Emmanouela Karaveti

Evaluation of the effect of season and raft location on the immune status and byssus production of mussels cultivated in Galicia (Rías Baixas)

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Declaração de Autoria

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Emmanouela Karaveti

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Abstract

Climate change presents a serious threat to marine ecosystems, impacting both wild and cultivated species. One of the most important cultivated species in Europe is the Mediterranean mussel, *Mytilus galloprovincialis*, which is primarily produced along the nutrient-rich Galician coastline in northwest Spain. This mussel species is crucial for the local economy and provides many jobs in the region. Due to their filter-feeding lifestyle, mussels constantly face environmental stress and depend on their innate immune system, primarily mediated by hemocytes, to survive. In this study, we investigated how ocean waves and seasonal changes affect the immune system of cultivated mussels and how these factors influence byssus production, the structure that helps mussels attach to surfaces. To evaluate the immune response, we measured oxidative activity-free radicals such as reactive oxygen species (ROS) and nitric oxide (NO). Besides, the gene expression of glutathione S-transferase (GST), lysozyme (LYS), and heat shock protein 70 (HSP70) was assessed. Our results showed that immune responses varied significantly with the seasons, peaking in the summer, likely due to higher temperatures and consequently the potential proliferation of pathogenic organisms, and increased metabolic demands. We also examined byssus production through the expression of the mussel foot protein Mgfp3 gene. Our findings indicated that the expression of the Mgfp3 varied seasonally, being probably affected by factors such as hydrodynamic forces and temperature, peaking in spring. Overall, this study highlights the complex relationship between climate factors and mussel performance. It emphasizes the importance of implementing sustainable aquaculture practices that consider seasonal variations and climate impacts. Understanding these dynamics is crucial for adapting mussel farming strategies to the challenges posed by climate change, ensuring the resilience of this vital species.

Key words: Aquaculture, *Mytilus galloprovincialis*, Climate change, Immune system, Byssus production, Ocean waves, Seasonality

Resumo

As alterações climáticas estão a ter um impacto crescente nos ecossistemas marinhos, afetando tanto as espécies selvagens como as cultivadas. Uma espécie particularmente importante para a aquicultura na Europa é o mexilhão do Mediterrâneo (*Mytilus galloprovincialis*), com a maior parte da sua produção concentrada ao longo da costa galega, no noroeste de Espanha. A criação de mexilhões nesta região é essencial para a economia local, proporcionando emprego e contribuindo para a subsistência da comunidade. No entanto, os mexilhões são filtradores e estão constantemente expostos a stresses ambientais que podem influenciar o seu sistema imunitário e a sua capacidade de produzir *byssus,* os fios fibrosos que os ajudam a fixar-se às superfícies. Neste estudo, pretendemos compreender de que forma as alterações sazonais e a ação das ondas do mar afetam a resposta imunitária dos mexilhões e a sua produção de *byssus*. O sistema imunitário dos mexilhões é simples mas crucial para a sua sobrevivência, dependendo dos hemócitos, um tipo de célula imunitária, que ajuda o mexilhão a combater os agentes patogénicos. Os hemócitos também produzem espécies reativas de oxigénio (ROS) e óxido nítrico (NO), moléculas que desempenham um papel na defesa dos mexilhões contra infeções e stress ambiental. Para avaliar a resposta imunitária, medimos a produção de ROS e NO nos mexilhões em diferentes condições. Examinámos também a expressão de genes relacionados com a função imunitária e as respostas ao stress: glutationa S-transferase (GST), lisozima (LYS) e proteína 70 de choque térmico (HSP70). Além disso, investigámos a expressão do gene Mgfp3, que está envolvido na produção de *byssus*, para ver como a capacidade dos mexilhões de se fixarem às superfícies era afetada pelas estações do ano e pela ação das ondas. O estudo foi realizado em quatro locais diferentes de cultivo de mexilhões na Ría de Arousa, na Galiza, onde as jangadas que contêm os mexilhões foram expostas a diferentes níveis de ação das ondas do mar. Os mexilhões foram amostrados sazonalmente - durante a primavera, o verão, o outono e o inverno - para que pudéssemos acompanhar a evolução do seu sistema imunitário e da produção de *byssus* ao longo do ano. A análise estatística foi utilizada para determinar se fatores como a localização da jangada, a profundidade da água e a estação do ano tinham impacto na saúde e na força de fixação dos mexilhões. Os nossos resultados mostraram que a resposta imunitária dos mexilhões foi fortemente afetada pela estação do ano. No verão, quando as temperaturas da água eram mais elevadas, os mexilhões produziam mais ROS e NO, que são indicadores de um sistema imunitário ativo. Isto deve-se provavelmente ao facto de as temperaturas mais quentes aumentarem as taxas metabólicas dos mexilhões e os tornarem mais suscetíveis a agentes patogénicos, exigindo uma resposta imunitária mais forte. Também descobrimos que os genes envolvidos na função imunitária - GST, LYS e HSP70 - estavam mais ativos durante os meses mais quentes. Por exemplo, o GST, que ajuda a gerir o stress oxidativo, estava mais ativo na primavera. Isto pode dever-se à fase de recuperação após a desova, quando os mexilhões precisam de reparar as células e os tecidos. Na primavera, as águas das Rias Galegas também tendem a ter mais algas, proporcionando uma fonte de alimento rica para os mexilhões, mas também aumentando o stress oxidativo a que estão sujeitos. O gene da lisozima (LYS), que é fundamental para quebrar as paredes celulares bacterianas, estava mais ativo no verão, provavelmente porque as águas mais quentes promovem o crescimento bacteriano. Os mexilhões precisam de reforçar as suas defesas antibacterianas para fazer face ao aumento da presença de agentes patogénicos. A proteína de choque térmico 70 (HSP70), que ajuda a proteger as células de danos relacionados com o calor, também mostrou a sua maior expressão nos meses de primavera e verão. Este facto pode dever-se à necessidade de proteção adicional dos mexilhões contra as temperaturas mais elevadas durante estes períodos. Para além de estudar o sistema imunitário, também analisámos a forma como a produção de *byssus* variava com as estações do ano. O *byssus* é vital para os mexilhões, pois permite-lhes fixarem-se às superfícies e resistir às forças das ondas do mar. Verificámos que a produção de *byssus*, tal como indicado pela expressão do gene Mgfp3, era mais elevada na primavera. Isto pode dever-se ao facto de as condições na primavera - temperaturas moderadas e menor stress ambiental - serem mais favoráveis à produção de *byssus*. Por outro lado, a produção de *byssus* foi mais baixa no verão. Durante este período, os mexilhões parecem dar prioridade à gestão do stress térmico e de outros desafios em detrimento da produção de fios de *byssus* fortes. Além disso, as condições hidrodinâmicas mais calmas no verão podem significar que os mexilhões não precisam de produzir tantos fios de byssal para se manterem agarrados às superfícies. É interessante notar que, embora a localização das jangadas e a profundidade a que os mexilhões foram cultivados não pareçam afetar significativamente a sua resposta imunitária ou a produção de *byssus*, os efeitos sazonais foram muito claros em todos os locais. Isto sugere que as alterações sazonais, particularmente as variações de temperatura e a carga patogénica, são os principais fatores que influenciam o sistema imunitário e a força de fixação dos mexilhões. O facto de a localização e a profundidade da jangada não terem desempenhado um papel importante pode indicar que os mexilhões são geralmente capazes de se adaptar a diferentes condições ambientais, desde que essas condições sejam adequadas. Globalmente, este estudo realça a forte influência que as alterações sazonais têm nas respostas imunitárias e na produção de bisso do *Mytilus galloprovincialis*. Estes resultados são importantes para a cultura de mexilhões, especialmente porque as alterações climáticas continuam a afetar os ambientes marinhos. O aumento das temperaturas, tempestades mais frequentes e outras alterações relacionadas com o clima são suscetíveis de colocar desafios à mitilicultura no futuro. Compreender a forma como os mexilhões respondem às pressões ambientais ao longo do ano pode ajudar os agricultores a adaptarem as suas práticas para melhor apoiarem a saúde dos mexilhões e manterem uma forte fixação do byssus, que é fundamental para evitar que os mexilhões sejam deslocados pelas ondas. Em conclusão, os resultados deste estudo sugerem que o ajustamento das práticas agrícolas para ter em conta as variações sazonais nas respostas imunitárias dos mexilhões e na força de fixação será crucial para o futuro da aquicultura de mexilhões. A investigação deve continuar a explorar os mecanismos moleculares subjacentes que conduzem a estas alterações sazonais, bem como a forma como outros fatores ambientais, como a disponibilidade de alimentos e a acidificação dos oceanos, podem influenciar a saúde dos mexilhões. Ao obter uma compreensão mais profunda destes processos, podemos trabalhar no sentido de desenvolver práticas de cultura de mexilhões mais sustentáveis e resistentes que ajudarão a mitigar os impactos das alterações climáticas.

Palavras-chave: Aquicultura, *Mytilus galloprovincialis*, Mudanças climáticas, Sistema imunológico, Produção de byssus, Ondas oceânicas, Sazonalidade.

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Abbreviation list

AMP Anti-Microbial Peptides

DO Dissolved Oxygen

FSW Filtered Sea Water

LPS Lipopolysaccharide

LTA Lipoteichoic Acid

Mgfp3 *Mytilus galloprovincialis* Foot Protein 3

NO Nitric Oxide

NOS Nitric Oxide Synthase

NTP Nitrotyrosine Protein

PAMP Pathogen-Associated Molecular Patterns

PI Propidium Iodide

PRR Pattern Recognition Receptors

RLU Relative Luminescence Units

RNS Reactive Nitrogen Species

ROI Oxygen Intermediate Reagents

ROS Reactive Oxygen Species

STT Sea Surface Temperature

UNFCCC United Nations Framework Convention on Climate Change

1. Introduction

According to the United Nations Framework Convention on Climate Change (UNFCCC), climate change is defined as "a change in the climate that is attributed directly or indirectly to human activity, which alters the composition of the global atmosphere and is in addition to natural climate variability observed over comparable time periods". Climate change can also be defined, in a simpler way, as the shift in climate patterns mainly caused by greenhouse gas emissions. Greenhouse gas emissions result in heat being trapped by the earth's atmosphere, making this the main driving force responsible for global warming (Fawzy et al., 2020). In the last decades, climate change has been causing major alterations in the Earth's climatic system. Such changes include increasing temperature and heat stress, extreme weather conditions, floodings, biodiversity loss, ocean acidification, species vulnerability, and migration (Chausali et al., 2023).

This global process of climate change and its manifestation by ocean warming and acidification, sea level rise, and extreme weather events, such as storms, winds, and waves, are all impacting marine ecosystems, affecting their resilience, functionality, and biodiversity dynamics (Des et al., 2020; Gissi et al., 2021). Among others, one of the highest effects on marine ecology is due to the stratification caused by the waves, which are therefore responsible for the distribution of nutrients, plankton, and pelagic ecology (Woolf and Wolf, 2013). All these changes have a significant impact not only on the natural marine species and habitats but also on the ones produced by the aquaculture sector. Therefore, climate change also has an important economic and social influence beyond the environmental impact. Directly, environmental disturbances can lead to the loss of cultured organisms, but in addition, aquaculture is also affected by an increase in operational costs, an unstable market, and financial risks, resulting in job losses and community displacement (Cochrane et al., 2009).

1.2. Mussel aquaculture

The most important mollusk aquaculture products in Europe are mussels, mainly the Mediterranean mussel (*Mytilus galloprovincialis*, Lamarck, 1819) and the blue mussel (*Mytilus edulis*) (Hough, 2022), with a production of 37% out of the total aquaculture volume (eca.europa, 2023) (Figure 1.1).

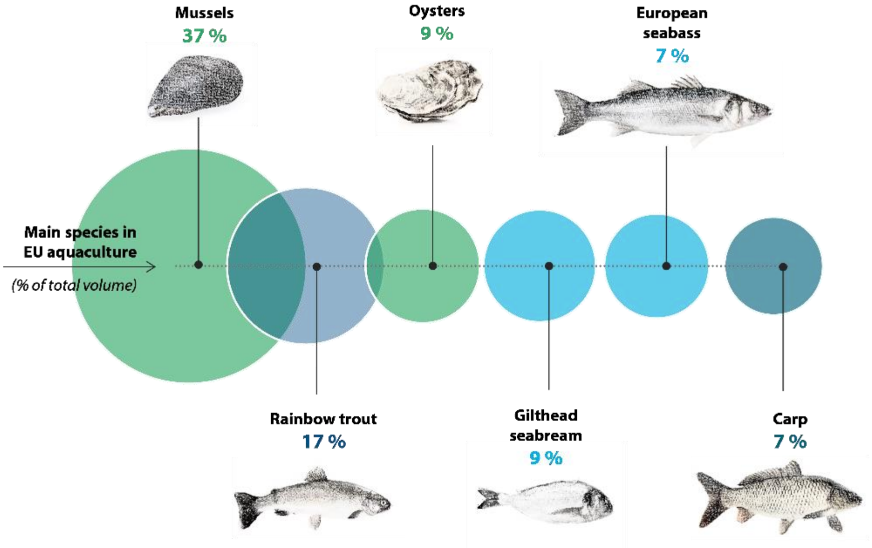


Figure 1.1: Main aquaculture species farmed in the EU, 2020 (Source: eca.europa, 2023)

Concretely, in Spain, a total of 206.4 thousand tons of mollusks was produced in 2021, being the first producing European country and obtaining a value in the first sale of € 150.4 million in 2021 according to FAO (APROMAR, 2023) (Figure 1.2).

Figure 1.1: Production of aquaculture mollusks in EU Member States by volume (tons) and value (million euros) in 2021 (based on FAO data) (Source: APROMAR, 2023).

The 97% of the total national Spanish mussel production takes place in Galicia (APROMAR, 2023). Galicia is a region located in the northwest area of the Iberian Peninsula and just north of Portugal (Figure 1.3). It is flanked to the north by the Cantabrian Sea and to the west by the Atlantic Ocean, which jointly accounts for more than 1,490 km of coastline (Garza-Gil et al., 2017). The Galician coastline is characterized by a system of drowned river valleys classified as inland waters (Rías) (Figure 1.2) fed by a dense network of rivers that benefit from nutrient-rich waters, and therefore phytoplankton growth, and providing a sheltered area, creating optimal environment and conditions for marine bivalve aquaculture (Fuentes-Santos et al., 2021; Fraga-Corral et al., 2022).

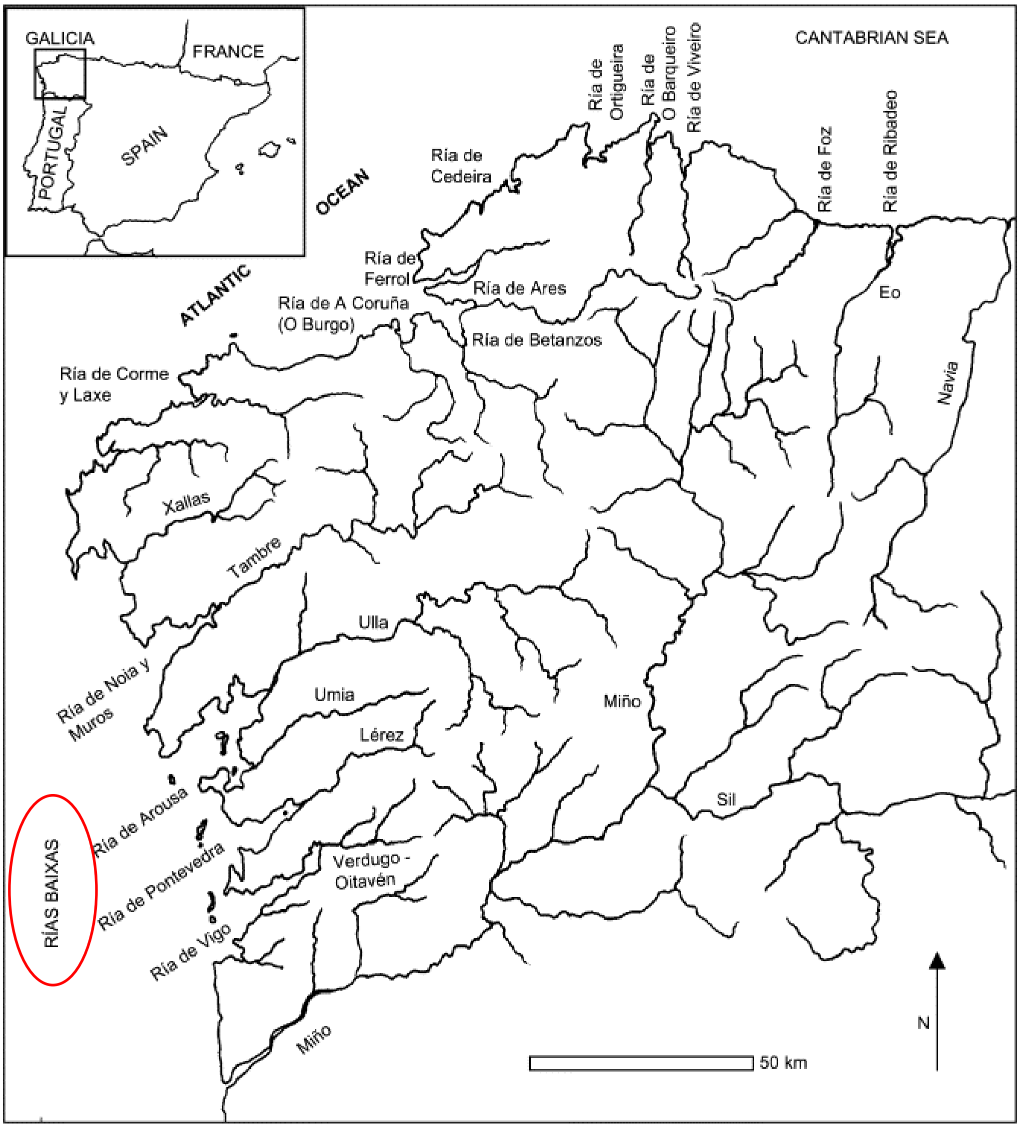


Figure 1.2: Galician coast and rias (Source: Geological antecedents of the Rías Baíxas (Galicia, northwest Iberian Peninsula) by Méndez, G., & Vilas (2005), F. Journal of Marine Systems, 54(1-4), 195-207.)

1.2.1. The importance of preserving mussel cultivation in Galicia

In Europe, there are several methods for mussel cultivation, including suspension culture, bottom or park culture, and bouchots. In the region of Galicia, Spain, the most commonly used method is suspension cultivation on rafts, known locally as "*bateas*" (Ferreira et al., 2013). The current number of rafts in Galicia is 3,387, based mostly on the Rías Baixas, and over 60% of them are in Ría de Arousa (Outeiro et al., 2020). According to FAO (2022), the mussel production reached almost 200,000 tons and a value of 164,000 €. The high number of rafts and the elevated production make mussel aquaculture an essential social and economic activity in Galicia with a direct contribution to employment (5,849 people depended directly on mussel aquaculture in 2021 (APROMAR, 2023)). All these data reflect the need to preserve this sector by studying all those factors that could reduce its production. The change in the climate conditions is producing significant variations in the temperatures, currents, waves, and other environmental factors, which could alter the success of this important economic and social activity. It is, therefore, crucial to know how mussels respond against these external factors, both by assessing the response of their immune system to these external aggressions and studying whether these can alter their ability to respond, and by analyzing whether these same aggressions can alter their ability to attach to the substrate (a key mechanism to ensure that cultivation on the rafts' ropes can function properly).

1.3. Immune system of the mussels

Bivalves, due to some specific aspects of their life (filter feeders, sedentary, presence of an open circulatory system…), are continuously exposed to sustained stress conditions, such as biotic environmental stressors (bacteria, viruses, protozoan…), xenobiotics such as chemical contaminants or abiotics, with a special exposition to currents and wave forcing (Söderhäll, 2011, Babarro and Carrington, 2013). Along the evolution, they have developed various effective strategies for protection against pathogens and environmental stresses (Song et al., 2010).

The bivalve defense system includes several physical and biological barriers (Andreyeva et al., 2022). Firstly, the shell provides the principal physical barrier, supporting and protecting the soft tissue from biological and physicochemical insults. Mucosal barriers constitute the second physical barrier, entrapping microbes and facilitating their elimination via ciliary activity (Allam and Raftos, 2015).

As invertebrates, bivalves rely exclusively on the innate immune system (Song et al., 2010), which detects the presence and the nature of infection and provides the first line of host defense (Medzhiton, 2001). This system cannot specifically discriminate between different foreign agents. However, it is capable of recognizing common pattern structures associated with pathogens known as Pathogen-Associated Molecular Patterns (PAMPs) that are present on the surface of different microorganisms (Costa, 2008), through pattern recognition receptors (PRRs) (Grinchenko and Kumeiko, 2022). Lipoteichoic acid (LTA), lipopolysaccharide (LPS), and DNA CpG motifs from bacteria; glucans or zymosan from yeast; and double-stranded RNA from viruses, among others, have been identified as PAMPs (Costa et al., 2009). Mussels' innate immunity relies on the active immune cells (hemocytes) in collaboration with the relevant humoral factors (Figure 1.4) aiming to activate an effective immune response (Bouallegui et al., 2019).

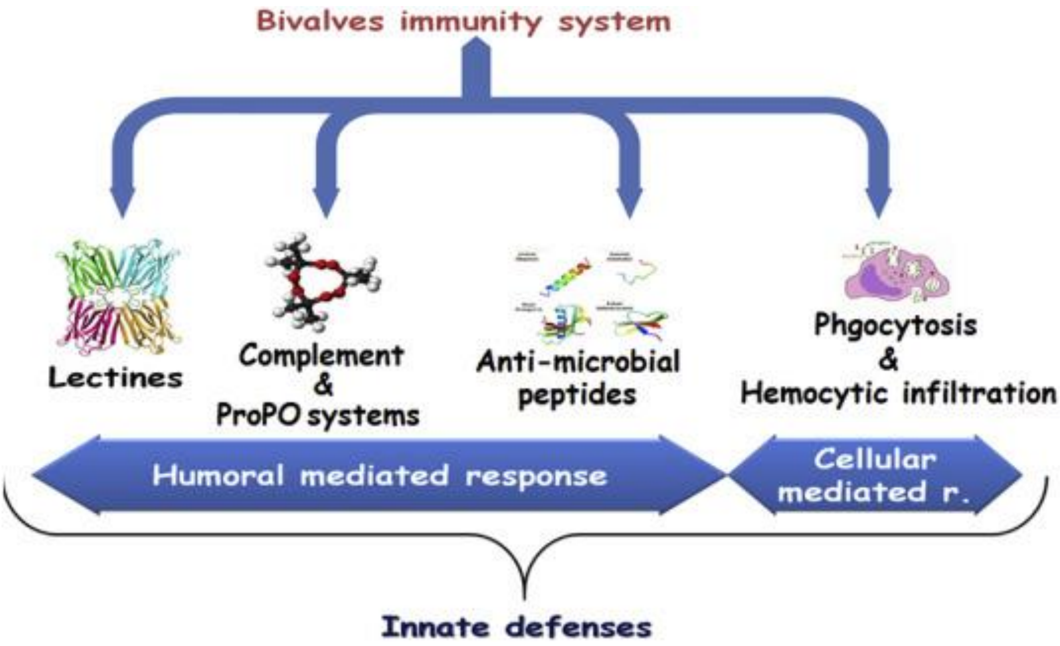


Figure 1.4: Diagram representing main types and components of bivalves' immune defenses (Source: Immunity in mussels: an overview of molecular components and mechanisms with a focus on the functional defenses by Bouallegui, Y. (2019). Fish & shellfish immunology, 89, 158-169.)

1.3.1.Cellular components of the immune system: the hemocytes

Hemocytes are the main immune cells and are responsible for the efficient protection against different invaders. These immune cells are circulating within the hemolymph functioning as phagocytes, recognizing and destroying pathogens and foreign particles (Burgos-Aceves et al., 2017). Regarding the bivalve species and according to the most common classification, the types of hemocytes are divided into two major categories: hyalinocytes and granulocytes (Grinchenko and Kumeiko, 2022). Hemocytes have several functions, some of which are phagocytosis and producing oxygen-free radicals (Bouallegui et al., 2019).

1.3.1.1. Phagocytosis

Phagocytosis is the most common and important mechanism for foreign agents' elimination (De la Ballina et al., 2022; Grinchenko and Kumeiko, 2022).

This process can be divided into five stages: (1) the recognition of the non-self-particle, (2) adhesion, (3) ingestion, (4) destruction, and (5) elimination. The first two can be caused by a random encounter between the phagocytic cell and the foreign particle or actively by chemotaxis. Recognition of foreign particles occurs through interaction between the hemocyte (specialized cellular receptors) and various PAMPs (Feng, 1988; Fawcett and Tripp, 1994).

Adhesion processes are the next stage of the phagocytic process. In this step, specialized receptors of the phagocyte's surface recognize and bind to molecules present on the surface of the microbe or target cell. The action of adhesion mechanisms is followed by the ingestion of foreign particles by the hemocytes (Uribe-Querol and Rosales, 2020).

The last steps of the phagocytic process are determined by the destruction and digestion of the pathogen. After the phagocyte engulfs the pathogen, the phagosome binds with lysosomes to form a phagolysosome (Gordon, 2016).

Lysosomes are membrane-enclosed organelles filled with a variety of enzymes capable of degrading all types of biological polymers—proteins, nucleic acids, carbohydrates, and lipids. Lysosomes function as the digestive system of the cell; they break down materials that are ingested from the outside and also digest old parts of the cell, degrading therefore the material derived from phagocytosis (Cooper and Adams, 2022). The interior of the lysosome is full of acidic hydrolytic enzymes that attack the newly formed vacuoles, releasing their contents inside them and destroying the pathogenic agent (Ratcliffe et al., 1985; López et al., 1997).

1.3.1.2. Oxidative Activity-Free radicals

After phagocytosis, the production of radicals with microbicidal activity is induced and may be used to evaluate the hemocytes' immunocompetence (De la Ballina et al., 2022).

Free radicals, such as reactive oxygen species (ROS) and nitric oxide (NO) include a range of small reactive and non-reactive molecules. Although they have extremely damaging potential, they also exhibit many physiological capabilities. In the last decades, they have attracted much attention, and it is now clear that most alterations of standard environmental conditions (e.g., biological and chemical contaminants, pathogens, alteration of physicochemical variables, etc.) can cause modifications in the oxidative radicals' production and therefore, on the cellular oxidative stress (Donaghy et al., 2015).

a. Reactive oxygen species

Reactive oxygen species (ROS) are cytotoxic agents produced by phagocytic cells as a response to membrane disturbance and are used to defend against infectious diseases because of their antimicrobial properties (Anderson, 2001).

During the process of phagocytosis, there is a high production of oxygen radicals, also known as "respiratory burst". This oxygen consumption is due to the action of NADPH-oxidase, which reduces molecular oxygen to oxygen intermediate reagents (ROIs). When there is a contact between a hemocyte with a non-self-particle, small disturbances are created at the cell membrane, causing a series of reactions (Donaghy et al, 2015). The initial ROS generated, the superoxide anion ($O_2•^-$), is converted to hydrogen peroxide (H_2O_2), which produces even more toxic products, like hydroxyl radical (•OH), hypochlorous acid (HOCl), and singlet oxygen ($^1O_2$) (Halliwell, 2006), Under normal and non-stress conditions, the formation and removal of ROS are balanced, due to their effective elimination by antioxidant defense systems (Giannetto et al., 2017). However, when ROS production exceeds the detoxification capacity of these systems, oxidative stress occurs, causing cellular damage and reducing the antioxidant defense (Georgieva, 2005).

b. Nitric oxide

Reactive nitrogen species (RNS) are a family of antimicrobial molecules. They are produced when nitric oxide (NO) is generated, either exogenously or endogenously, and then interacts with reactive oxygen species like superoxide ($O_2\bullet^-$) and hydrogen peroxide (H_2O_2) (Alhasawi et al., 2019).

RNS and nitric oxide (NO) are also produced by molluskan hemocytes as a defense mechanism. NO is a small, reactive molecule, generated from L-arginine by the nitric oxide synthase family (NOS) of enzymes. NOS catalyzes the oxidation of L-Arg via N-Hydroxy-L-arginine, an unstable intermediate, to citrulline and NO (Groves and Wang, 2000). NO is produced by three isoforms of NOS: the endothelial (eNOS) and the neuronal (nNOS), which are calcium-dependent, and the inducible (iNOS), which is calcium-independent (Förstermann and Sessa, 2012; Donaghy et al, 2015).

NO reacts with the $O_2\bullet^-$ produced by NADPH oxidase and generates peroxynitrite (ONOO-), an intermediate molecular species formed with high oxidizing power and great cytotoxic activity (Beckman and Koppenol, 1996). In mollusks, NO is produced and released after stimulation of hemocytes with a variety of particles, such as phorbol-myristate-acetate (PMA), lipopolysaccharide (LPS), or zymosan (Costa, 2008).

1.3.2. Humoral immunity

Humoral immunity refers to the components of the immune system that act quickly to defend the body against pathogens, using soluble factors present in bodily fluids (Smith et al., 2019). Hemocytes have also an important role in mussels' defenses by acting as indirect mediators of the humoral defenses. Innate mechanisms consist of a variety of molecules secreted by hemocytes, such as lectins, molecules of the complement system, and anti-microbial peptides (AMPs). These components are released in the hemolymph, activating the hemocytes and aiming to neutralize and/or eliminate foreign invaders, often accompanying the cellular response (Bouallegui et al., 2019).

1.3.2.1. Lectins

Lectins are glycoproteins that can bind to specific carbohydrates or carbohydrate moieties on glycoconjugates in a reversible manner without affecting their structure (Vasta, 2016). Lectins are essential molecules for recognizing foreign substances. They primarily serve as agglutinins and opsonins, substances that bind together particles or cells to signal immune cells to destroy them. Lectins are essential to a wide range of biological processes, including the regulation of protein-carbohydrate interactions throughout an organism's body, cell-to-cell communication, immune responses, and host-parasite interactions (Grinchenko and Kumeiko, 2022).

1.3.2.2. Complement system

The complement system consists of a group of proteins that collaborate to identify and eliminate pathogens to prevent infections (Merle et al., 2015). It works by triggering a chain of reactions that eventually eliminate pathogens. There are three pathways of complement activation: the classical pathway, which is activated directly by antibodies or by direct binding of complement component C1q to the pathogen surface; the lectin pathway, which is triggered by mannan-binding lectin, a normal serum constituent that binds some encapsulated bacteria; and the alternative pathway, which is triggered directly on pathogen surfaces (Janeway et al., 2001). It functions through opsonization, which marks pathogens for destruction. Inflammation and the formation of membrane attack complexes that directly lyse invading cells are all processes that can occur when the complement system is activated (Chen et al., 2018; Grinchenko and Kumeiko, 2022).

1.3.2.3. Antimicrobial Peptides (AMPs)

Antimicrobial peptides are major humoral components of the innate immune system. They are small, positively charged, and cysteine-rich molecules. (Liao et al., 2013). They are stored in granules as active forms (Mitta et al., 2000) and secreted by hemocytes, showing antifungal, antibacterial, or antiviral effects and acting via binding to the invading agents' membrane, by an electrostatic interaction between cell wall or membrane residues, resulting in their elimination (Grienke et al., 2014). They are considered natural antibiotics due to their ability to directly attack and kill microbes (Brown and Hancock, 2006; Balseiro et al., 2011). AMPs in mussels have been classified into four families, based on their structure (defensins, myticins, mytilins, and mytimycins) (Mitta et al., 2000).

1.4. Climate effect on the immune system

Mussels, due to their exposure to natural stressors, result in an increased variability of molecular and tissue responses (Carella et al., 2018). Changes in abiotic factors, such as temperature, salinity, and water acidification, due to climate change, can influence significantly the immune system of mollusks (Matozzo and Marin, 2011).

For instance, elevated temperatures enhance the immune response, increasing total hemocyte count (THC), phagocytosis lysozyme activity, and antioxidant enzyme activity (Monari et al., 2007). Additionally, salinity changes can result in suppression of the immune response (Gagnaire et al., 2006; Green and Barnes, 2010), leading to decreasing hemocyte activity and down-regulation of the immune response genes, indicating stress and decreased immune capacity. Furthermore, reduced pH levels, caused by ocean acidification, can compromise mollusks' immunity by decreasing hemolymph cytotoxicity and phagocytic activity, making them more susceptible to pathogens. These environmental changes can alter mollusks' immune capacities and increase their vulnerability to diseases, potentially impacting their populations (Matozzo et al., 2012).

1.5. Mussel attachment

The mussel development consists of three primary stages: blastula, free swimming larva, and settled juvenile/adults attaching to the substrate (Dyachuk and Odintsova, 2009). Adult mussels live in a range of habitats attached to the substrate and are strongly influenced by the motion of water. The velocity of the water motion varies as well, in a range of 1-10 m/s on protected shores and bays and on wave-exposed outer coasts, respectively (Carrington et al., 2008). Moving water generates forces on mussels, leading them to maintain a strong byssal attachment that withstands hydrodynamic challenges to persist (Carrington, 2002). For that reason, they have developed a particular holdfast system, the byssus, to attach to different hard substrates (Wang and Scheibel, 2018). The byssus is a fibrous, proteinaceous, and extracellular attachment structure. It is secreted by the mussel foot and is composed of numerous byssal threads, each connecting proximally to a common stem that is rooted within the byssus gland of the foot and ultimately connects to the byssus retractor muscles (Carrington, 2002) (Figure 1.5).

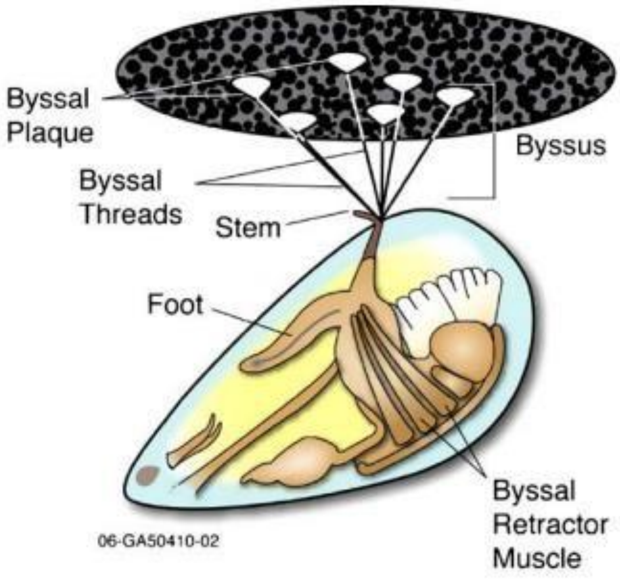


Figure 1.5: Anatomy of M. edulis mussel and byssus structures (Source: 'Understanding marine mussel adhesion' by Silverman and Roberto (2007), Marine Biotechnology, 9, 661-681.)

1.5.1. Climate effect on the attachment and byssus production

According to a study conducted by Newcomb et al. (2022), environmental factors such as temperature, salinity, tidal fluctuations submergence, agitation, water velocity, and seasonality appear to have some kind of effect on the byssus production and strength, directly or indirectly.

Hydrodynamics have a significant role in influencing mussel population dynamics. Specifically, disturbances created by wave-generated hydrodynamic forces control the structuring of mussel bed communities (Babarro and Carrington, 2013).

A significant reason for the loss of mussels in the intertidal zone and the shallow subtidal zone (for mussels overgrown by algae) is the dislodgment by waves. In patches where mussels are tightly packed, they provide a shield for each other from hydrodynamic forces acting along the flow and physical support for resisting these forces. However, the difference in pressure between the top and bottom of a raft can potentially cause mussel dislodgement (Hunt and Scheibling, 2001). The risk of dislodgment increases with flow speed and mussel size and decreases with mussel tenacity or attachment strength (Babarro and Carrington, 2013).

Objectives

The main objective of this study is to assess the effect of some consequences of climate change on mussel aquaculture. For that, a study to assess the impact of ocean waves and seasonality on the immune system of cultivated mussels and to investigate its influence on byssus production was conducted. This was achieved through the development of the following specific objectives.

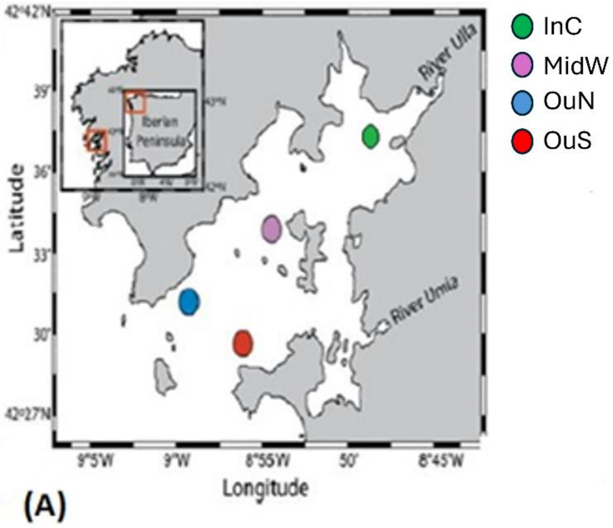
1. The evaluation of the immune condition and oxidative stress to establish the potential relation between wave forcing and temperature variations on the mollusk cellular stress response.

2. The evaluation of the specific gene expression of foot protein 3 (Mgfp3) to infer risk assessments for the maintenance of the biomass in the cultivation systems in combination with spatial (raft location) and temporal (seasonality) parameters.

2. Materials and Methods

Four distinct raft locations were selected according to the natural variability for the Ría de Arousa within the range of rafts highly exposed to waves, assuming the outer zones of the bay (OuN and OuS) as the most exposed, and more sheltered and protected areas towards the interior of the Ría (InC). In the middle section of the Ría, another site was used (MidW) with intermediate environmental conditions between the most exposed and the most sheltered rafts (Figure 2.1A).

The sampling was conducted by boat at the 4 rafts sites described above. Six mussels (three at 0-6 m depth and three at 6-12 m depth) were taken per raft (a total of 24 mussels per seasonal sampling) and brought to the aquarium facilities for further processing. Seasonal sampling was carried out in spring, summer, autumn, and winter (Figure 2.1B).



(A)

DATE	SEASON	Nº MUSSELS
7/6/2023	Spring	24 (1M-24M)
7/9/2023	Summer	18 (25M-42M)*
22/11/2023	Autumn	24 (43M-66M)
29/2/2024	Winter	24 (67M-90M)

*Sampling not possible at raft 2 (OuS)

(B)

Figure 2.1: (A) Map of Ría de Arousa (NW Spain) showing the experimental zones where mussel sampling was conducted. OuN (blue) outer exposed northern site, OuS (red) outer exposed southern site, MidW (purple) middle section of the Ría, InC (green) inner and sheltered site (B) Sampling dates and the total number of individuals sampled per season.

2.2. Experimental setup

In each sampling, a hemolymph sample was taken from each mussel (Figure 2.2) to analyze the production of ROS and NO, and viability of hemocytes. The hemocyte viability was measured to reference the values obtained in ROS and NO production with regard to the number of live cells. Mussels were notched in the shell and hemolymph was extracted from the anterior adductor muscle with 0.5 mm diameter disposable needles. Afterward, on the day of sampling, a piece of gill and 3 pieces of the foot corresponding to the proximal, middle, and distal parts of each mussel were collected (Figure 2.3) and preserved in RNAlater at -80°C until their processing for gene expression analysis.

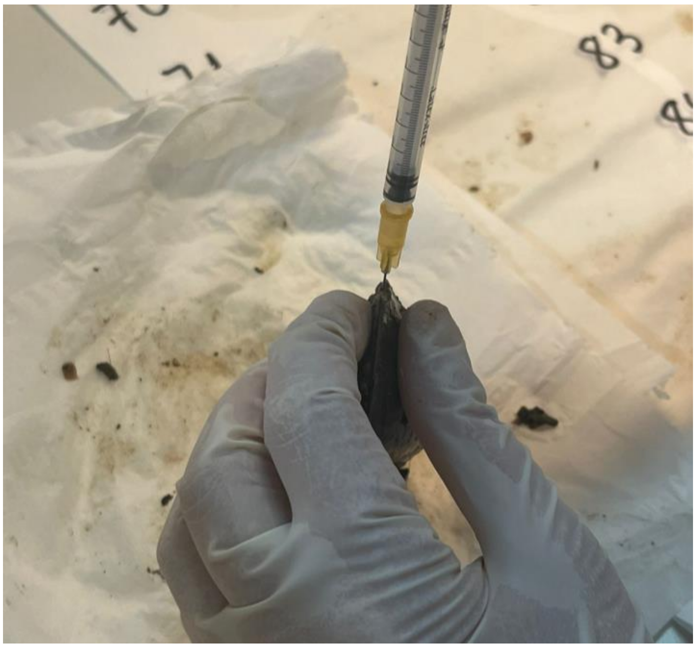


Figure 2.2: Hemolymph extraction from adductor muscle after notching the shell.

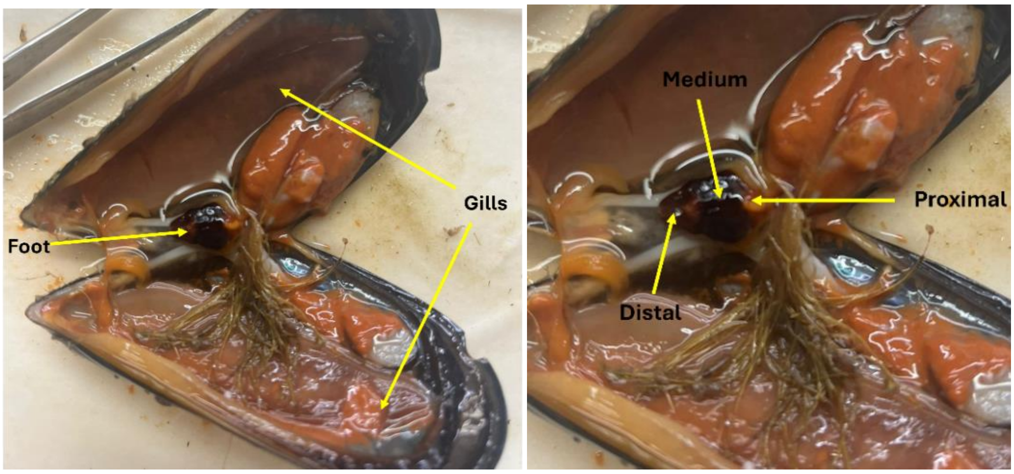


Figure 2. 3:Internal anatomy of the mussel. The sampled tissues (gills and the different sections of the foot) are shown.

2.2.1. Reactive Oxygen Species (ROS) Production

The emission of relative luminescence units (RLU) for ROS production was determined after stimulation of the cells with zymosan A (Sigma) and amplified by the addition of luminol. Briefly, 100 μL of hemolymph per mussel were dispensed into 96-well plates in duplicate. After 30 min of incubation at 15°C in the dark for cell adhesion, 100 μL of zymosan A per well (final concentration 1 mg/mL) diluted in the working solution of luminol were added into the wells. 100 μL of filtered seawater (FSW) were added to the controls. The maximum luminescence value was immediately read on a luminometer. The ROS production was normalized regarding the percentage of live cells (see 2.2.3), which are the cells producing ROS.

2.2.2. Nitric Oxide (NO) production - Griess reaction

The Griess reaction (Green et al., 1982) quantifies the concentration of nitrite in the supernatant. Nitric oxide (NO) is an unstable molecule that is degraded to nitrite and nitrate. The concentration of nitrite in the supernatants was estimated by comparing the absorbance obtained with known concentrations of $NaNO_2$ (0.1, 0.5, 1, 5, 10, 25, 50, and 100 μM) for a standard line. Briefly, 100 μL of hemolymph per mussel were dispensed into 96-well plates in duplicate. After 30 min of incubation at 15°C in the dark for cell adhesion, 100 μL of zymosan A solution was added to get a final concentration of 1 mg/mL into the wells mimicking an infection. The same volume of FSW was added to the controls and incubated for 2 h. 50 μL of the supernatants were removed from individual wells and placed in a new 96-well plate. A 100 μL aliquot of 1% sulphanilamide (Sigma) in 2.5% phosphoric 12 acid (Sigma) was added to each well, followed by 100 μL of 0.1% N-naphthyl-ethylenediamine (Sigma) in 2.5% phosphoric acid. The absorbance was determined using a spectrophotometer at 540 nm (Multiskan™GO, Thermo Fisher Scientific). The nitrite concentration (μM) in the sample was determined from standard curves generated using known concentrations of sodium nitrite (Sigma). Zymosan A was previously boiled for 30 min, washed twice in FSW, and resuspended in FSW (20 mg/mL). The nitrite concentration (μM) was normalized regarding the percentage of live cells (see 2.2.3), which are the cells producing NO.

2.2.3. Measurement of hemocyte viability

For hemocyte viability, the percentage of viable hemocytes (live cells) was determined by flow cytometry using Propidium Iodide (PI, Invitrogen). PI is a fluorescent agent, which only penetrates into dead cells that have lost their membrane integrity.

Hemolymph was dispensed into flow cytometry tubes (100 µL per mussel) and 2 µL of PI (1.0 mg/mL) were then added and incubated for 15 min in the dark at room temperature. After stopping the labeling by placing the tubes on ice, cellular suspensions were diluted by the addition of 200 µL of PBS 1X. Once the PI intercalates with DNA, it emits fluorescence that is detected on the FL2-H channel, providing the percentage of dead cells out of 100% and consequently, reflecting the percentage of viable cells, since PI is not permeable into live cells. A total of 100,000 events were measured per sample.

2.2.4. RNA Extraction

Total RNA from gill and foot samples was extracted using Trizol (Invitrogen), by aqueous-organic separation and Zymo Research kit (Direct-zol RNA Miniprep), following the manufacturer's recommended instructions. In summary, the tissue obtained was homogenized with Trizol using a potter with a specific device (Figure 2.4). After vortexing and incubation, chloroform was added, and the sample was loaded onto Phase Lock columns. Following centrifugation, the aqueous phase was transferred to a new tube, and ethanol was added for further RNA purification using a Zymo kit column. A DNase treatment was performed with DNase I enzyme to eliminate any DNA contamination from the samples. The RNA was eluted, kept on ice during quantification, and stored at -80°C.

2.2.5. RNA quantification

The quantification of RNA was conducted with the use of a Thermo Scientific NanoDrop 2000c Spectrophotometer. 2 µL of each sample were used for the quantification. Absorbance measurements made on a spectrophotometer included the absorbance of all molecules in the sample that absorb at the wavelength of interest. The ratio of absorbance at 260 nm and 280 nm (maxima absorbance of nucleic acids and proteins, respectively) was used to assess the purity of RNA (Table 2.1). A ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm.

Table 2.1: RNA concentrations measured by Nanodrop for samples belonging to two animals of the total samples analyzed.

Sample ID	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
12M Gills	1129.5	ng/µl	28.237	13.279	2.13	2.24	RNA	40
12M Proximal	1057.4	ng/µl	26.434	12.453	2.12	2.12	RNA	40
12M Medial	1108.8	ng/µl	27.72	13.296	2.08	2.1	RNA	40
12M Distal	1388.9	ng/µl	34.723	16.159	2.15	2.19	RNA	40
13M Gills	705.7	ng/µl	17.642	8.481	2.08	2.01	RNA	40
13M Proximal	1261	ng/µl	31.524	14.822	2.13	2.17	RNA	40
13M Medial	1058	ng/µl	26.449	12.328	2.15	2.24	RNA	40
13M Distal	1272.5	ng/µl	31.813	14.84	2.14	2.16	RNA	40

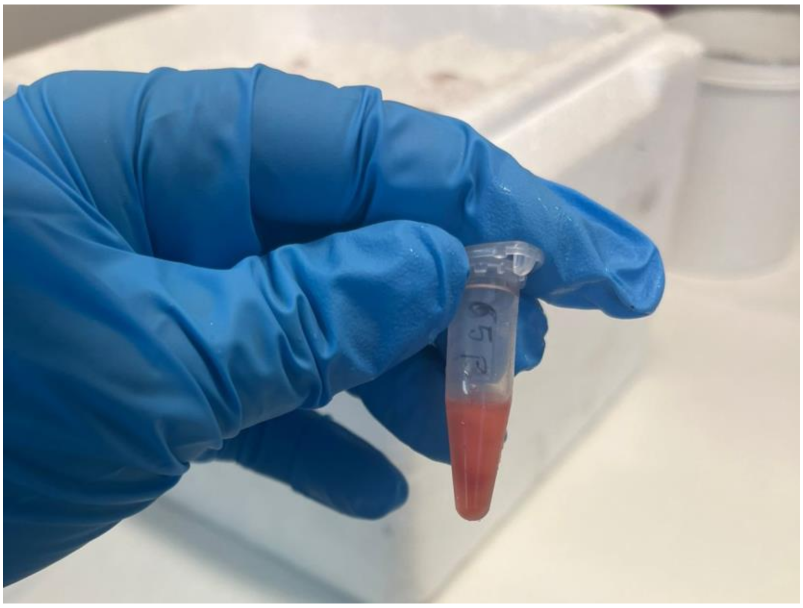


Figure 2.4: Gill tissue homogenized with Trizol

2.2.6. First Strand cDNA Synthesis (RNA Retro-Transcription)

The First-strand cDNA was synthesized using 4 µL of 5X Reaction Mix, 2 µL of Maxima Enzyme Mix, RNA (1,000 ng/µL), and RNase-free water calculated based on the RNA concentration up to a final reaction volume of 20 µL. The mixture was incubated for 10 minutes at 25°C, followed by 15 minutes at 50°C. The reaction was terminated by heating at 85°C for 5 minutes. The products of the first strand cDNA synthesis were stored at -20°C until use.

2.2.7. Conventional PCR to check the efficiency of retro-transcription

For the PCR, a total volume of 25 µL reaction mixture was prepared consisting of 12 µL Taq Master Mix (DreamTaqMM), 1 µL Forward primer (10 µM), 1 µL Reverse primer (10 µM), 10 µL nuclease-free water and 1 µL cDNA. 18S gene was used as reference for cDNA transcription efficiency. The mixture was placed in a thermal cycle following the cycle: 94°C for 5 minutes and then at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, for 35 cycles. The reaction was then terminated by incubating at 72°C for 7 more minutes to allow for complete elongation of all amplified fragments. Negative control was always included using the reaction mixture without the addition of cDNA.

2.2.8. Gel electrophoresis

Agarose electrophoresis was performed in 2% agarose gels containing 1 × TAE buffer (0.4 M Tris-acetate, 0.01M EDTA), which was also used as a running buffer and green safe as intercalating dye. 8 µL of PCR products obtained in 2.2.7. were then loaded on the gel. 5 µL of GeneRuler 100bp Plus DNA Ladder were added as a size reference. Electrophoresis was performed at a voltage of 100V, for 30 minutes. The gel photos were taken on Image Lab™ Software, like the example shown in Figure 2.5.

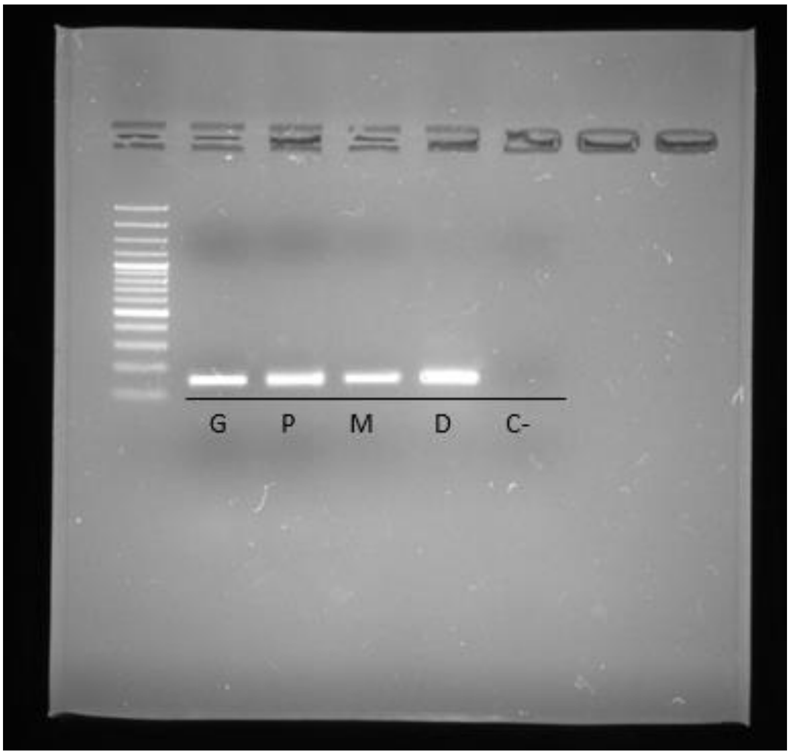


Figure 2.5: Gel showing the 18S PCR to evaluate the efficiency of the retro-transcription of the sample "Mussel 9". The first line indicates the ladder, and the samples in order: (G: Gills, P: Proximal Foot, M:

Medial Foot, D: Distal Foot). The last one (band not visible) is the negative control. The gel shown in this photo is an example of one sample.

2.2.9. Gene expression by qPCR

Regarding the samples for the gene expression analysis, only 2 rafts were considered, in particular the ones with the most extreme conditions (OuN and InC). Gene expression analysis by qPCR was conducted using 3 genes: GST for oxidative stress, HSP70 for both heat shock and protein folding, and lysozyme for immunity evaluation. Also, the foot protein 3 (Mgfp3), which is a component of the adhesive plaque of the byssus was evaluated in the distal parts of the foot. Previous qPCR expression analyses (data not shown) displayed a higher expression of Mgfp3 in this part of the foot compared to the medial or proximal parts. Specific Real-time PCR (qPCR) primers (Table 2.2) were designed using the PrimerQuest™ Tool from the Integrated DNA Technologies (IDT) website, according to known qPCR restrictions (amplicon size, Tm difference between primers, GC content, and self-dimer or cross-dimer formation). Then, the efficiency of the primer pairs was analyzed with seven serial ten-fold dilutions of cDNA and calculated from the slope of the regression line of Cts versus the relative concentration of cDNA (Pfaffl, 2001). A melting curve analysis was also performed to verify that no primer dimers nor unspecific products were amplified. qPCR reactions were performed in a QuantStudio3 (Applied Biosystems) sequence detector. One microliter of 10-fold diluted cDNA template was mixed with 0.5 µL of each primer (10 µM) and 12.5 µL of SYBR green PCR master mix (Thermo Fisher Scientific) in a final volume of 25 µL. The standard cycling conditions were: 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplification of a single PCR product (a single sharp peak) from each experiment was monitored by a melting curve analysis. All reactions were performed as technical triplicates. The expression of the selected genes was normalized using the 18S as a housekeeping gene and analyzed following the Pfaffl method.

Table 2.2: Primers used for the qPCR analysis.

Primer name	Gene name	Sequence 5'-3'
Mussel 18S-F	18S small subunit ribosomal RNA gene	CACTGAAGGAATCAGCGTGTCT
Mussel 18S-R		CGTAATCAACGCGAGCTTATGA
Mgfp3B-F	Foot protein-3B	ATCAGTGTCGCAGTGTTG
Mgfp3B-R		CAACGTCTTGAAGGACCATAG
MgGSTS2-F	Glutathione S transferase sigma 2	TGAAGCAAAGAAGGCAGAGG
MgGSTS2-R		CAAGCCTTTACATCCGGGTA
MgHSP70-F	HSP70	CCTTCACAGACACCGAAAG
MgHSP70-R		TGTACTGTAGCGTCATCAAAC
MgLYS-F	Lysozyme	TCGACTGTGGACAACCAAAA
MgLYS-R		AGGGTTTGTGCATCCTCTTG

2.3. Statistical analysis

Statistical analysis was performed using the software STATISTICA v.7.0.61. ROS and NO production, and gene expression were analyzed by Factorial ANOVA with season, raft position, rope depth, and treatment as independent variables. Before analyses, normality and homogeneity of variances were checked by Shapiro-Wilk and Levene’s tests, respectively. In cases of violation of the assumptions, rank-transformed data were considered to run the ANOVA tests. Results were considered significant at $p \leq 0.05$. In the cases of significant differences, post-hoc analysis by Tukey’s HSD were made for multiple comparisons. The values were expressed as mean ± SEM.

3. Results

3.1. Immune system evaluation

3.1.1. ROS production

Regarding ROS production, the individuals stimulated with zymosan showed a statistically significant response, compared to the controls, for all the variables (season of the year, raft position, and ropes depth) (data not shown). This suggests that even though the mussels were exposed to the potential stress caused by the different variables of the experiment, their immune response seemed to respond to this particular stimulus.

Among all the analyzed variables, significant differences were only found for the season ($p \leq 0.05$) with different detected responses on ROS production according to the moment of the year. The stimulation index was calculated to measure how high or low the hemocytes' response was regarding the controls. As is shown in Figure 3.1, a significantly higher stimulation index of ROS, and therefore stimulated hemocyte's response, was observed during summer and autumn, with very similar values (18 ± 1.98 and 19 ± 1.76, respectively). In spring and winter, the hemocytes' response stayed much lower (3 ± 0.38 and 2 ± 0.35, respectively).

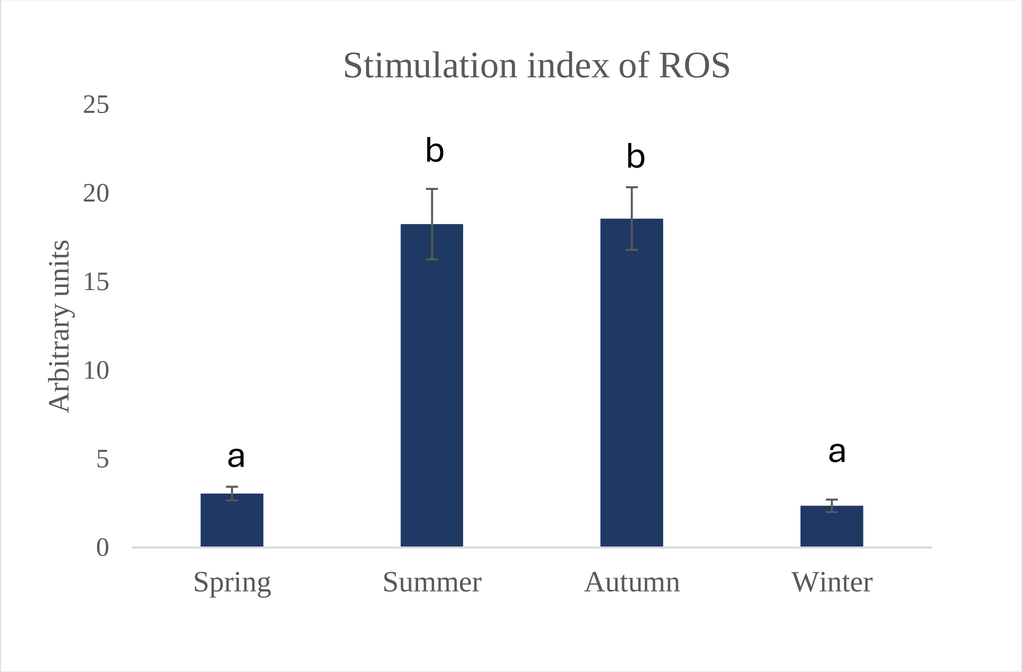


Figure 3.1:Stimulation index of Reactive Oxygen Species (ROS) production of mussel hemocytes after stimulation by zymosan (Mean ± SEM). Bars indicate the mean of the ROS production of 24 mussels at each sampling season (18 for summer). The same letter indicates no significant differences ($p > 0.05$)

3.1.2. NO Production

Regarding the NO production, the effect of zymosan in the NO response (*in vitro* stimulated hemocytes) was statistically significant regarding controls (non-stimulated hemocytes) for all the variables (season of the year, raft position, and ropes depth) (data not shown). This seems to indicate that the potential stress caused by the different variables of the experiment does not limit the ability of the mussels' immune system to respond to a direct stimulus.

However, significant differences were found among seasons ($p \leq 0.05$). As it was conducted for the production of ROS, the stimulation index was calculated to understand the NO response among the different seasons and detect the differences between the stimulated hemocytes and the controls. As shown in Figure 3.2, a statistically significant stimulation of the hemocytes was observed during summer (12.79 ± 0.75) concerning the other seasons, followed by a significantly decreased expression in spring and autumn in similar levels (4.2 ± 0.27 and 4.88 ± 0.32, respectively). Finally, winter showed a significantly lower production (1.5 ± 0.09).

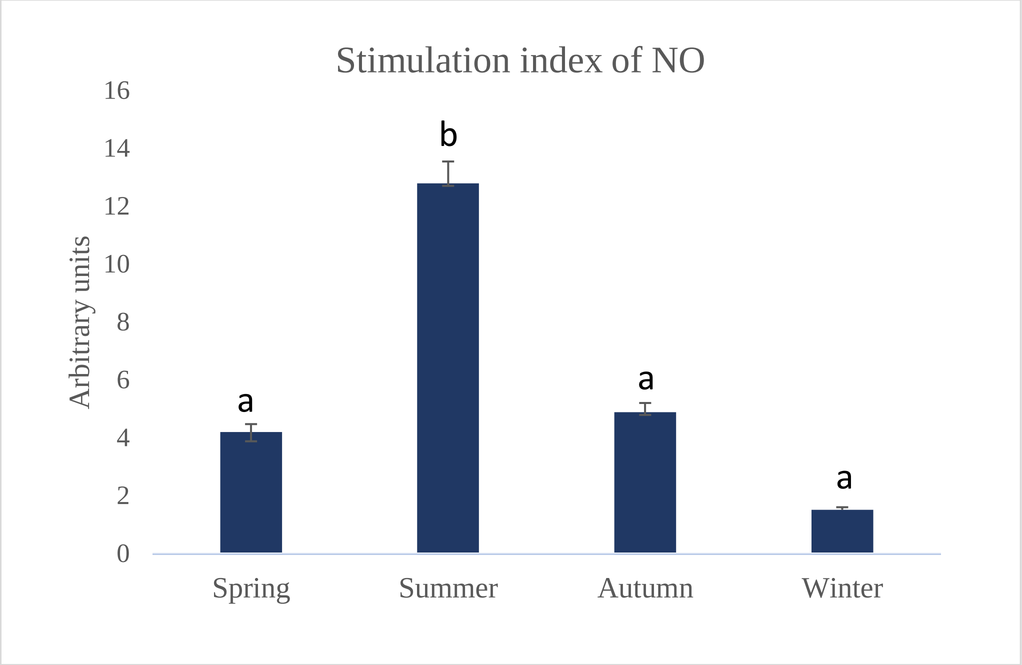


Figure 3.2: Stimulation index of Nitric Oxide (NO) production of mussel hemocytes after stimulation by zymosan (Mean ± SEM). Bars indicate the mean of NO production of 24 mussels at each sampling season (18 for summer). The same letter indicates no significant differences ($p > 0.05$)

A significant difference was also found in the comparison raft position × season ($p \leq 0.05$) (data not shown). However, it is likely that this results from the greater weight that the significance of the season *per se* has concerning the location of the raft. Thus, when looking at the combination of raft position and season of the year, differences between the variables are shown, but there is no specific pattern that can be concluded from these results.

3.1.3. Gene expression

The immune status of the mussels was also evaluated by the analysis of three specific immune-related genes for the variables season of the year, raft position, and ropes depth. GST, LYS, and HSP70, genes involved in oxidative stress, innate defense, and heat shock/protein folding respectively, were the genes selected for the analysis by qPCR.

Besides, to evaluate the byssus' production and therefore the mussel's ability to attach to the substrate, the expression of the gene Mgfp3 was assessed by qPCR differentiating among the variables of season of the year, raft position, and ropes depth.

3.1.3.1. Immunity evaluation (GST, LYS, and HSP70 gene expression)

Considering the GST expression, statistically significant differences were found between the seasons and also for the interaction season × position ($p \leq 0.05$) (Table 3.1). According to Figure 3.3, the highest expression was observed in spring and a decrease in the gene expression was shown with the passing of the seasons, leading to a winter expression close to zero, although the statistical differences concerning the highest point (spring) were not observed until autumn. The decrease in expression was also significantly different between autumn and winter. Regarding the interaction season x position, the significant difference is influenced mostly by the seasons ($p \leq 0.05$), however, there is a particular decline in the more exposed raft location (data not shown). As shown in Table 3.1, neither the raft position nor the rope depth had a significant difference regarding the GST expression.

Table 3.1: Univariant results for GST expression

Effect	SS	d.f	MS	F	p
Season	**5291.39**	**3**	**1763.80**	**27.6453**	**0.000000**
Position	97.58	1	97.58	1.5295	0.225482
Depth	121.34	1	121.34	1.9018	0.177744
Season × Position	**675.43**	**3**	**225.14**	**3.5288**	**0.026160**
Season × Depth	308.72	3	102.91	1.6129	0.206383
Position × Depth	84.05	1	84.05	1.3173	0.259857
Season × Position × Depth	97.96	3	32.65	0.5118	0.677118
Error	1977.83	31	63.80		

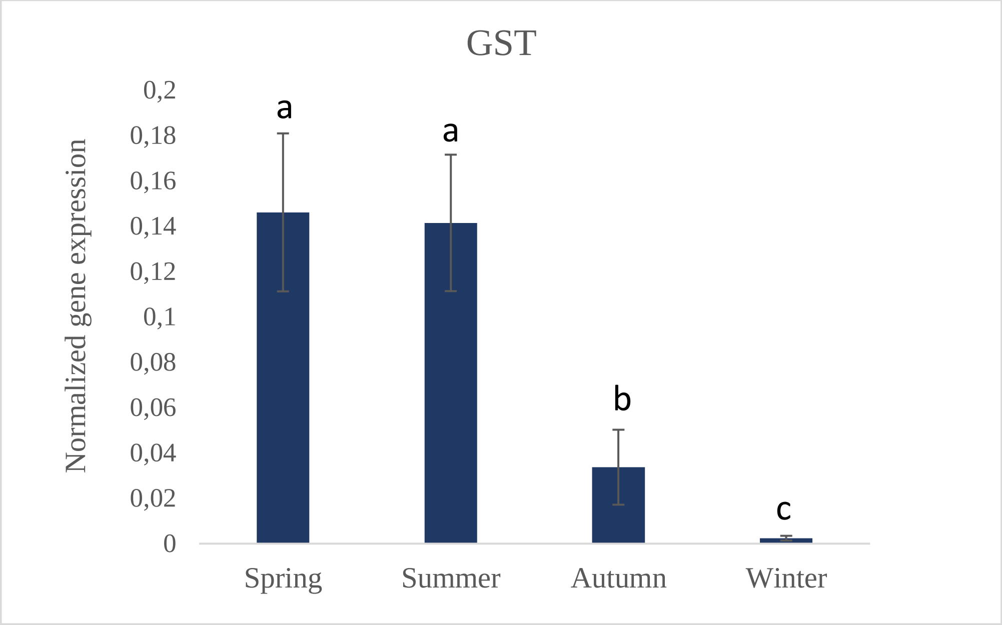


Figure 3.3: Expression of glutathione S transferase (GST) in mussels' gill (Mean ± SEM). The bars indicate the mean of 12 mussels at each sampling season. The y-axis represents the relative expression of GST for each individual normalized to the housekeeping gene (18S). The same letter indicates no significant differences ($p > 0.05$)

Regarding LYS expression, there was a statistically significant difference among the seasons ($p \leq 0.05$). As illustrated in Figure 3.4, the expression increased significantly in summer. A decrease followed with autumn, and the expression level decreased even more in winter.

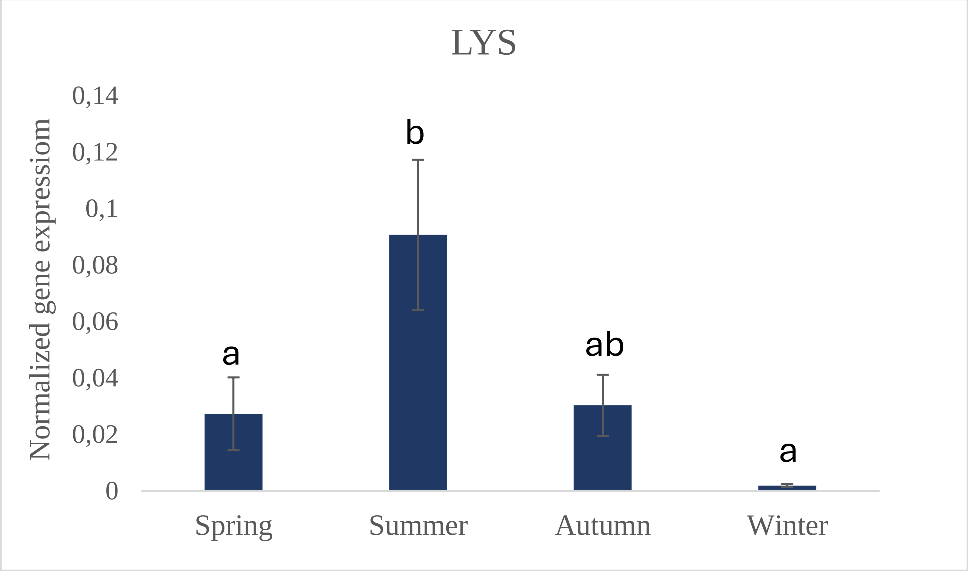


Figure 3.4: Expression of lysozyme (LYS) in mussels' gill (Mean ± SEM). The bars indicate the mean of the relative expression of LYS of 12 mussels at each sampling season. The y-axis represents the relative expression of LYS for each individual normalized to the housekeeping gene (18S). The same letter indicates no significant differences ($p > 0.05$)

A statistically significant difference in HSP70 expression was observed across the different seasons ($p \leq 0.05$) (Table 3.2). As it is shown in Figure 3.5, the enzyme's highest expression was found in spring. Following spring, there was a progressive decline of the HSP70 expression, with the lowest levels observed in winter. However, no differences were found in spring concerning summer. Significant differences were also found in the interaction between season × position ($p \leq 0.05$). This significant difference is mainly affected by the seasonality ($p \leq 0.05$), however, the more sheltered raft appeared to have higher gene expression (data not shown).

Table 3.2: Univariant results for HSP70 expression

Effect	SS	d.f	MS	F	p
Season	**5411.58**	**3**	**1803.86**	**30.8409**	**0.000000**
Position	84.05	1	84.05	1.4369	0.239719
Depth	137.50	1	137.50	2.3509	0.135357
Season × Position	**740.32**	**3**	**246.77**	**4.2191**	**0.012989**
Season × Depth	152.43	3	50.81	0.8687	0.467769
Position × Depth	176.61	1	176.61	3.0195	0.092193
Season × Position × Depth	160.39	3	53.46	0.9141	0.445493
Error	1813.17	31	58.49		

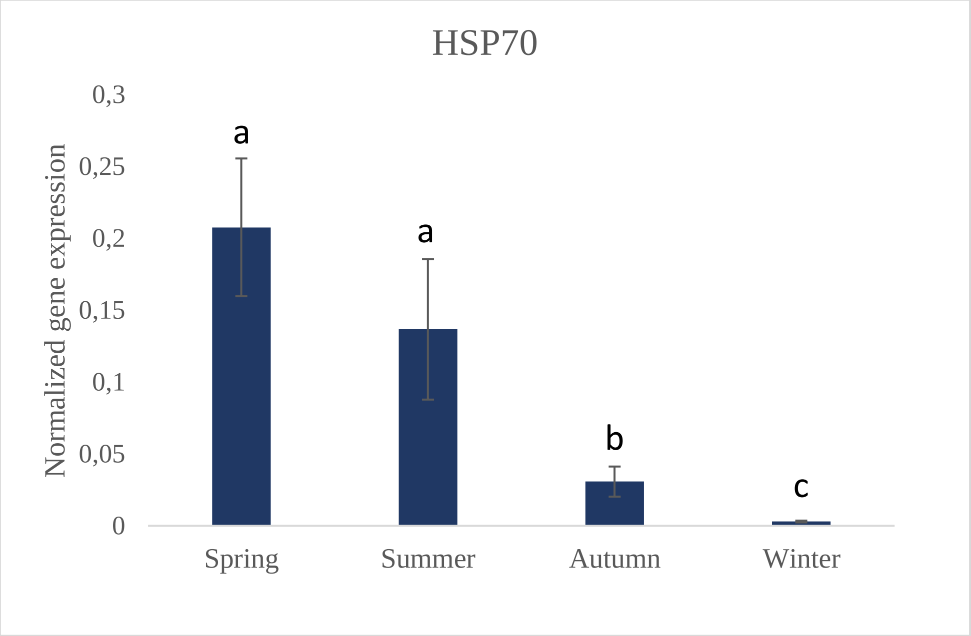


Figure 3.5: Expression of Heat Shock Protein 70 (HSP70) in mussels' gill (Mean ± SEM). The bars indicate the mean of the relative expression of HSP70 of 12 mussels at each sampling season. The y-axis represents the relative expression of HSP70 for each individual normalized to the housekeeping gene (18S). The same letter indicates no significant differences ($p > 0.05$)

3.2. Byssus production (Mgfp3 gene expression)

The byssus production and therefore the mussel's ability to attach to the substrate was evaluated through the expression of the foot protein 3 gene. Significant differences were only observed between seasons ($p \leq 0.05$). From Figure 3.6 it is shown that the maximum gene expression occurred in spring, followed by autumn and winter. The expression of the protein remained extremely low during the summer.

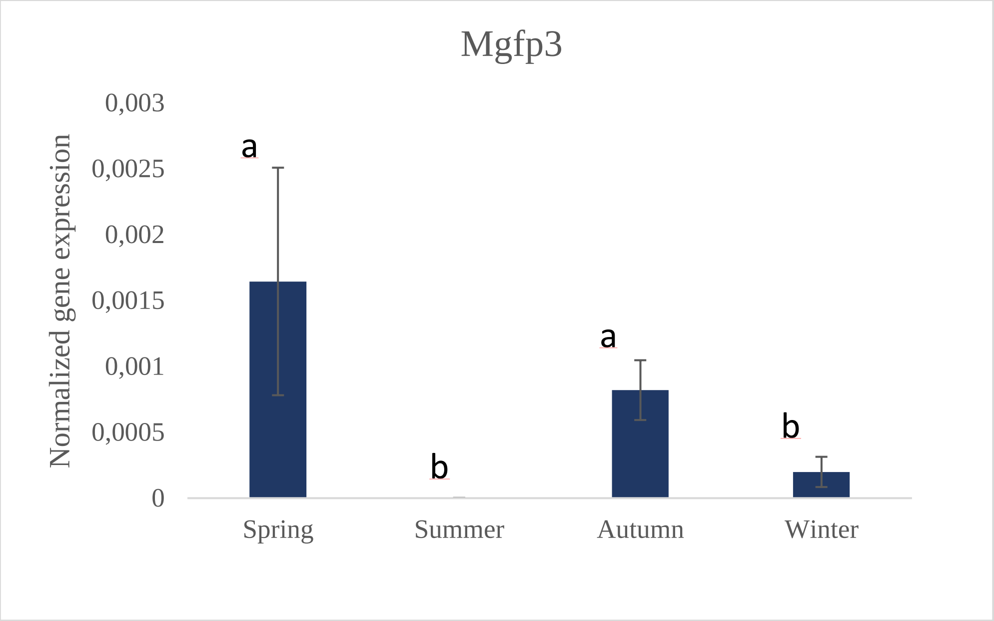


Figure 3.6: Expression of foot protein (Mgfp3) in mussels' foot (Mean ± SEM). The bars indicate the mean of the relative expression of Mgfp3 of 12 mussels at each sampling season . The y-axis represents the relative expression of Mgfp3 for each individual normalized to the housekeeping gene (18S). The same letter indicates no significant differences ($p > 0.05$)

4. Discussion

Mussel cultivation is an essential industry in Spain, especially in the Galicia (NW Spain) region, which has a crucial socio-economic role, in supporting the local economy and providing employment opportunities. However, mussels, due to their lifestyle, are frequently exposed to a variety of environmental stressors, such as pathogens and weather conditions (Bouallegui, 2019), which could affect both at a defensive level and conditionate their survival in the medium and at a physical level provoking the dislodgment of the animals from the ropes in the rafts.

It is, therefore, essential (i) to know how their immune system functions and responds to several environmental challenges and (ii) to evaluate the byssus' generation to attach to the substrate, whose strength, growth, and production are also affected by the environmental conditions.

The present study is an attempt to evaluate the immune condition and byssus production of mussels cultivated in Galicia, to establish the association between the wave impact, depth of the ropes, and seasonality with the cellular molluskan stress response and the byssus production.

Initially, the immune response was evaluated by the measurement of ROS production, which is considered an important internal defense mechanism, and it has been described in several mussel species (Wu et al., 2016; Rahman et al., 2019). In the present study, mussels' hemocytes showed a baseline production of ROS and demonstrated a consistent response to zymosan stimulation. Similar findings have been reported by Moss and Allam (2006), Brokordt et al. (2019), and Han et al., (2021), suggesting that the hemocytes still keep their ability to respond to this stimulus independently of their position in the bay, season, or depth into the raft rope. In our study, the levels of ROS production after stimulation varied significantly between seasons, suggesting that the immune response is influenced by seasonal changes. The highest ROS response was found in summer and autumn with similar levels, and the lowest in spring and winter. This variation could be caused by seawater temperatures, which cause higher metabolic demands, enhancing their immune responses, including ROS production. Similar findings were noted by Rahman et al. (2019), who observed higher ROS activity in *M. galloprovincialis* and *Crassostrea gigas* at elevated temperatures.

Pathogen exposure is another important factor noticed in the summer and can influence ROS levels by increasing them. Elevated temperatures enhance bacterial proliferation both in the water column and within the mussel tissues, leading to a stressful condition for the mussels. Azizan et al. (2024) also noted the positive correlation between increased temperatures and vulnerability to diseases. Additionally, there is a possibility that higher temperatures occurred in autumn than in spring, possibly explaining the lower ROS levels in spring and higher in autumn, extending the mussels’ exposure to summer heat stress. However, it is important to note that in our analysis we have not measured temperature as a variable, but the season.

And, as described above, other factors could be conditioning these variations in ROS production. The increase in ROS production in autumn could also be related to the reproductive cycle and spawning, as was observed by Wu et al. (2016), indicating a link between the physiological demands of the mussels during the transition to the different reproductive phases and the occurrence of a variety in the environmental conditions.

The immune system was also evaluated by the measurement of NO production, which can be naturally produced in cells and tissues from L-arginine by the activity of NOS, which can be found in marine, freshwater, and land mollusks (Malanga et al., 2004). In our study, similar to ROS production, all the mussels produced NO when they were *in vitro* stimulated with zymosan. However, this response was different in the different sampling points, with the season being the most significant variable. The highest NO response was detected in summer and the lowest was in winter. These results suggest that seasonal variations of factors such as temperature, pathogen load, and metabolic activity could have a significant influence on NO production, and the increase of these factors could lead to higher immune responses to help the mussels defend against these stressors. These results are consistent with those reported by Balbi et al. (2017), who described a significant positive correlation between NO production in gills and seasonal changes in *M. galloprovincialis*, especially during gametogenesis and spawning, aligning with the peak of NO levels that we detected during summer. Considering that the gills are irrigated by hemocytes, which are actively involved in the immune response, there can be an indicator that the seasonal changes in NO production reflect an immune response to the stressors occurring particularly during the summer. Additionally, the metabolic activity can be enhanced by the elevated temperatures during the summer, similar to the study of Malanga et al. (2007) where a summer increased metabolic activity caused higher NO production in the digestive glands of the gastropod *Nacella (P.) magellanica*. On the other hand, the reduction of the NO production in winter observed in our study could be explained by the lower metabolic demand and energy shift, and pathogen exposure of the season, which was also suggested by Romero et al

(2014). Lower temperatures are likely to reduce immune defenses such as NO production, which was also noted by Rahman and Rahman (2021), who observed a decrease in the production of nitrotyrosine protein (NTP) expression, a marker of NOS activity, during colder seasons in *C. virginica*.

The immune status of the mussels was also evaluated by the expression of three genes: GST, LYS, and HSP70. Regarding GST, an oxidative stress indicator, the highest expression was found in spring, followed by summer. This could suggest that oxidative stress can be more distinct in spring, probably due to high temperatures or the redirection of the energy to post-spawning recovery of the mussels, when the needs for cellular recovery are higher, after the physiological stress that the organisms have been exposed to during spawning. Additionally, in the Galician Rias, algae blooms have been routinely reported in spring (but not recorded by us in the context of this study) (Broullón et al., 2020; Rodríguez et al., 2024), which can be linked to increased food availability for mussels, resulting in higher oxidative metabolism. Bocchetti and Regoli (2006), Balbi et al., (2017), Nahrgang et al. (2013), and Schmit et al. (2013) have all reported elevated GST activity in mussels during spring, particularly in the presence of elevated phytoplankton production or after spawning events. Similarly, Pinheiro and Oliveira (2016) observed increased GST activity in crayfish in spring, supporting the idea of the post-spawning phase and elevated temperatures contributing to increased oxidative stress and higher GST levels in this season. On the contrary, colder temperatures result in decreasing metabolic demands and oxidative stress, keeping the GST activity at lower levels.

Regarding the second immune gene, in our study, when the lysozyme (LYS) expression was measured, seasonality showed significant differences, with the highest expression in summer, lower in spring and autumn, and finally, the lowest gene expression was found in winter. The elevated lysozyme levels during summer can be explained by a potential increase in the pathogen load of the season since increased temperatures promote bacterial proliferation, therefore requiring stronger immune defenses. Li et al. (2009), Luna-González (2008), and Khan et al. (2020) support these results, since their studies showed increased lysozyme expression in the summer, highlighting a positive relationship between temperature and lysozyme activity. However, extensive exposure to environmental stress causes a decline in lysozyme activity (Khan et al., 2020), which could explain the lower expression levels observed in autumn in our results. The recovery of mussels after spawning could also be related to elevated lysozyme expression levels in summer, which can also be supported by Santarem et al. (1994) whose observations link our data of lysozyme expression with reproductive stress. On the contrary, the lower expression that occurred in autumn and winter could indicate a reduced immune activity, probably due to lower pathogen load in colder seasons and lower metabolic activity, as was also mentioned by Auguste et al. (2024). This seasonal variation highlights the seasonal-dependent immune responses in mussels, with increased defense responses occurring in periods of elevated stress, such as higher temperature, pathogen accumulation, and reproductive cycle.

Finally, after the analysis of HSP70, a thermal stress protein, a significant difference between the seasons was found, with a similar pattern as GST. The highest activity of HSP70 occurred in spring and summer, where the temperatures are usually higher, and the lowest one was in autumn and winter. These findings suggest increased HSP70 expression in mussels during warmer months to help them cope with thermal stress, since this protein has a crucial role in protecting the cells from heat damage and assisting in their recovery. Elevated temperatures could also lead to increased HSP70 production to help mussels deal with the metabolic challenges correlated with heat stress. Additionally, the availability of food increases throughout the summer could play a role in the increase of HSP70 activity, providing more energy to the mussels, enhancing their growth and the protein's support on cellular recovery (Minier et al. 2000). Ioannou et al. (2009) and Valenzuela-Castillo et al. (2015) have also noted higher HSP70 expression during the summer, highlighting the relationship between the temperature, the food availability, and the energy demands to cope with growth and environmental stress. The reduced HSP70 expression observed in autumn and winter could be explained by the decreased demands for thermal protection since there are usually lower temperatures and therefore less challenges to cause stress to the organisms.

Concerning the second part of the study, the byssus production was measured through the expression of Mgfp3, which showed a significant seasonal variation, with the highest Mgfp3 expression occurring in spring and the lowest in summer.

During higher temperatures, and particularly in summer, the organism's energy can shift, as we observed in the immune response, to managing heat stress and other environmental stresses instead of byssal production. Furthermore, in summer the need to produce more byssus to hold more tightly to the substrate might be minimized since it is usually a calmer time concerning hydrodynamic forces. Lachance et al. (2008) reported also significantly decreased byssus production during the summer, due to mussels' prioritization for survival to stress exposure. On the other hand, during spring, environmental conditions seem to be more favorable for the byssus production, since the temperature and physiological stress are lower, allowing the mussels to invest more energy into the byssus strengthening. Moeser and Carrington (2006) come in agreement with this, since they observed stronger and more extensible byssal threads in spring, probably due to the energy's allocation towards the attachment. Additionally, the elevated expression of Mgfp3 in spring could reflect the stronger attachment strength as a "preparation" to handle the seasonal changes (Carrington et al., 2015). The reduced Mgfp3 expression observed in our study in summer, apart from the thermal stress, can be explained by the shift in energy demands into supporting the reproductive processes, with mussels building large stores of gametes before releasing them in one massive energy expenditure in late spring (Carrington 2002).

To sum up, in the context of this study, we investigated the impact of environmental stresses on the immunological status and byssus production of *M. galloprovincialis* cultivated in Galicia, such as season, rope depth, and raft location. Our findings suggest the distinct influence of seasonality and environmental factors, such as temperature, algae bloom, food availability, and pathogens, on the immune defenses and byssus production of *M. galloprovincialis*. Temperature is crucial, but not the only one factor that could explain our observed results, since increased bacterial proliferation, harmful algal bloom, and pathogen pressure on the mussels tend to appear in spring and summer, the seasons that showed an overall peak of the mussels' immune activity. Spawning and food availability can also contribute to the high metabolic and immune responses. Although in our study the raft location did not significantly affect the mussels' functionality and defense, it is still likely that there was a potential influence by the wave activity, especially across different seasons. It is also worth noting that the lack of significant effects on the immune response of the mussels from the other parameters that were tested could be due to the mussels' ability to adapt to varying conditions, and the changes that occurred in these factors could be within the mussels' range of tolerance.

In conclusion, this study highlights how seasonality significantly influences the immune responses and byssus production of *M. galloprovincialis*, showcasing the complexity of mussel physiology in adapting to environmental changes. While raft location and rope depth did not show significant effects, seasonal variations were clear. ROS and NO levels were highest in summer and autumn, likely reflecting oxidative stress caused by increased metabolic activity and pathogen pressures. Immune gene expression peaked in spring and summer, suggesting a seasonal defense response influenced by temperature, pathogen exposure, or reproductive cycles. Byssus production, as indicated by Mgfp3 expression, was highest in spring when attachment conditions were most favorable but declined in summer as mussels appeared to prioritize managing stress. These findings emphasize the importance of adjusting mussel farming practices to account for seasonal and climate-driven changes, ensuring optimal mussel health and attachment strength while minimizing stress during critical periods. Further research is needed to fully understand how environmental factors interact and influence the sustainability of mussel aquaculture.

5. References

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