suggest that climate change and other human activities could have severe impacts on marine ecosystems and biodiversity, including the potential loss of important species like *L. clathrata*.

2.7   References

Adamo, S. A. (2012). The effects of the stress response on immune function in invertebrates: an evolutionary perspective on an ancient connection. *Hormones and Behavior*, 62(3), 324–330. https://doi.org/10.1016/j.yhbeh.2012.02.012

Ader, R., & Cohen, N. (1993). Psychoneuroimmunology: conditioning and stress. *Annual Review of Psychology*, 44(1), 53-85. 10.1146/annurev.ps.44.020193.000413

Ardor, Bellucci, L. M., & Smith, N. F. (2019). Crawling and righting behavior of the subtropical sea star *Echinaster (Othilia) graminicola*: effects of elevated temperature. *Marine Biology*, 166, 1-9. https://link.springer.com/article/10.1007/s00227-019-3591-4

Ballarin, L., Karahan, A., Salvetti, A., Rossi, L., Manni, L., Rinkevich, B., Rosner, A., Voskoboynik, A., Rosental, B., Canesi, L., Anselmi, C., Pinsino, A., Tohumcu, B. E., Jemec Kokalj, A., Dolar, A., Novak, S., Sugni, M., Corsi, I., & Drobne, D. (2021). Stem Cells and Innate Immunity in Aquatic Invertebrates: Bridging Two Seemingly Disparate Disciplines for New Discoveries in Biology. *Frontiers in Immunology*, 12, 688106. https://doi.org/10.3389/fimmu.2021.688106

Ben Khadra, Y., Ferrario, C., Di Benedetto, C., Said, K., Bonasoro, F., Carnevali, M. D., & Sugni, M. (2015). Wound repair during arm regeneration in the red starfish *Echinaster sepositus*. Wound repair and regeneration: official publication of the *Wound Healing Society and the European Tissue Repair Society*, 23(4), 611–622. https://doi.org/10.1111/wrr.12333.

Cooper, E. L. (Ed.). (2018). *Advances in Comparative Immunology*. Springer.

Coteur, G., Corriere, N., & Dubois, P. (2004). Environmental factors influencing the immune responses of the common European starfish (*Asterias rubens*). *Fish & Shellfish Immunology*, 16(1), 51-63. https://doi.org/10.1016/S1050-4648(03)00030-5

Ellis, R. P., Parry, H., Spicer, J. I., Hutchinson, T. H., Pipe, R. K., & Widdicombe, S. (2011). Immunological function in marine invertebrates: responses to environmental perturbation. *Fish & Shellfish Immunology*, 30(6), 1209–1222. https://doi.org/10.1016/j.fsi.2011.03.017

Fast, M. D., Ross, N. W., Mustafa, A., Sims, D. E., Johnson, S. C., Conboy, G. A., Speare, D. J., Johnson, G., & Burka, J. F. (2002). Susceptibility of rainbow trout *Oncorhynchus mykiss*, Atlantic salmon *Salmo salar* and coho salmon *Oncorhynchus kisutch* to experimental infection with sea lice *Lepeophtheirus salmonis*. *Diseases of Aquatic Organisms*, 52(1), 57–68. https://doi.org/10.3354/dao052057

Guillard, R. R., & Sieracki, M. S. (2005). Counting cells in cultures with the light microscope. *Algal Culturing Techniques*, Elsevier, 239-252.

Hamel, J. F., Jobson, S., Caulier, G., & Mercier, A. (2021). Evidence of anticipatory immune and hormonal responses to predation risk in an echinoderm. *Scientific Reports*, 11(1), 10691.). https://doi.org/10.1038/s41598-021-89805-0

Hatanaka, R., Sekine, Y., Hayakawa, T., Takeda, K., & Ichijo, H. (2009). Signaling pathways in invertebrate immune and stress response. *Invertebrate Survival Journal*, 6(1), 32-43. https://www.isj.unimore.it/index.php/ISJ/article/view/177/93

Holm, L., Reitelseder, S., Pedersen, T. G., Doessing, S., Petersen, S. G., Flyvbjerg, A., Andersen, J. L., Aagaard, P., & Kjaer, M. (2008). Changes in muscle size and MHC composition in response to resistance exercise with heavy and light loading intensity. *Journal of Applied Physiology*, 105(5), 1454–1461. https://doi.org/10.1152/japplphysiol.90538.2008.

Kohl, W. T., McClure, T. I., & Miner, B. G. (2016). Decreased Temperature Facilitates Short-Term Sea-star Wasting Disease Survival in the Keystone Intertidal Sea-star *Pisaster ochraceus*. *PloS One*, 11(4), e0153670. https://doi.org/10.1371/journal.pone.0153670.

Lang, B. J., Donelson, J. M., Caballes, C. F., Uthicke, S., Doll, P. C., & Pratchett, M. S. (2022). Effects of elevated temperature on the performance and survival of pacific crown-of-thorns starfish (*Acanthaster cf. solaris*). *Marine Biology*, 169(4), 43. https://doi.org/10.1007/s00227-022-04027-w

Lang, B.J., Donelson, J.M., Caballes, C.F. *et al.* Effects of elevated temperature on the performance and survival of pacific crown-of-thorns starfish (*Acanthaster* cf. *solaris*). *Marine Biology,* 169, 43 (2022). https://doi.org/10.1007/s00227-022-04027-w

Lavine, M. D., & Strand, M. R. (2002). Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology*, 32(10), 1295–1309. https://doi.org/10.1016/s0965-1748(02)00092-9

Lawrence, J. M. (2010). Energetic Costs of Loss and Regeneration of Arms in Stellate Echinoderms. *Integrative and Comparative Biology*, 50(4), 506–514. http://www.jstor.org/stable/40863400

Leclerc, M., & Bajelan, M. (1992). Homologous antigen for T cell receptor in axial organ cells from the asteroid *Asterias rubens*. *Cell Biology International Reports*, 16(5), 487–490. https://doi.org/10.1016/s0309-1651(06)80068-8.

McClintock, J. B., & Lawrence, J. M. (1985). Characteristics of foraging in the soft-bottom benthic starfish *Luidia clathrata* (Echinodermata: Asteroidea): prey selectivity, switching behavior, functional responses and movement patterns. *Oecologia*, 66, 291-298. https://doi.org/10.1007/BF00379867

Mundim, K. C., Baraldi, S., Machado, H. G., & Vieira, F. M. (2020). Temperature coefficient (Q10) and its applications in biological systems: Beyond the Arrhenius theory. *Ecological Modelling*, 431, 109127. https://doi.org/10.1016/j.ecolmodel.2020.109127

Pinsino, A., Thorndyke, M. C., & Matranga, V. (2007). Coelomocytes and post-traumatic response in the common sea star *Asterias rubens*. *Cell Stress & Chaperones*, 12(4), 331–341. https://doi.org/10.1379/csc-288.1

Poloczanska, E.S., Brown, C.J., Sydeman, W.J., Kiessling, W., Schoeman, D.S., Moore, P.J., Brander, K., Bruno, J.F., Buckley, L.B., Burrows, M.T. and Duarte, C.M. (2013). Global imprint of climate change on marine life. *Nature Climate Change*, 3(10), pp.919-925.https://doi.org/10.1038/nclimate1958

Roeder,, T. (1999). Octopamine in invertebrates. *Progress in Neurobiology*, 59(5), 533-561. https://doi.org/10.1016/S0301-0082(99)00016-7

Shimizu, M., Kohno, S., Kagawa, H., & Ichise, N. (1999). Lytic activity and biochemical properties of lysozyme in the coelomic fluid of the sea urchin *Strongylocentrotus intermedius*. *Journal of Invertebrate Pathology*, 73(2), 214–222. https://doi.org/10.1006/jipa.1998.4808.

L. Courtney Smith, Vincenzo Arizza, Megan A. Barela Hudgell, Gianpaolo Barone, Andrea G. Bodnar, Katherine M. Buckley, Vincenzo Cunsolo, Nolwenn M. Dheilly, Nicola Franchi, Sebastian D. Fugmann, Ryohei Furukawa, Jose Garcia-Arraras, John H. Henson, Taku Hibino, Zoe H. Irons, Chun Li, Cheng Man Lun, Audrey J. Majeske, Matan Oren, Patrizia PagliaraAnnalisa Pinsino, David A. Raftos, Jonathan P. Rast, Bakary Samasa, Domenico Schillaci, Catherine S. Schrankel, Loredana Stabili, Klara Stensväg, Elisse Sutton (2018). Echinodermata: the complex immune system in echinoderms. *Advances in Comparative Immunology*, 409-501. https://doi.org/10.1007/978-3-319-76768-0_13

Stickle, W. B., & Diehl, W. J. (1987). Effects of salinity on echinoderms. *Echinoderm Studies*, 2, 235-285.

Tort, L., Cockrem, J. F., & Narayan, E. J. (2020). Editorial: comparative endocrine stress responses in vertebrates. *Comparative Endocrine Stress Responses in Vertebrates*. https://doi.org/10.3389/fendo.2019.00652

Watts, S. A., & Lawrence, J. M. (1990). The effect of temperature and salinity interactions on righting, feeding and growth in the sea star *Luidia clathrata* (Say). *Marine & Freshwater Behaviour & Physiology*, 17(3), 159-165. https://doi.org/10.1080/10236249009378765

Zhan, Y., Li, J., Sun, J., Zhang, W., Li, Y., Cui, D., Hu, W., & Chang, Y. (2019). The Impact of Chronic Heat Stress on the Growth, Survival, Feeding, and Differential Gene Expression in the Sea Urchin *Strongylocentrotus intermedius*. *Frontiers in Genetics*, 10, 301. https://doi.org/10.3389/fgene.2019.00301

Zhukova, N. V. (2022). Fatty Acids of Echinoderms: Diversity, Current Applications and Future Opportunities. *Marine Drugs*, *21*(1), 21. https://doi.org/10.3390/md21010021.

Pei, S., Dong, S., Wang, F., Tian, X., & Gao, Q. (2012). Effects of density on variation in individual growth and differentiation in endocrine response of Japanese sea cucumbers (*Apostichopus japonicus*). *Aquaculture*, 356, 398-403. https://doi.org/10.1016/j.aquaculture.2012.04.032.

CHAPTER 3.   ANTIBACTERIAL ACTIVITY OF SEA STAR (*LUIDIA CLATHRATA*) TISSUES EXTRACTS AGAINST SELECTED PATHOGENIC BACTERIA

3.1   Abstract

As resistance to traditional antibiotics has become a major issue, it is essential to explore natural sources for new antimicrobial agents. The marine environment offers a variety of natural bioactive compounds. In this study, we examined the antibacterial potential of *Luidia clathrata*, a tropical sea star species. The experiment was conducted against both gram-positive (*Bacillus subtilis*, *Enterococcus faecalis, Staphylococcus aureus, Bacillus cereus* and *Mycobacterium smegmatis*) and gram-negative (*Proteus mirabilis*, *Salmonella typhimurium, Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) bacteria using disk diffusion method. Specifically, we extracted the body wall and gonad using methanol, ethyl acetate, and hexane. Our findings show that the body wall extract using ethyl acetate (1.78μg/ml) was particularly effective against all tested pathogens, while the gonad extract (0.107μg/ml) showed activity against six out of ten selected pathogens. This is a crucial and new discovery that suggests *L. clathrata* may be a useful source for discovering antibiotics and more research is required to pinpoint and comprehend the active ingredients.

3.2   Introduction

Multi-drug resistance (MDR) pathogens that have arisen over the past decade are a considerable threat to patients' health (Magiorakos et al., 2012; Tacconelli et al., 2018). These MDR microorganisms evolve through mutation and gene transfer in response to the prolonged use and misuse of certain drugs (Magiorakos et al., 2012; Tacconelli et al., 2018: Yoneyama and Katsumata, 2006). Numerous adverse side effects possessed by conventional antibiotics are another problem related to health (Cuhna, 2001;Magiorakos et al., 2012; Iredell, 2016). It is crucial to develop a sustainable solution to mitigate the limitations of existing antibiotics. Exploration of

nutraceuticals in natural sources could serve as an effective way to develop such a solution (Andersson, 2003; WHO, 2015; Yoneyama and Katsumata, 2006;). Marine environments are one potentially overlooked resource for nutraceuticals (Choudhary et al., 2017).

The oceans, which cover almost 70% of the earth's surface, offer a myriad of organisms rich in secondary metabolites that can be exploited for pharmaceutical purposes (Choudhary et al., 2017; Moloney, 2016). Secondary metabolites are organic compounds produced by plants and animals which are not essential for their survival and growth but are utilized in defense responses (Manivasagan et al., 2014; Pereira et al., 2021; Reinisch and Bang, 1971). These compounds include but are not limited to echinochrome A, complement-like protein, antimicrobial peptides (AMP), steroidal glycosides, asterosaponin, and sulfated steroidal compounds (Diehl et al., 1984; Guenther et al., 2007; Hennbert et al., 2015; Kim et al., 2018; Leonard et al., 1990; Manivasagan et al., 2014; Reinisch and Bang, 1971). All have been previously isolated from the echinoderms and studied for their medicinal importance (Diehl et al., 1984; Guenther et al., 2007; Hennbert et al., 2015; Kim et al., 2018; Leonard et al., 1990; Manivasagan et al., 2014; Reinisch and Bang, 1971). The results from those studies imply that such compounds have diverse medicinal properties including anti-microbial, anti-inflammatory, antioxidant, and anticancer effects (Diehl et al., 1984; Guenther et al., 2007; Hennbert et al., 2015; Kim et al., 2018; Leonard et al., 1990; Manivasagan et al., 2014; Reinisch and Bang, 1971; Venkatesan et al., 2019).

The sea star is a keystone predator in marine ecosystems full of bioactivities and nutraceutical properties, but they have been poorly studied compared to other echinoderms such as sea cucumber, sea urchins, and brittle stars (Sumitha et al., 2017; Popov et al., 2022). This benthic free-living creature is well documented for its distinctive defensive mechanism to mitigate the disadvantage and ecological cost associated with other commensal or parasitic surface associated organisms. (Guenther et al., 2007; Li et al., 2015). The surface microtopography of some tropical sea stars has demonstrated the presence of a unique cuticle overlying the epidermis. This cuticle is rich in highly extended glycocalyx and chondroitin sulfate proteoglycans, which are pericellular glycoproteins that cover the cell and act as a physical barrier (Guenther et al., 2007). These surface-associated bioactive compounds provide good protection from pathogens by modulating the adhesive properties of the surface (Guenther et al., 2007; Sumitha et al., 2022). Although existing research has well documented the bioactivity and pharmaceutical potential of

various sea star species, *Luidia clathrata,* a tropical slender armed sea star, has been barely studied for its antibacterial potential.

In this experiment, we investigated the antimicrobial potential *of L. clathrata.* We analyzed the inhibitory properties of different body tissues (body wall and gonad) with respect to diverse pathogenic bacteria. We used three different solvents (methanol, ethyl acetate, and hexane) exhibiting different properties to extract different bioactive compounds from these tissues. We used the Kirby Bauer Disk Diffusion method to assess the inhibitory potential of extracted tissues (Jorgensen et al., 2015). The results showed that the body wall extracted with ethyl acetate possesses inhibitory properties across all tested pathogens, while gonad extract only inhibits the activity of a few pathogens. Methanol and hexane extracts did not produce any activity. Methanol extract of the body wall demonstrated hemolytic activity on red blood cells. This encouraging finding implies that the body wall and gonad of *L. clathrata* could serve as an important source of antibiotics for pathogenic bacteria.

3.3   Materials and methods

3.3.1   Species acquisition and maintenance

24 Healthy sand sifting sea star adults (24.41±1.50gm) were procured from a certified animal vendor (Gulf Specimen Marine Lab, Panacea, Florida, USA). Upon arrival, the species were maintained in optimal water conditions (temperature: 68-70ºF, salinity: 28±1ppt, ammonia: 0-0.25mg/L, pH:7.8-8.0) in the invertebrate lab. The specimens were thoroughly cleaned with de-ionized water to remove any adherent sediments and contaminants before dissection. 24 sea stars were dissected to collect the body wall, gut, and gonad. The different components were then pooled separately. Due to their fragility, the gonads were homogenized using a tissue homogenizer, while the body wall tissues were finely ground using a coffee grinder (Hamilton Beach® Fresh Grind™).

3.3.2   Preparation of extracts

The extraction procedure was carried out by following the methods described by Shuchizadeh et al. with some modifications (Shushizadeh et al., 2019). The gonad (5gm), and body wall (84.3gm) were submerged in reagent grade (99%) methanol, hexane, and ethyl acetate (PRA grade, ≤ 99.5%, Sigma-Aldrich) in 1:3 (w/v) ratio and constantly agitated on orbit shaker (Lab-

line Orbit Shaker, Model 3520) for 96 hours at room temperature. The flasks were covered with aluminum foil to avoid photolysis and thermal degradation of secondary metabolites prior to extraction. The extract was then decanted and filtered with Whatman® Grade 3 Filter Paper (diameter 12.5cm). The resulting filtrate was concentrated using a rotary evaporator (BU-R134 Rotary Vap System, Switzerland) at reduced pressure and temperature (40-45°C). The concentrated crude residues were stored at 4ºC for the subsequent investigations.

3.3.3   Determination of extract concentration

The volume of concentrated crude extract was measured and transferred to the previously weighted empty dish. The total weight of the crude extract with the dish was taken. The concentration was calculated using the following formula (Walag and Khawar et al., 2021):

$$Concentration = \frac{\left(Weight_{Extract+dish} - Weight_{empty\ dish}\right)}{Volume\ of\ crude\ extract\ in\ ml} X \frac{1000mg}{g}$$

3.3.4   Test microorganism and culture medium

Five gram-positive bacteria [*Bacillus subtilis*[X], *Enterococcus faecalis* (ATCC 25922), *Staphylococcus aureus* (ATCC 27659), *Bacillus cereus*[x] and *Mycobacterium smegmatis*[x]) and five gram-negative [(*Proteus mirabilis*[x], *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 11229), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (ATCC 13883)] were examined in this experiment ([x] denotes that ATCC number is not available). All the bacteria, except *E. faecalis* were sub-cultured on Tryptic soy agar (TSA) media at 37ºC for 24 hours. *E. faecalis* was grown on 5% sheep blood agar media. These subcultures were kept at 4ºC to guarantee bacterial viability and purity.

3.3.5   Antibacterial assay

Antibacterial activity was assessed by the disk diffusion method (Jorgensen and Turnidge, 2015). Petri plates (100mm and 150mm) were prepared by pouring 20 ml and 60 ml of Muller Hinton Agar (MHA) respectively. The plates were swabbed aseptically with fresh bacterial suspension prepared from the subculture maintained at 4ºC and standardized with 0.5 McFarland standard. A sterile filter paper disk (6mm) was impregnated with the extracted samples and placed

on the agar surface along with positive and negative controls at an appropriate distance and incubated for 24 hours at 37ºC. The extraction solvents were employed as negative controls, whereas antibiotics appropriate to the organism (gentamicin, vancomycin, penicillin, streptomycin, and SXT) were utilized as positive controls. The zone of inhibition was characterized by the formation of a clear zone around the disk. For the haemolytic activity, 5% sheep blood agar plate inoculated with *E. faecalis* was used. The zone of haemolysis was interpreted as a clear zone formed by destruction of red blood cells around the disk. The diameter of the zone of inhibition and haemolysis were measured in millimeters.

3.3.6 Statistical analysis

The assays were maintained in triplicates and data obtained are presented as means± standard error of the mean (SEM). The assumption of normality was met. Comparison between negative control and sample extracted was performed by analysis of variance (ANOVA, $p<0.05$) followed by Bonferroni correction.

3.4 Results

3.4.1 Ethyl acetate extracts exhibit broad-spectrum antibacterial activity

The antimicrobial activity of ethyl acetate extract of *L. clathrata* body wall (1.78μg/ml) and gonad tissues (0.107μg/ml) is summarized in Table1. Ethyl acetate extract of the body wall exhibited significant antibacterial activity against all tested pathogens. Gonad extracted with ethyl acetate exhibited inhibitory activity against six out of the ten selected pathogens. Activity was not observed for the gonad extract against *Pseudomonas aeruginosa*, *Enterococcus faecalis, Bacillus cereus,* and *Mycobacterium smegmatis*. Overall antibacterial activity was also lower than that observed in the body wall extract. We did not observe the zone of haemolysis for any of the tissues extracted with ethyl acetate Table 3.1.

Table 3.1 Antibacterial activity demonstrated by the ethyl acetate extracts of Luidia clathrata tissues (body wall and gonad) on selective pathogenic bacteria achieved by the disk diffusion method. Values are presented as the mean diameter of inhibition zones (mm) ± standard error of the means (n=3). GM (Gentamicin), SXT (Sulfamethoxazole-Trimethoprim), P (Penicillin), ST (Streptomycin) & VA (Vancomycin). '-' = no activity against the bacteria. All other interactions were significantly changed from the negative control (p<0.05).

Pathogens	Antibiotics (µg)	Zone of Inhibition (Diameter in mm)			
		Positive control (Antibiotics) (Mean± SEM)	Negative Control (Ethyl acetate) (Mean± SEM)	Body wall (Mean± SEM)	Gonad (Mean± SEM)
Gram Negative					
Proteus mirabilis [x]	GM (10)	27.33±1.33	0	34.00±0.88	12.00±0.66
Salmonella typhimurium (ATCC 14028)	SXT (10)	36.33±1.33	0	35.66±2.96	12.66±1.85
Escherichia coli (ATCC 11229)	GM (10)	30.00±0.55	0	34.66±1.45	12.33±1.33
Pseudomonas aeruginosa (ATCC 27853)	ST (10)	17.66±0.33	0	26.33±0.88	-
Klebsiella pneumoniae (ATCC 13883)	GM (10)	25.00±1.00	0	29.66±0.33	9.00±1.00
Gram Positive					
Bacillus subtilis [x]	P (10)	33.66±2.18	0	32.33±1.20	12±1.52
Enterococcus faecalis (ATCC 25922)	GM (10)	20.66±0.33	0	18.66±0.33	-
Staphylococcus aureus (ATCC 27659)	P (10)	38.33±0.33	0	37.66±2.33	11.33±0.33
Bacillus cereus [x]	VA (30)	19.00±0.00	0	20.66±0.33	-
Mycobacterium smegmatis [x]	ST (10)	28.00±1.72	0	44.66±2.90	-

[x] denotes that ATCC number is not available.

3.4.2 Methanol and hexane extracts do not exhibit antibacterial activity

Methanol extract (1.78μg/ml) of any of the tissues exhibited no inhibitory activity against the selected pathogens. We did observe significant ($p<0.05$) beta-haemolysis, a complete destruction of red blood cells by the methanol extract of body wall Table 3.2. Because beta haemolysis was not observed in ethyl acetate extract, the responsible compound must be specifically soluble in methanol. Hexane extract of any of the tissues exhibited no inhibition against the selected pathogens. Because of the nonpolar nature of the hexane, any polar bioactive compounds would not be extracted (Uli et al., 2016). Haemolytic activity was also not observed with the tissues extracted with hexane.

Table 3.2 Haemolysis activity of *L. clathrata* extract of body wall (1.78μg/ml) and gonad tissues (0.107μg/ml) extracted with different solvent by disk diffusion method. Results are illustrated as the mean diameter of haemolysis zones (mm)±standard error of the means (n=3). '-' = no activity against the bacteria. All other interactions were significantly changed from the negative control ($p<0.05$).

Solvent Used	Zone of Haemolysis		
	(Diameter in mm)		
	Control (solvents only) (Mean± SEM)	**Body wall** (Mean± SEM)	**Gonad** (Mean± SEM)
Methanol	0	14±1.00	-
Ethyl acetate	0	-	-
Hexane	0	-	-

3.5 Discussion

The emergence of antibiotic resistant organisms has made treating the diseases they cause difficult (Magiorakos et al., 2012). Discovery of new therapeutic agents from natural sources could provide a potential solution. In this experiment, we aimed to determine the antibacterial activity of *L. clathrata* against selected pathogenic bacteria (Tacconelli et al., 2018: Yoneyama and Katsumata, 2006).

Existing literature has shown the wide range of bioactivity from a variety of marine invertebrates, but little information is available about the sea star antibacterial activity (Pereira et al., 2021; Reinisch and Bang, 1971; Li et al., 2015). In our study, ethyl acetate extract of the body wall showed a significant ($p<0.05$) zone of inhibition in all tested pathogens compared to the negative control. The zone of inhibition was highest against *M. smegmatis* (44.66±2.90mm) and smallest against *E. faecalis* (18.66±0.33mm). Our finding is supported by Bryan et al. (Bryan et al., 1996). They discovered body wall extract of *L. clathrata* that potentially inhibited the attachment of a marine bacteria *Luteo violaceato* from the wells of microtiter plates, indicating the defense mechanism of the body wall which could potentially be antibacterial in nature. However, they did not explain in detail the antimicrobial potential of the body wall (Bryan et al., 1996). Similarly, ethanolic extract of whole-body tissue from *L. maculata* partially purified using liquid partition and column chromatography exhibited antimicrobial activity against five bacterial and five fungal pathogens (Suguna et al., 2014). The antibacterial activity of *Astropecten indicus* and found that crude methanol and ethyl acetate tissue extract exhibited high inhibitory activity against the tested pathogens including *P. aeruginosa, K. pneumoniae* and moderate activity against species like *Streptococcus* and *E. coli* (Chamundeeswari et al., 2012). In our case high activity was observed on all the tested pathogens. Previous research primarily focused on whole body tissues and the body wall (Bryan et al., 1996; Saguna et al., 20114). In the present study, we have explored the antibacterial potential of the gonad as well for the first time along with the body wall. Gonad extracted in ethyl acetate was able to inhibit some of the tested pathogens. It is likely that the ethyl acetate extract of the body wall was more effective than the gonad extract because of discrepancies in concentration. The concentration of body wall extract is about 16X higher than gonad extract. Another possibility could be due to the difference in the chemical nature of compounds present in two tissue types. This also explains the fact that gonad is likely more effective against the gram-negative pathogens compared to the gram-positive ones. Out of six pathogens being inhibited by

gonad extract, four of them are gram-negative and two are gram-positive. The greater inhibitory activity against gram-negative pathogens could be because they have an extra lipopolysaccharides layer. Fatty compounds from gonads may dissolve the lipopolysaccharides and thus likely destroy gram-negative pathogens more readily than gram-positive (Costerton et al., 1974).

The methanol extract of none of the tissues showed activity against tested pathogens. This is interesting because methanol is a widely used polar solvent due to its ability to extract a diverse range of compounds and proven to have good extraction yield (Dhawan and Gupta, 2017; Bimakr et al., 2011). However, we noticed beta-haemolytic activity of the body wall extracted with methanol on 5% sheep blood agar. The haemolytic activity by methanolic extract of the body wall observed in the present experiment could be due to the presence of saponin in body wall (Southeeswaran and Kenchingoton, 1989; Segaram and Chua, 2020). Saponin, a polar secondary metabolite mostly found in plants and lower invertebrates is well characterized by its ability to breakdown red blood cells. This property is used as a screening test to determine whether saponin is present in natural substances (Southeeswaran and Kenchingoton, 1989; Segaram and Chua, 2020).

In this Experiment, the complete destruction of erythrocytes by the methanol extract of body wall suggests that body wall of *L. clathrata* is rich in saponin. The hexane extracts did not show any positive activity because hexane, as a non-polar solvent, is not able to extract the polar compounds present in the sample (Malekzadeh et al., 2016). Since the ethyl acetate extract produced the majority of the positive results in the present studies, we anticipate that ethyl acetate is the proper solvent to extract the bioactive compounds with antibacterial nature from *L. clathrata*. Our results are in line with Darya et al., who reported the ethyl acetate extract of different body parts of *Holothuria leucospilota* and had more antibacterial activity than n-hexane, and methanol extract (Darya et al., 20220. The present result of our study suggests that the antimicrobial compound(s) found in the body wall and gonad of *L. clathrata* is likely polar or partially polar.

3.6 Conclusion

In this research, we analyzed the antibacterial potential of *L. clathrata* tissues using diverse types of extracts of different polarities on selected pathogens. We found that ethyl acetate extracts of body wall and gonad tissues exhibit significant inhibitory activity. This indicates the studied species, *L. clathrata,* could be an excellent source for discovering antibiotics to treat various types of diseases. This work can be expanded through the isolation, characterization and purification of the specific compounds responsible for the antibacterial potential.

3.7 References

Andersson, D.I. (2003). Persistence of antibiotic resistant bacteria. *Current Opinion in Microbiology*, 6(5), pp.452-456. https://doi.org/10.1016/j.mib.2003.09.001.

Bimakr, M., Rahman, R.A., Taip, F.S., Ganjloo, A., Salleh, L.M., Selamat, J., Hamid, A. and Zaidul, I.S.M. (2011). Comparison of different extraction methods for the extraction of major bioactive flavonoid compounds from spearmint (*Mentha spicata L.*) leaves. *Food and Bioproducts Processing*, 89(1), pp.67-72. https://doi.org/10.1016/j.fbp.2010.03.002.

Bryan, P.J., Rittschof, D. and McClintock, J.B. (1996). Bioactivity of echinoderm ethanolic body-wall extracts: an assessment of marine bacterial attachment and macroinvertebrate larval settlement. *Journal of Experimental Marine Biology and Ecology*, 196(1-2), pp.79-96. https://doi.org/10.1016/0022-0981(95)00124-7.

Capita, R. and Alonso-Calleja, C. (2013). Antibiotic-resistant bacteria: a challenge for the food industry. *Critical Reviews in Food Science and Nutrition*, 53(1), pp.11-48. https://doi.org/10.1080/10408398.2010.519837.

Chamundeeswari, K., Saranya, S. and Rajagopal, S. (2012). Exploration of potential antimicrobial activity of sea star Astropecten indicus. *Journal of Applied Pharmaceutical Science*, 2(7), pp.125-128. 10.7324/JAPS.2012.2716.

Choudhary, A., Naughton, L.M., Montánchez, I., Dobson, A.D. and Rai, D.K. (2017). Current status and future prospects of marine natural products (MNPs) as antimicrobials. *Marine Drugs*, 15(9), p.272. https://doi.org/10.3390/md15090272.

Costerton, J.W., Ingram, J.M. and Cheng, K.J. (1974). Structure and function of the cell envelope of gram-negative bacteria. *Bacteriological Reviews*, 38(1), pp.87-110. https://doi.org/10.1128/br.38.1.87-110.1974.

Cunha, B.A. (2001). Antibiotic side effects. *Medical Clinics of North America*, 85(1), pp.149-185.. https://doi.org/10.1016/S0025-7125(05)70309-6.

Darya, M., Sajjadi, M.M., Yousefzadi, M., Sourinejad, I. and Zarei, M. (2020). Antifouling and antibacterial activities of bioactive extracts from different organs of the sea cucumber *Holothuria leucospilota. Helgoland Marine Research*, 74, pp.1-13. 10.1186/s10152-020-0536-8

Dhawan, D. and Gupta, J. (2017). Research article comparison of different solvents for phytochemical extraction potential from datura metel plant leaves. *International Journal Biological Chemistry*, 11(1), pp.17-22. 10.3923/ijbc.2017.17.22.

Diehl, W.J. and Lawrence, J.M. (1984). The effect of salinity on coelomic fluid osmolyte concentration and intracellular water content in *Luidia clathrata* (Say) (Echinodermata: Asteroidea). *Comparative Biochemistry and Physiology Part A: Physiology*, 79(1), pp.119-126. https://doi.org/10.1016/0300-9629(84)90718-7.

Guenther, J. and De Nys, R. (2007). Surface microtopographies of tropical sea stars: lack of an efficient physical defense mechanism against fouling. *Biofouling*, 23(6), pp.419-429. https://doi.org/10.1080/08927010701570089.

Hennebert, E., Leroy, B., Wattiez, R. and Ladurner, P. (2015). An integrated transcriptomic and proteomic analysis of sea star epidermal secretions identifies proteins involved in defense and adhesion. *Journal of Proteomics*, 128, pp.83-91. https://doi.org/10.1016/j.jprot.2015.07.002.

Iredell, J., Brown, J. and Tagg, K. (2016). Antibiotic resistance in Enterobacteriaceae: mechanisms and clinical implications. *British Medical Journal*, 352. https://doi.org/10.1136/bmj.h6420.

Jorgensen, J.H. and Turnidge, J.D. (2015). Susceptibility test methods: dilution and disk diffusion methods. *Manual of Clinical Microbiology*, pp.1253-1273. https://doi.org/10.1128/9781555817381.ch71.

Kim, C.H., Go, H.J., Oh, H.Y., Park, J.B., Lee, T.K., Seo, J.K., Elphick, M.R. and Park, N.G. (2018). Identification of a novel antimicrobial peptide from the sea star *Patiria pectinifera*. *Developmental & Comparative Immunology*, 86, pp.203-213. https://doi.org/10.1016/j.dci.2018.05.002.

Leonard, L.A., Strandberg, J.D. and Winkelstein, J.A. (1990). Complement-like activity in the sea star, *Asterias forbesi. Developmental & Comparative Immunology*, 14(1), pp.19-30. https://doi.org/10.1016/0145-305X(90)90004-X.

Li, C., Blencke, H.M., Haug, T. and Stensvag, K. (2015). Antimicrobial peptides in echinoderm host defense. *Developmental & Comparative Immunology*, 49(1), pp.190-197. https://doi.org/10.1016/j.dci.2014.11.002.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B. and Paterson, D.L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*, 18(3), pp.268-281. https://doi.org/10.1111/j.1469-0691.2011.03570.

Malekzadeh, M., Najafabadi, H.A., Hakim, M., Feilizadeh, M., Vossoughi, M. and Rashtchian, D. (2016). Experimental study and thermodynamic modeling for determining the effect of non-polar solvent (hexane)/polar solvent (methanol) ratio and moisture content on the lipid extraction efficiency from *Chlorella vulgaris*. *Bioresource Technology*, 201, pp.304-311. https://doi.org/10.1016/j.biortech.2015.11.066.

Manivasagan, P., Venkatesan, J., Sivakumar, K. and Kim, S.K. (2014). Pharmaceutically active secondary metabolites of marine actinobacteria. *Microbiological Research*, 169(4), pp.262-278. https://doi.org/10.1016/j.micres.2013.07.014.

Moloney, M.G. (2016). Natural products as a source for novel antibiotics. *Trends in Pharmacological Sciences*, 37(8), pp.689-701. https://doi.org/10.1016/j.tips.2016.05.001.

Pagare, S., Bhatia, M., Tripathi, N., Pagare, S. and Bansal, Y.K. (2015). Secondary metabolites of plants and their role: Overview. *Current Trends in Biotechnology and Pharmacy*, 9(3), pp.293-304.

Pereira, R.C., Sudatti, D.B., Moreira, T.S. and Ventura, C.R.R. (2021). Chemical defense in developmental stages and adult of the sea star *Echinaster (Othilia) brasiliensis*. *PeerJ*, 9, p.e11503. https://doi.org/10.7717/peerj.11503.

Popov, R.S., Ivanchina, N.V. and Dmitrenok, P.S. (2022). Application of MS-based metabolomic approaches in analysis of starfish and sea cucumber bioactive compounds. *Marine Drugs*, *20*(5), p.320. https://doi.org/10.3390/md20050320.

Reinisch, C.L. and Bang, F.B. (1971). Cell recognition: reactions of the sea star (*Asterias vulgaris)* to the injection of amebocytes of sea urchin (*Arbacia punctulata*). *Cellular Immunology*, 2(5), pp.496-503. https://doi.org/10.1016/0008-8749(71)90058-X.

Segaran A, Chua LS. (2020). Saponins Rich Fractions from Eurycoma longifolia Extract. In: *Third International Conference on Separation Technology 2020 (ICoST 2020). Atlantis Press;* 2020. p. 57–61. https://doi.org/10.2991/aer.k.201229.008.

Shushizadeh, M.R., Nasiri, M.B., Ameri, A.G., Ghatrami, E.R. and Tavakoli, S. (2019). Preparation of the Persian Gulf Echinometra mathaei organic extracts and investigation of their antibacterial activity. *Jundishapur Journal of Natural Pharmaceutical Products*, 14(4). 10.5812/jjnpp.57093.

Southeeswaran, S. and Kenchington, W. (1989). Hemolysis test for saponins: A caution. *Journal of Chemical Education*, 66(12), p.1058. https://doi.org/10.1021/ed066p1058.

Suguna, A., Bragadeeswaran, S., Natarajan, E. and Mohanraj, M. (2014). Studies on antioxidant properties of starfish *Luidia maculata* (Muller & Troschel, 1842) off Parangipettai, Southeast coast of India. *Journal of Coastal Life Medicine*, 2(9), pp.694-698.

Sumitha, R., Banu, N. and Parvathi, V.D. (2017). Novel natural products from marine sea stars. *Current Trends in Biomedical Engineering & Biosciences*, 2(4), pp.59-63.

Sumitha, R., Banu, N. and Parvathi, V.D. (2017). Novel natural products from marine sea stars. *Current Trends in Biomedical Engineering & Biosciences*, 2(4), pp.59-63. https://doi.org/10.1016/0022-0981(95)00124-7.

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D.L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y. and Ouellette, M. (2018). Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *The Lancet Infectious Diseases*, 18(3), pp.318-327. https://doi.org/10.1016/S1473-3099(17)30753-3.

Uli, H., Noor, A., Mandey, F.W. and Sapar, A. (2016). Isolation, identification and bioactivity test of non-polar compounds on N-hexane extract of *Haliclona (Reniera) fascigera* from Samalona Island-spermonde Archipelago. *Marina Chimica Acta*, 17(2). 10.20956/mca.v17i2.1125.

Venkatesan, G.K., Kuppusamy, A., Devarajan, S. and Kumar, A.K.K. (2019). Review on medicinal potential of alkaloids and saponins. *Pharamacologyonline*, 1, pp.1-20.

Walag, A.M.P. And Kharwar, R.N. (2021). Assessment Of crude extract yield and In-vitro antioxidant activity of Sea Star from Philippines. *Uttar Pradesh Journal of Zoology*, 42(22), Pp.68-76. https://www.mbimph.com/index.php/UPJOZ/article/view/2567.

World Health Organization, (2015). Antibiotic resistance: multi-country public awareness survey. https://apps.who.int/iris/handle/10665/194460.

Yoneyama, H. and Katsumata, R. (2006). Antibiotic resistance in bacteria and its future for novel antibiotic development. *Bioscience, Biotechnology, and Biochemistry*, 70(5), pp.1060-1075. https://doi.org/10.1271/bbb.70.1060.

CHAPTER 4.   MOLECULAR RESPONSES OF SEA STAR, *LUIDIA CLATHRATA,* EXPOSED TO PHYSICAL AND THERMAL STRESS

4.1   Abstract

The impact of rising global temperatures and human activity on living organisms can be detrimental. *Luidia Clathrata*, a sea star species found in shallow waters along coastlines, provides an ideal system for studying how organisms respond at the molecular level to physical and thermal stress, which is becoming more frequent due to anthropogenic activities and climate change. We conducted an experiment in which we gradually raised the temperature up to 7°C to stress *L. clathrata*, to determine the molecular mechanisms activated in the sea stars under stress. First, we measured the RNA/DNA ratio, a common biochemical index used to assess the condition of marine organisms, but the results were inconclusive due to contamination in the samples. Second, we analyzed the expression levels of the heat shock protein 90 gene in different experimental groups. Although we did not observe significant changes in hsp90 expression levels among the groups, we did notice a trend of slightly higher expression levels in the stressed groups, which suggests that 7°C rise in temperature in future could be lethal to *L. clathrata*, and global warming could lead to a loss of important species like this starfish, thereby affecting biodiversity. This is the first study to document the stress response of *L. clathrata* at the molecular level, and we believe it provides a foundation for further research. However, additional research is recommended over a longer period of time with a larger sample size.

4.2   Introduction

Human activities and global warming have had a detrimental impact on marine creatures, particularly stenothermal echinoderms such as starfish (Bose, 2010). However, these organisms have developed various physiological and molecular adaptations to cope with environmental stress caused by factors like temperature. Some species have been observed to produce peptides and polyunsaturated fatty acyl chains to slow down their metabolism rate and survive in extreme environmental conditions (Gonzalez et al., 2016; Morgan et al., 2006). Although this evolutionary adaptation has helped them survive in harsh environments, it has limitation to adapt to changing

environmental conditions in echinoderms like *L. clathrata*, which has narrow range of tolerance to salinity and temperature (Ingels et al., 2012; Peck et al., 2014).

Molecular techniques have become increasingly crucial in marine ecology research, especially in the study of the physiological and nutritional state of aquatic organisms (Chicharo and Chicharo 2008; Hussna et al., 2020). PCR and nucleic acid derived indices, such as the RNA:DNA ratio, have been proven useful in this regard. The RNA:DNA ratio has been widely used for nearly 30 years as a biochemical indicator of the physiological and nutritional state of aquatic organisms in their natural habitats (Chicharo and Chicharo 2008; Bulow 1984; Buckly 1984). Generally, it indicates cells' ability to produce proteins and is closely related to their nutritional condition. The RNA:DNA ratio is based on the idea that DNA remains constant in somatic cells despite changes in the environment, while RNA levels vary with protein synthesis. Higher RNA:DNA ratios usually indicate a better organismal condition (Chicharo and Chicharo 2008; Rocker and Holt 1996). Studies have shown that organisms living in good environmental conditions tend to have higher RNA:DNA ratios than those in poor conditions, suggesting that RNA production may be an indicator of overall organismal health. However, extreme overexpression of RNA can be associated with stressed conditions and abnormal physiological functioning. (Chicharo and Chicharo 2008; Dortch et al., 1983; Rocker and Holt). This index has been used to investigate numerous marine species and has proven to be valuable in assessing their physiological state in natural settings (Chicharo and Chicharo 2008; Rocker and Holt). Furthermore, it is a reliable and sensitive index of metabolic activity and growth rate, with a high correlation to traditional measures of condition such as weight and size (Chicharo and Chicharo 2008; Dortch et al., 1983; Hussna et al., 2020; Rocker and Holt).

The exposure to extreme temperatures, such as heat or cold, can lead to thermal stress and adversely affect an organism's performance from a physiological standpoint. Nevertheless, organisms can respond to changes in temperature by adjusting their molecular and cellular structures, enabling them to sustain their performance in different environments (Gonzalaz et al., 2018; Giglio et al., 2021; Wiens et al., 2000). One of the critical responses to thermal stress at the molecular level is the production of heat shock proteins (HSPs). HSPs are a group of highly conserved molecular chaperones that range in size from 10 kDa to 170 kDa and are present in almost all eukaryotic organisms (Arribas et al., 2022; Pinsino et al., 2007; Vergara et al., 2017). Although originally identified during heat stress and named accordingly, HSPs have been shown

to have various functions in many cellular mechanisms, including regulating protein folding, maturation and stability, signal transduction, protein degradation, and DNA repair (Gonzalez et al., 201; Lang et al., 2022; Yusof at al., 2022).

The HSP response plays an important role in helping organisms tolerate thermal stress, but the way different species respond to such stress can vary widely. Some marine organisms are capable of generating a thermal stress response, while others lack the necessary physiological mechanisms to do so (Gonzalez et al., 201; Lang et al., 2022; Yusof at al., 2022). *L. clathrata*, a species commonly found in estuaries, has been greatly impacted by environmental changes, making it important to understand how it responds to temperature and physical stress at the molecular level. Our study used a transcriptomic approach to investigate the effects of elevated temperature and arm amputation on *L. clathrata*, analyzing RNA and DNA ratios and measuring the expression of HSP90 in the different stress groups.

4.3 Materials and Methods

4.3.1 Animal maintenance

In May 2021, healthy adult *L. clathrata* were purchased from Gulf Specimen Marine Lab, Panacea, Florida, USA. These starfish were brought to the invertebrate laboratory at Purdue University Fort Wa yne, where they were acclimated to their new environment. This was done by carefully transferring them to separate ten-gallon aquaria with three individuals in each. The acclimation process involved floating the bag containing the starfish in the aquarium for 15 to 20 minutes, then gradually releasing half of the water into the tank through small holes in the bag. The starfish were finally introduced into the aquariums after half the water was released. They were left in optimal water conditions with a temperature of 68-70oF, salinity of 28±1 ppt, ammonia levels of 0-0.25mg/L, and a pH of 7.8-8.0, and subjected to a 12:12 light-dark cycle in the laboratory. The acclimation period lasted for 24 hours.

4.3.2 Experimental design

Following the 24-hour acclimation period, the organisms were divided into four experimental groups, each containing two technical replicates with three individuals, as shown in Figure 4.3.1. The stress groups were then subjected to various stressors, including thermal stress,

physical stress, and a combination of both (thermal stress + arm amputation). To induce thermal stress, the temperature-stressed group was subjected to a 1°C increase in temperature per day for seven days (Mundim et al., 2020). To induce physical stress, the arm amputation group had one of their arm tips (1-1.5 cm) cut off aseptically using a scalpel (Fan, 2011). After arm amputation, the starfish were carefully returned to the aquarium and allowed to bury themselves in the sand. The combined stress group had their arm tip excised on day 1 and were then exposed to the gradual temperature rise for seven days. Throughout the experimental period, daily monitoring and assessment of water quality parameters were carried out using an API Saltwater Aquarium Master Kit, and the parameters were maintained at optimal levels. To compensate for water evaporation caused by the high temperatures in the temperature-stressed groups, sufficient water additions were made to maintain constant salinity and water volume. The organisms were fed daily with chopped frozen shrimp (10mm size) ad-libitum, and any remaining food remnants were promptly removed once feeding had ceased.

4.3.3 Sample preparation

organisms were dissected and the gonads were collected aseptically. The gonad was chosen as the study tissue due to its ease of collection. The collected samples from each group were flash frozen in liquid nitrogen, then stored in -80 until needed.

4.3.4 DNA and RNA extraction

The Trizol/Chloroform extraction method was used to extract nucleic acids from 100 mg of frozen gonad tissue. Initially, 100 µL of trizol reagent (Invitrogen, USA) was added to the sample, which was then homogenized with a dounce homogenizer to ensure complete cell disruption. Subsequently, the samples were resuspended in 900 µL of trizol reagent and subjected to extraction, resulting in the isolation of RNA in the upper aqueous layer and DNA in the interphase layer. Each layer was collected without disturbing the others. The resulting DNA and RNA samples were then measured for their concentration and purity using the Nanodrop, and their concentrations were recorded in ng/µL, along with their A 260/280 and A260/A230 values.

4.3.5   cDNA Synthesis

After extracting and measuring the total RNA, 1 μg of the RNA was used to produce single-stranded cDNA. This was achieved through a 10 μl reaction volume following the instructions provided by the manufacturer (Invitrogen™ SuperScript™ Double-Stranded cDNA Synthesis Kit, 11917020). The resulting samples were stored at a temperature of -80 degrees Celsius for preservation.

4.3.6   Primer selection and validation

To obtain a partial sequence of HSP90, conserved regions of the European sea star Asterias rubens (accession no. XM_033775919) were used to determine appropriate primers. The forward primer selected was 5′-GACGTTCACAGACCCACAGA-3′, while the reverse primer was 5′-TTGCATGGCTCCAACGAAAC-3′. We used 18s ribosomal RNA (18s RNA) to normalize the target gene. The forward primer selected was 5′-GTGGAGCGATTTGTCTGGTT-3′, while the reverse primer used was 5′-AAGGGCATCACAGACCTGTT-3′. Primers were designed with the aid of primer-BLAST, which is a tool provided by the National Center for Biotechnology Information (Ye et al., 2012). To validate the integrity and find the appropriate annealing temperature, we ran PCR on cDNA obtained from the gonad sample with temperatures ranging from 52°C-62°C. The obtained primers were validated by running them through the two-dimensional gel electrophoresis. Both primers were found to be efficiently working at 60 °C annealing temperature.

4.3.7   QPCR

To measure the expression level of HSP90, qPCR was employed and the expression stabilities were evaluated using the ΔΔCt method. The assays were performed in triplicate, and the relative levels of the target gene were calculated as the ratio of the target gene to the reference gene.

4.3.8 Statistical analysis

The relative fold change in gene expression for various experimental groups was compared using ANOVA followed by Bonferroni correction after normalizing the target gene (hsp90) with the 18s gene. The results were reported as Mean ± Standard error of Mean (SEM).

4.4 Results

4.4.1 RNA: DNA ratios

The RNA: DNA ratios observed in the different experimental groups are summarized in table 4.1. We also measured the purity of DNA and RNA samples. Majority of the RNA samples had A260/A230 less than 1.9 indicating the phenol and guanidine contamination. In the case of DNA samples, we noticed some of the samples had lower than 1.8 A260/A230 suggesting acid and protein contamination. Looking at each individual organism, the majority had RNA/DNA ratios more than 1. Due to the contamination in the samples, it is difficult to draw any conclusions about how the stressor group differs from the control group in terms of their nucleic acid indices.

4.4.2 HSP90 expression patterns

Figure 4.1 displays the relative fold change in HSP90 gene expression for different experimental groups. Our analysis did not reveal any significant differences in HSP90 expression between the control group and any of the experimental groups. However, there was a trend of slightly increased expression of HSP90 in all stressed groups, suggesting a possible upregulation of the gene under thermal and physical stress. *L. clathrata* exposed to temperature stress exhibited a 2.7-fold increase in HSP90 gene levels compared to the control group, while the group with amputated arm showed a 2.37-fold increase. However, the combination group showed a lower fold change in HSP90 expression than the group exposed to individual stressors. This may be due to variation within the individual in the same group, making it difficult to draw a conclusive explanation. As a result, additional research is required to examine this phenomenon further.

Table 4.1 RNA: DNA ratios of the *L.clathrata* from different experimental groups.

| Experimental groups	RNA (ng/µL)		DNA (ng/µL)		RNA/DNA ratios	Reference
	RNA (ng/µL)	RNA (A260/A230)	DNA (ng/µL)	DNA (A260/A280)		
Control	3181.4	1.46	0.37	1.22	308.873	
	915.4	3.44	2.04	1.85	4.432	
	4389.4	1.91	-0.1	-0.13	-21947	Unknown contamination
Arm amputation	467.9	1.25	2.14	1.83	3.863	Guanidine contamination
	487.6	1.44	2.05	1.78	10.981	
	296.4	0.45	1.25	1.77	1.52	Phenol contamination
Temperature	874.1	2.18	2.24	1.73	20.713	
	766.9	1.71	2.19	1.87	1.887	
	696.9	1.38	2.12	1.85	2.682	
Combination	1109.19	1.47	1.38	0.52	924.325	Unknown contamination
	406.1	0.5	2.08	1.82	0.698	Unknown contamination
	1100.4	1.75	1.86	1.72	27.578	
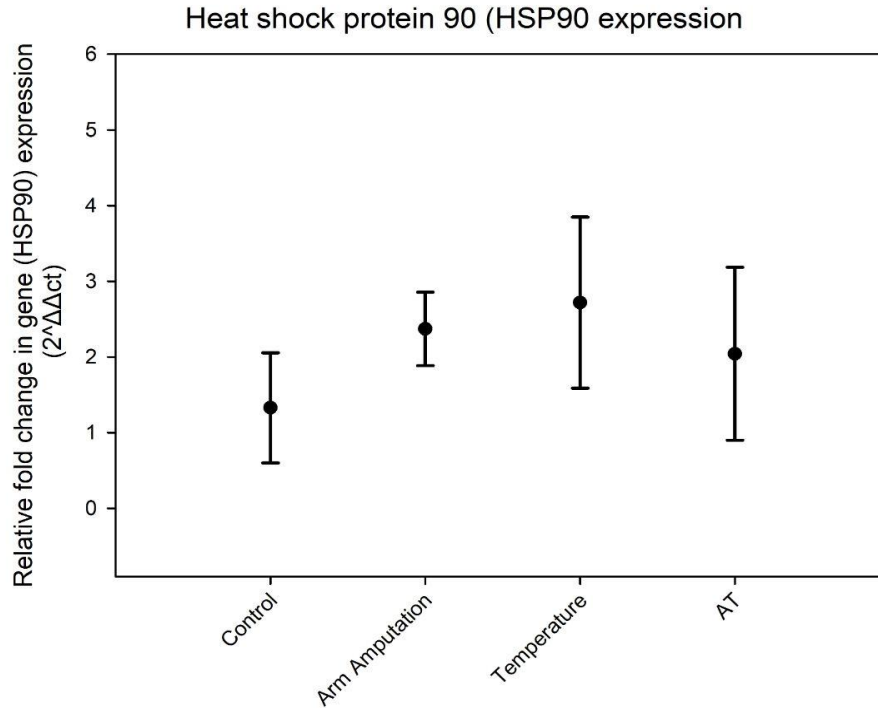
Figure 4.1 Relative mean fold change in HSP90 gene expression in different experimental groups. Data are presented as mean ± SEM.

4.5   Discussion

Environmental stressors, such as thermal and physical stress, have a significant impact on the physiological and biochemical processes of organisms, and play a vital role in determining their distribution and abundance. This is especially true for invertebrates and other ectothermic organisms (Fangue et al., 2006). While comparative studies across divergent species have provided valuable insights into thermal adaptation, understanding the role of temperature in establishing fine-scale patterns of thermal tolerance within closely related species or populations can provide further insights into the nature of adaptive variation in thermal tolerance.

Heat shock proteins are known to be encoded by multiple genes and are classified into families based on their molecular mass and sequence similarity. Some of these proteins are constitutively expressed under normal physiological conditions, while others are induced in response to protein-denaturing stress (Zhan et al 2019). In a previous study, we found that subjecting organisms to thermal stress of around 7°C for a duration of seven days resulted in significant physiological and immunological changes. To further investigate the effects of such stressors at the molecular level, we conducted a study to quantify the tolerance of *L. clathrata* to

thermal and physical stress, and examined the RNA:DNA ratio as well as the mRNA expression patterns of heat shock protein 90 (HSP90).

The RNA:DNA ratio was inconclusive due to multiple contaminants, but upon examining individual organisms, most had RNA:DNA ratios greater than 1, indicating no adverse effects on their survival with the exception of two organisms which apparently exhibited contamination. However, the majority of RNA samples had A260/A230 less than 1.9, indicating contamination with phenol and guanidine, while some of DNA samples had A260/A230 lower than 1.8, indicating acid and protein contamination. To mitigate the contamination, a potential future direction involves using a DNA or RNA clean-up procedure over a column. This technique typically involves passing the sample through a column containing a resin or matrix that selectively binds to the nucleic acids, allowing other contaminants to pass through. This can be an effective way to remove contaminants and improve the purity of the DNA or RNA samples. Additionally, washing the RNA precipitates twice with 70% ice-cold ethanol before drying the pellet could potentially eliminate any residual contaminants (Toni et al., 2018).

The relative fold change in the expression of the HSP90 gene was not significantly different across the experimental groups, but there was a slight increase in all stressed groups. Comparing our results with previous studies, we found supporting evidence that echinoderms exposed to different stressors such as heat, ocean acidification, oxidative stress, and changes in salinity show significant changes in cellular responses. For instance, when *Asterias rubens* were subjected to trauma, their coelomocytes exhibited a time-dependent increase in HSP70 levels, as detected by immunocytochemistry and immunoblotting, peaking at 6 hours after amputation (Pinsino et al., 2007). Similarly, *Apostichopus japonicus* also showed time-dependent expression patterns of heat shock protein when exposed to heat shock (Zhao et al., 2011). Wiens et al. (2000) found that heat shock proteins, particularly HSP90, play a significant role in helping organisms survive heat stress. They observed a 4.5-fold increase in HSP90 levels in thermally stressed *Dendronephthya klunzingeri* compared to those collected from non-stressed areas. This indicates that HSP90 could be a useful biomarker for monitoring environmental stress on corals (Wiens et al., 2000). Our study also showed a similar pattern of increased HSP90 expression when stressed, suggesting that this protein may serve as a useful molecular marker for studying stress responses. However, in order to draw firm conclusions from our findings, further research using a larger sample size and longer time period is necessary. Additionally, as a potential future direction, examining the expression of

HSP90 in response to acute exposure (within 24 hours) and chronic exposure can provide a more comprehensive understanding of how HSP90 is involved in stress response.

4.6   Conclusion

Protein secretion is crucial for various cellular processes, including the movement of proteins in response to cellular changes or signals from other cells, which can affect biological functions. In our study, we investigated the changes in RNA:DNA ratios among different experimental groups to understand how nucleic acids change in response to stressors, followed by quantification of HSP90 gene expression. However, due to multiple contamination issues, we could not draw any conclusions regarding RNA:DNA ratios. Despite lacking statistical significance, the organisms subjected to environmental stressors such as thermal and physical stress showed a slight increase in HSP90 gene expression, indicating that temperature stress may impact their normal physiology, leading to upregulation of HSP90 as a stress response. Therefore, we believe that HSP90 could be an effective molecular marker for stress studies in invertebrates. However, given the small sample size and multiple contamination issues, we recommend further research with a larger sample size and longer study period to obtain robust conclusions in the future.

4.7   References

Morgan-Kiss, R. M., Priscu, J. C., Pocock, T., Gudynaite-Savitch, L., & Huner, N. P. (2006). Adaptation and acclimation of photosynthetic microorganisms to permanently cold environments. *Microbiology and Molecular Biology Reviews*, 70(1), 222-252. https://doi.org/10.1128/MMBR.70.1.222-252.2006

Bose, B. K. (2010). Global warming: Energy, environmental pollution, and the impact of power electronics. *IEEE Industrial Electronics Magazine*, 4(1), 6-17. 10.1109/MIE.2010.935860

Peck, L. S., Morley, S. A., Richard, J., & Clark, M. S. (2014). Acclimation and thermal tolerance in Antarctic marine ectotherms. *Journal of Experimental Biology*, 217(1), 16-22. https://doi.org/10.1242/jeb.089946.

Ingels, J., Vanreusel, A., Brandt, A., Catarino, A. I., David, B., Ridder, C. D., Dubois, P., Gooday, A.J., Martin, A., Pasotti, F., & Robert, H. (2012). Possible effects of global environmental changes on Antarctic benthos: a synthesis across five major taxa. *Ecology and Evolution*, 2(2), 453-485.https://doi.org/10.1002/ece3.96

González, K., Gaitán-Espitia, J., Font, A., Cárdenas, C. A., & González-Aravena, M. (2016). Expression pattern of heat shock proteins during acute thermal stress in the Antarctic sea urchin, Sterechinus neumayeri. *Revista Chilena de Historia Natural*, 89(1),1-9.https://doi.org/10.1186/s40693-016-0052-z

Toni, L. S., Garcia, A. M., Jeffrey, D. A., Jiang, X., Stauffer, B. L., Miyamoto, S. D., & Sucharov, C. C. (2018). Optimization of phenol-chloroform RNA extraction. *MethodsX*, 5, 599–608. https://doi.org/10.1016/j.mex.2018.05.011

Hussna, I. A., Asmi, O., Shah, F., Bhat, B., Hussain, T., Hafeez, M., Rasid, M., Razak, N., & Hussain, R. A. (2020). RNA: DNA ratio as an indicator of growth, nutritional status and condition of fish: A review. *Journal of Entomology and Zoology Studies*, 8, 654-8.

Rooker, J. R., & Holt, G. J. (1996). Application of RNA: DNA ratios to evaluate the condition and growth of larval and juvenile red drum (*Sciaenops ocellatus*). *Marine and Freshwater Research*, 47(2), 283-290.https://doi.org/10.1071/MF9960283

Chícharo, M. A., & Chícharo, L. (2008). RNA: DNA ratio and other nucleic acid derived indices in marine ecology. *International Journal of Molecular Sciences*, 9(8), 1453-1471.https://doi.org/10.3390/ijms9081453

Bulow, F. J. (1970). RNA–DNA ratios as indicators of recent growth rates of a fish. *Journal of the Fisheries Board of Canada*, 27(12), 2343-2349.https://doi.org/10.1139/f70-262

Buckley, L. J. (1984). RNA-DNA ratio: an index of larval fish growth in the sea. *Marine Biology*, 80(3), 291-298.https://link.springer.com/article/10.1007/BF00392824

Dortch, Q., Roberts, T. L., Clayton Jr, J. R., & Ahmed, S. I. (1983). RNA/DNA ratios and DNA concentrations as indicators of growth rate and biomass in planktonic marine organisms. *Marine Ecology Progress Series. Oldendorf*, 13(1), 61-71. https://www.int-res.com/articles/meps/13/m013p061.pdf

Fangue, N. A., Hofmeister, M., & Schulte, P. M. (2006). Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*. *Journal of Experimental Biology*, 209(15), 2859-2872.https://doi.org/10.1242/jeb.02260

Wiens, M., Ammar, M. S., Nawar, A. H., Koziol, C., Hassanein, H. M., Eisinger, M., Müller, I. M., & Müller, W. E. (2000). Induction of heat-shock (stress) protein gene expression by selected natural and anthropogenic disturbances in the octocoral *Dendronephthya klunzingeri*. *Journal of Experimental Marine Biology and Ecology*, 245(2), 265–276. https://doi.org/10.1016/s0022-0981(99)00167-7

González-Aravena, M., Calfio, C., Mercado, L., Morales-Lange, B., Bethke, J., De Lorgeril, J., & Cárdenas, C. A. (2018). HSP70 from the Antarctic sea urchin *Sterechinus neumayeri*: molecular characterization and expression in response to heat stress. *Biological Research*, 51(1), 8. https://doi.org/10.1186/s40659-018-0156-9

Giglio, S., Agüera, A., Pernet, P., M'Zoudi, S., Angulo-Preckler, C., Avila, C., & Dubois, P. (2021). Effects of ocean acidification on acid-base physiology, skeleton properties, and metal contamination in two echinoderms from vent sites in Deception Island, Antarctica. *Science of the Total Environment*, 765, 142669.https://doi.org/10.1016/j.scitotenv.2020.142669

Vergara-Amado, J., Silva, A. X., Manzi, C., Nespolo, R. F., & Cárdenas, L. (2017). Differential expression of stress candidate genes for thermal tolerance in the sea urchin *Loxechinus albus*. *Journal of Thermal Biology*, 68(Pt A), 104–109. https://doi.org/10.1016/j.jtherbio.2017.03.009

Pinsino, A., Thorndyke, M. C., & Matranga, V. (2007). Coelomocytes and post-traumatic response in the common sea star *Asterias rubens. Cell stress & Chaperones*, 12(4), 331–341. https://doi.org/10.1379/csc-288.1

Arribas, L. P., Alfaya, J. E., Palomo, M. G., Giulianelli, S., Vilela, R. A. N., & Bigatti, G. (2022). Ocean warming lead to heat shock protein expression and decreases in the feeding rate of the Patagonian sea star *Anasterias minuta*. *Journal of Experimental Marine Biology and Ecology*, 546, 151661.https://doi.org/10.1016/j.jembe.2021.151661

González, K., Gaitán-Espitia, J., Font, A., Cárdenas, C. A., & González-Aravena, M. (2016). Expression pattern of heat shock proteins during acute thermal stress in the Antarctic sea urchin, *Sterechinus neumayeri*. *Revista Chilena de Historia Natural*, 89(1), 1-9.https://doi.org/10.1186/s40693-016-0052-z

Lang, B. J., Donelson, J. M., Caballes, C. F., Uthicke, S., Doll, P. C., & Pratchett, M. S. (2022). Effects of elevated temperature on the performance and survival of pacific crown-of-thorns starfish (*Acanthaster solaris*). *Marine Biology*, 169(4), 43.https://doi.org/10.1007/s00227-022-04027-w

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC bioinformatics*, *13*, 134. https://doi.org/10.1186/1471-2105-13-134

Yusof, N. A., Masnoddin, M., Charles, J., Thien, Y. Q., Nasib, F. N., Wong, C. M. V. L., Murad, A. M. A., Mahadi, N. M., & Bharudin, I. (2022). Can heat shock protein 70 (HSP70) serve as biomarkers in Antarctica for future ocean acidification, warming and salinity stress?. *Polar Biology*, 45(3), 371-394.https://doi.org/10.1007/s00300-022-03006-7

Zhan, Y., Li, J., Sun, J., Zhang, W., Li, Y., Cui, D., Hu, W., & Chang, Y. (2019). The Impact of Chronic Heat Stress on the Growth, Survival, Feeding, and Differential Gene Expression in the Sea Urchin *Strongylocentrotus intermedius*. *Frontiers in Genetics*, 10, 301. https://doi.org/10.3389/fgene.2019.00301

Zhao, H., Yang, H., Zhao, H., Chen, M., & Wang, T. (2011). The molecular characterization and expression of heat shock protein 90 (Hsp90) and 26 (Hsp26) cDNAs in sea cucumber (*Apostichopus japonicus*). *Cell Stress & Chaperones*, 16(5), 481–493. https://doi.org/10.1007/s12192-011-0260-z

CHAPTER 5.   OVERALL CONCLUSIONS

In conclusion, our study highlights the potential impacts of climate change and human activities on marine ecosystems and biodiversity, including the potential loss of important species like *L. clathrata*. Our findings suggest that continuous prolonged exposure to thermal stress could be harmful to the starfish's physiology, as evidenced by the observed changes in immunological parameters (phagocytic capacity and total coelomic protein), behavior and increased mortality rates. Short-term exposure to thermal stress may trigger the immune response, but chronic exposure could have adverse effects.

Furthermore, our research highlights the therapeutic potential of *L. clathrata* and the need for conservation efforts to protect this species and other marine life. Future research should focus on understanding the underlying molecular mechanisms of stress response in *L. clathrata*, including the role of HSP90 genes. Additionally, further investigation is needed to isolate, purify, and characterize the specific compound responsible for the antimicrobial properties of *L. clathrata*, which could lead to the discovery of new drugs. Overall, our study underscores the importance of preserving marine ecosystems and the need for continued research to inform conservation efforts.