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TESI DI LAUREA

Antarctic organisms in relation to rising seawater temperature, exploring immunological responses of *Trematomus bernacchii*

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ABSTRACT:

During the past decades, the climate has undergone multiple transformations, with rising temperatures becoming one of the most important issues affecting marine ecosystems. Even the Southern Ocean and the Antarctic continent are not safe from these changes, and the increase in water temperatures is a major concern for the scientific community. This research aimed to analyse the physiological responses of the Antarctic fish species *Trematomus bernacchii* to rising seawater temperatures. Specifically, we focused on how its immune system can adjust its responses when facing higher-than-normal temperatures. We conducted experiments to investigate the expression levels of four different immune-related genes, *igm, igt, trl2*, and *trl9*, in the liver and spleen, the primary organs generating immune responses. Our study involved the analysis of adult specimens of *T. bernacchii*, sampled from Terra Nova Bay (Ross Sea), experimentally exposed to a temperature range from 0°C to +3°C. The results were compared with a control group maintained at a constant temperature of 0°C. This research provides valuable insights into the adaptive potential of the immunological systems of Antarctic fish in response to global warming. These findings can help address the Antarctic ecosystem's conservation challenges and unique species.

1. INTRODUCTION

Antarctica is Earth's southernmost continent, containing the geographic South Pole. It is the fifth largest continent at about 14 million square kilometres, almost wholly covered by ice. It becomes strikingly different among the continents because of its extreme environment, isolation, and significant role in global climate regulation (Convey, 2009).

GEOGRAPHICAL POSITION AND PHYSICAL FEATURES

Antarctica is almost entirely positioned in the south of the Antarctic Circle. It is within a region bounded by the Southern Ocean. That right at the centre of the South Pole extends from about 60°S latitude to the pole at 90°S. Also, the elevation in this area is significantly high, with an elevation average of about 2,500 meters above sea level, which ranks it the highest continent on Earth (Morlighem, 2020). The East Antarctic Ice Sheet, which covers most of the continent, is on a bedrock in some places below sea level due to the immense weight of the ice over it (Tewari, 2021).

Antarctica contains around 60% of the world's fresh water in its ice sheets, covering 98% of the continent. Again, it has been divided into two major parts: the East Antarctic Ice Sheet and the West Antarctic Ice Sheet, where the former is stable and thick, and the latter is less stable and is more likely to melt (Figure 1) (Rignot, 2011). Recent studies and satellite data have shown that the ice loss in Antarctica has increased, with dramatic melting in the West Antarctic Ice Sheet and Antarctic Peninsula (Shepherd, 2018). Although there is a lot of interannual variability in the extent of sea ice around the continent, recently, a negative trend in sea ice extent can be seen, which has resulted in the lowest sea ice coverage ever recorded from the 2022-2023 data (Böning, 2008).

Antarctica is known to have the most extreme climate on Earth. During the winter period from March to September, the Antarctic land remains in absolute darkness, whereas in summer, from October to February, there is absolute daylight. Temperatures in Antarctica usually range up to 60°C (76°F) in winter in the interior and might rise around – 2°C (28°F) near the coasts during summers. However, over the past few decades, there has been a clear warming tendency, more precisely in the Antarctic Peninsula, where temperatures have increased by nearly 3°C (5.4°F) since the 1950s, turning this into one of the most rapidly warmed places on Earth (Convey, 2009).

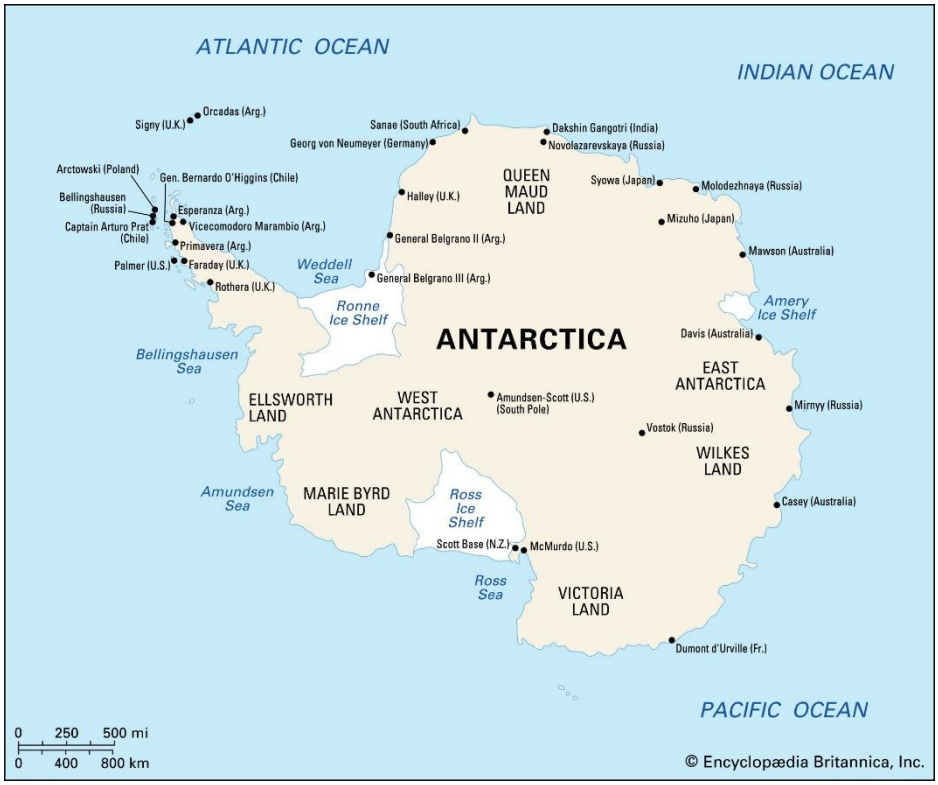


Figure 1: The Antarctic continent *(Watt, 2024)*

The nature of Antarctica has been identified as one of these very important ecosystems in understanding general biological processes. Its unique characteristics are the hosts of several organism types that have developed definite adaptations to life under extreme living conditions. The Antarctic continent's influence on global climate, ocean systems, and biodiversity has significantly made it a gold mine as a resource for studying biological mechanisms and adaptation strategies (Wynn-Williams, 1996).

MARINE LIFE IN ANTARCTICA

The Antarctic marine environment represents one of Earth's most unique and extreme ecosystems. Cold water, powerful currents, and large areas covered with sea ice characterize the Southern Ocean surrounding the Antarctic continent. Also included under these conditions are very productive habitats for diverse marine life, such as microorganisms and large marine mammals. It has cold temperatures, mostly below -2°C, with a strong circumpolar current around the continent. Those processes, powered by the most energetic current on Earth (the Antarctic Circumpolar Current), participate in mixing water masses and are involved in the distribution of nutrients over the Southern Ocean (Harris, 2003). These processes result in a constant churning of water, bringing deep waters rich in nutrients to the surface and fuelling the growth of phytoplankton, which forms the base of the food web.

Seasonal sea ice also plays an important role in the Southern Ocean's ecosystem. During winter, it advances by millions of square kilometres and melts in summer to release nutrient-rich waters. This seasonal cycle of freezing and thawing profoundly impacts the availability of habitats and resources for marine organisms.

The harsh conditions of this Antarctic marine environment have led to the evolution of species specially adapted to survive in extreme cold and variable conditions. This contrasts sharply with the temperate regions, where the ecosystems are normally much more complex and varied, allowing the Southern Ocean ecosystem to be surprisingly simple but very effective (Griffiths, 2010).

GLOBAL CHANGES AND ANTARCTICA

The alteration of the climate, known as climate change, is a quantifiable and systematic shift in the state of the atmosphere due to natural events or anthropogenic activities that modify the atmospheric composition. One of the most critical problems is the rise in temperature caused by these changes. The collective impact of anthropogenic activities like the emission of greenhouse gases (GHGs), altering consistently the formation of the atmosphere and natural elements, has led to shifts in temperature trends (Hansen, 2006).

INCREASING SEAWATER TEMPERATURE

Both human-caused processes and natural occurrences can produce an increase in seawater temperature. Natural sources like changes in solar variations, in which more solar energy reaches the earth's surface, and shifts in the natural ocean currents like El Niño-Southern Oscillation (ENSO) affect global warming (Lean, 2008). Regarding human actions, manufacturing and industrial activities increase the production of CO_2 by burning and utilizing fossil fuels (oil, coal, and gases), along with the production of greenhouse gases (GHGs). These gases trap heat, warming the atmosphere and increasing the temperature of seawater and oceans (Kaufmann, 2011).

The exact geological timeframe in which the Southern Ocean reached its current temperature range is still one of the main topics of discussion and disagreement. Antarctica has been in freezing water for millions of years (Hubold, 1994). The impacts of climate change are notably amplified in the Antarctica regions, where ice is melting and exposed to absorbing heat. Furthermore, warming ocean currents transfer heat and increase the speed of melting ice.

Likewise, Figure 2 illustrates a general decrease in sea ice in Antarctica regions during summer over the past years, a consequence of climate change.

Increasing temperatures would impact the biotic and abiotic parts of the environment. Antarctic living organisms that adapted and survived in frigid conditions with summer times that do not even go above zero are terribly at risk. For example, the most sensitive ones, stenothermal species, could tolerate only a narrow temperature threshold. This temperature rise is stress-inducing for such fish. It incites direct and indirect effects on the body and most biological functions, including heart rate, metabolism, reproduction, behaviour, and immune responses

(Stern, 2014; Turner, 2014). Investigating the immune systems of these fish can help us understand the future challenges Antarctic fish may face and provide valuable insights into other fish's responses to climate change.

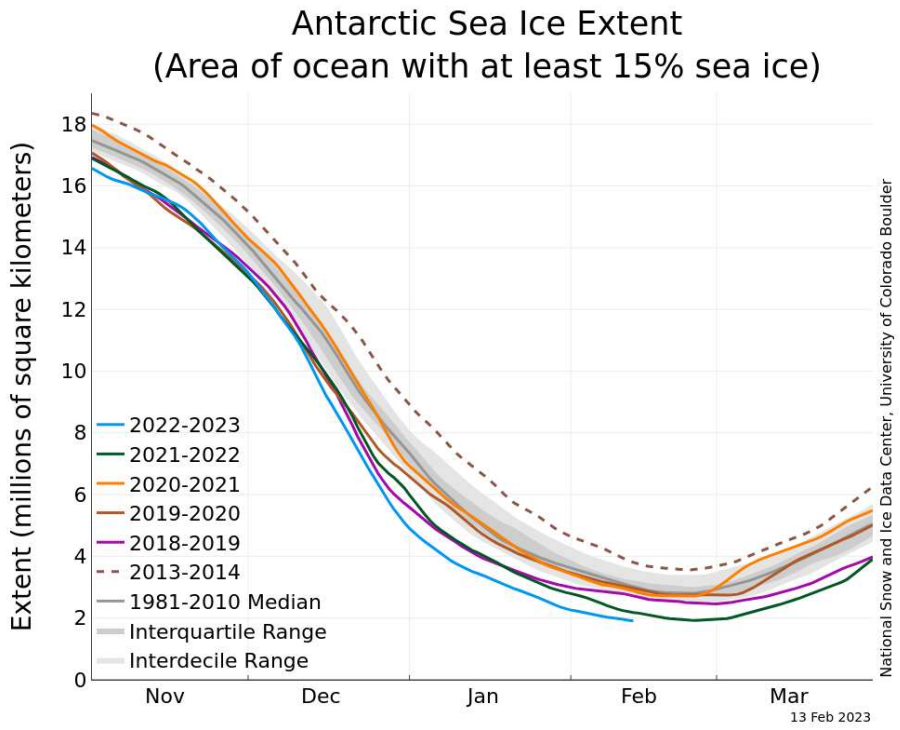


Figure 2: Antarctic Sea ice extent has dropped significantly in the past years; the 2022-2023 season spans down to very low levels. That can indicate changes that climate change is causing and is reflected in reduced sea ice during the summertime.

ANTARCTIC FISH

Physiological adaptation to extreme conditions of the Southern Ocean is one of the most impressive developments in Antarctic fish. The biochemical and physiological mechanisms these fish, mainly suborder Notothenioid, have developed define their ability to survive under extremely low temperatures. One of the most famous adaptations is antifreeze glycoproteins produced by fish that prevent their body fluids from freezing at very low temperatures (Giordano, 2012). Another range of unique blood characteristics in fish from Antarctica includes low haemoglobin levels or, as in the case of icefish, its complete absence. These evolutionary adaptations optimize oxygen transport under cold water conditions when oxygen is more easily dissolved, and metabolism is low (Verde, 2011).

On the immunological front, Antarctic fish develop immune adaptations to function at low temperatures. Cellular specialization in fish has reportedly occurred in Antarctic species like *Trematomus bernacchii* (Romano, 2002). The immune

systems of such fish should also be restructured to reduce the higher oxygen levels, as cold waters might increase oxidative stress. These adjustments at different levels are important in maintaining immune function in an environment where even minor disruptions in homeostasis could mean death. These organisms have also modified their enzyme performance to work best at low temperatures, which raises sensitivity to temperature increases despite having fundamental biochemical processes (Ciardiello, 1999).

IMMUNE SYSTEM IN FISH

Growing interest is bred concerning the temperature-increasing immunological responses of Antarctic fish, particularly gene expression. Recently, the interest in this field of study has grown because the Antarctic fish have adapted to extremely cold environments and are potentially vulnerable to rises in temperature through climate change. As already noted, some studies have been performed on related topics, suggesting how these organisms respond to temperature stress at the molecular level.

The latest research into the Antarctic plunder fish (*Harpagifer antarcticus*) concerns transcriptional responses to acute temperature stress. Although the loss of classical heat shock response was noted in this fish, exposure to high temperatures nevertheless results in a typically vertebrate acute inflammatory response, coupled with extensive changes in energy metabolism and oxidative stress pathways. (Thorne, 2010).

Although the subject of temperature changes in Antarctic fish's immune and gene expression responses is relatively new, it has attracted significant scientific interest. From this growing science of polar ecosystems, information regarding species' adaptation to their changing environment and, more generally, climate change repercussions for these ecosystems is now being gained.

The immune system in fish is complex and includes generally two separate systems: 1) innate immune system and 2) adaptive immune system. Figure 3 demonstrates the common features of these two compartments (Fischer, 2013). These systems help fish avoid infections and cope with numerous pathogens in their aquatic environments (Bruce, 2017). Environmental factors like temperature, salinity, pH, dissolved oxygen, and pollution could act as stressors to the immune system and cause interruptions or weaken it (Magnadóttir, 2006).

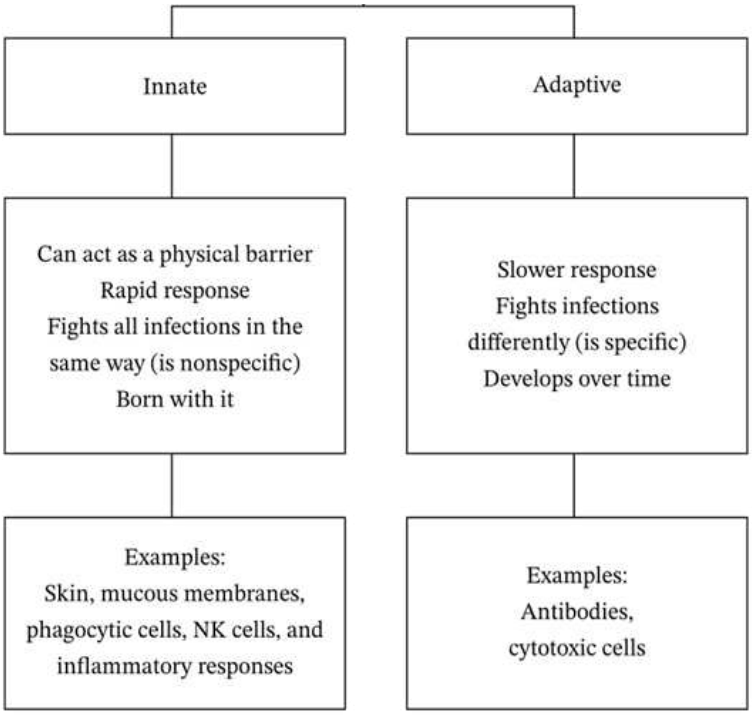


Figure 3: Dividing the immune system into innate and adaptive immunities.

INNATE IMMUNE SYSTEM

As in other organisms, the innate immune system is the first line of defence against any pathogen or infection in fish. It is a physical barrier to pathogens and other invasions; it contains humoral and cellular parts. The main role of this system is to prevent objects and pathogens from entering the body (Abbas, 2018). Due to its ancient origins and high conservation across species, this system may play a fundamental role in ensuring species survival. Physical barriers of this system include the skin and mucous membranes that prevent any non-native pathogens from entering, and cellular components consist of the phagocytic cells, such as macrophages and neutrophils, that engulf digestive pathogens (Magnadóttir, 2006).

Environmental factors also can act as triggers to activate immune responses in fish. Fluctuations in temperature in aquatic environments may affect the normal physiological performance of the fish, leading to an increase in the production of stress-related hormones like cortisol, which may trigger several elements involved in the innate immune system (Bly, 1997).

Usually, the innate system activates rapidly without differentiating between various pathogens, and the defence mechanism is almost the same for all external pathogens (Magnadóttir, 2006).

ADAPTIVE IMMUNE SYSTEM

The adaptive immune system of fish is more complicated than the innate system but less developed than in mammals. It can provide long-term immunity against pathogens (Cooper, 2006; Iwasaki, 2015). It has different parts, such as lymphocytes, including B and T cells. In B cells, antibody production occurs, and immunoglobulin M (IgM) makes a bond with antigens and defeated pathogens (Uribe, 2011).

Conversely, T cells provide cellular immunity by first recognizing and destroying infected cells. Somehow, it assists B cells in the production of antibodies (Uribe, 2011). Some organs, like the spleen and thymus, are crucial for maturing and storing lymphocytes. The spleen is a secondary lymphoid organ that generates immune responses (Lewis, 2019). The adaptive responses take more time to develop and are specific to pathogens (Zan-Bar, 1993). As previously mentioned, environmental factors could directly affect adaptive immune reactions in fish.

The table below categorizes the humoral and cellular elements of both innate and adaptive immunities that are involved in generating immunological responses (Chaplin, 2010):

Table 1: key components of the innate and adaptive immune system.

Immune System Component	***Innate Immune System***	***Adaptive Immune System***
Humoral	Antimicrobial peptides Lectins Lysozyme Complement Cytokines Interferons	Antibodies IgM IgD IgT/IgZ IgA IgE IgG
Cellular	Macrophages Neutrophils Eosinophils Basophils	B-lymphocytes T-lymphocytes Plasma cells Memory B cells
Receptors and Recognition	Pattern recognition receptors (PRRs) Toll-like receptors (TLRs) B cell receptors (BCRs)	Antigen receptors T cell receptors (TCRs)

GENES INVOLVED IN IMMUNOLOGY

Multiple genes modulating immunological responses are key factors, especially when these fish are challenged in environmental situations (Canosa, 2023). Based on their mechanisms of action and the stimulator, some could be active, and others deactivate. Among them, *ig* genes play a significant role in enhancing the adaptive immune response of fish through the production of antibodies. These genes are very important for immune protection under changing conditions. Besides, toll-like receptors, important elements of the innate immune system, are powerfully engaged in pathogen recognition and priming of immune responses (Chaussabel, 2014).

Excluding *ig*s and *tlr*s genes, additional immune-related genes involved in the TLR signalling pathways have been recognized to have a master role in shaping immune responses. These genes have been shown to control the expression of pro-inflammatory cytokines and other immune mediators crucial for immune homeostasis and mounting appropriate responses against external insults (Sundaram, 2012).

IMMUNOGLOBULINS (Igs)

Immunoglobulins, Igs, or antibodies, are glycoproteins B cells produce. They have a main role in identifying and neutralizing various groups of pathogens of bacteria, viruses, and toxins. The structure of immunoglobulins consists of two heavy and two light polypeptide chains, which bind together by disulfide bonds and create a Y-shaped (Figure 4) (Goldsby, 2003). By this Y arms, these molecules function as antigen-binding sites and connect to their specific antigens and other immune molecules. Based on the heavy chain structure and their biological features, Igs are classified into IgG, IgA, IgM, IgE, and IgD groups, which can be found in different body parts such as extracellular fluids and mucosal surfaces (Janeway, 2001; Kindt, 2007). Knowing how they work is a helpful way to diagnose diseases and produce vaccines.

A specific antigen targeted by immunoglobulins with the help of their variable regions is created by a somatic process unique to each immunoglobulin. After binding, they try to neutralize the pathogens directly and opsonize them so they will collapse by phagocytes or activate other immune system compartments (Keyt, 2020).

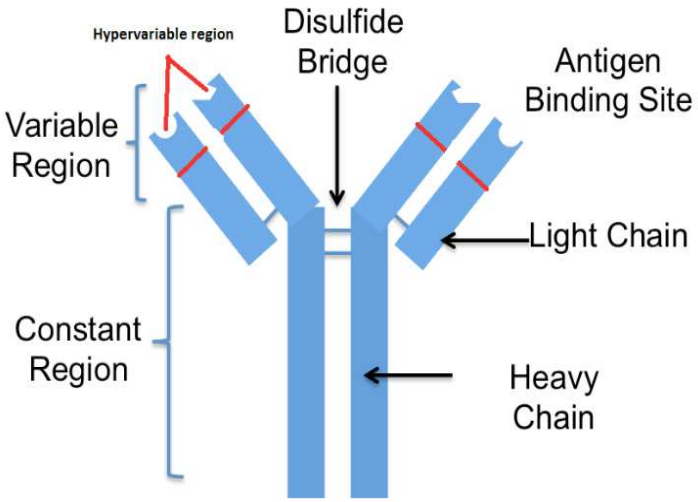


Figure 4: Basic Immunoglobulins (antibodies) structure *(Oruba, 2018)*.

IMMUNOGLOBULIN M (IgM)

Immunoglobulin M (IgM) is one of the most important immunoglobulins active in immune responses in fish at systemic and mucosal levels, and it has unique structural and functional roles as an antibody (Rombout, 2014). Different types could act as receptors on B cells or circulate in the body fluid as an immune component. It has light (κ or λ chain) and heavy (μ chain) chains. The light chain binds with the heavy chain and has regions to bond with antigens. Also, the heavy chain has various regions for binding with antigens and constantly helps in immune reactions (Xia, 2023). These chains are held together by disulfide bonds and create various shapes with a joining (J) chain that helps IgM to transport across epithelial cells into mucosal secretions (Abós, 2013). Whereas mammals' IgM are pentamers, IgM forms a tetramer in bony fish (for instance, in *T. bernacchii* and some other species). Each of those four parts contains two light chains and two heavy chains (Coscia, 2014). This structure is indicated by molecular mass analysis, which shows the relative molecular mass of the polymeric form to be about 830 kDa, which is consistent with a tetrameric arrangement (Figure 5) (Pucci, 2003). It involves systemic immunity and its distribution in different body parts, mainly in lymphoid organs like the spleen, kidney, or blood, liver, gills, and intestine of fish (Abós, 2013).

During infection, the IgM has an up-regulation, and its expression increases to detect and neutralise the pathogens. So, after an immune challenge like an infection, its level rises significantly to combat the new condition (Yang, 2020).

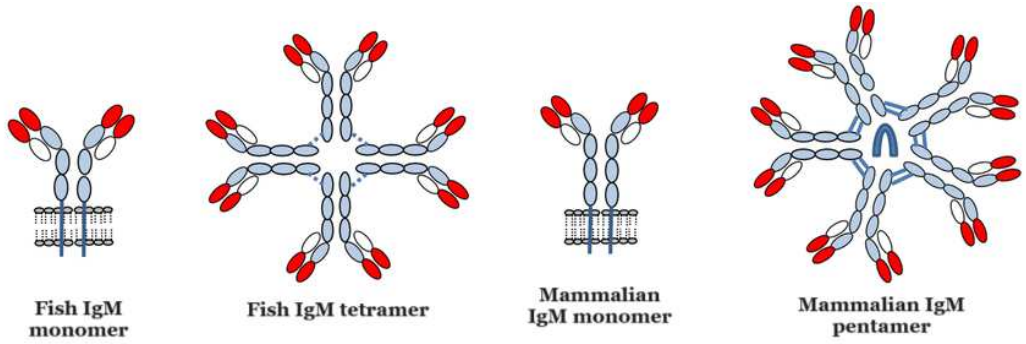


Figure 5: Differences between IgM structure in mammals and fish. *(Bilal, 2021)*

IMMUNOGLOBULIN T (IgT)

In fish, Immunoglobulin T (IgT) plays a crucial role in adaptive immunity and is analogous to the *IgA* in mammalians mucosal immunity (Zhang, 2011). IgT was first discovered in teleost fish, similar to IgD and IgM (Mirete-Bachiller, 2021). Its structure includes heavy chains with constant regions, and light chains make bonds by disulfide (Zhang, 2017), which could be expressed exclusively in mucosal tissues like gills, skin, and the intestine. It is also in lymphoid organs like the spleen and kidney (Piazzon, 2016). Unlike IgM, IgT generally exists as a monomer with a molecular weight of approximately 180 kDa (Du, 2016).

Its primary role could be considered in the mucosal immunity system on mucosal surfaces and helping to maintain mucosal homeostasis, but it also participates in systemic immunity (Du, 2016). In the presence of pathogens or infections such as parasites, bacteria, and viruses, these immunoglobulins experience an up-regulation (Xu, 2016), and their level elevates, which shows their role in activating immunity responses when facing a challenge (Zhang, 2010).

TOLL-LIKE RECEPTORS (TRLs)

Toll-like receptors are a class of proteins that belong to the pattern recognition receptors (PRRs) family, and they are divided into various groups (TRL2, TRL4, TRL9, etc.) according to their genetic nature and the specific pathogens that they can identify (Flo, 2005). Like other proteins, they comprise long amino acid chains that fold in a specific shape based on their functions (Lee, 2012). They might be found in different parts of the cells. Firstly, in the cell membrane, the surface of immune cells recognizes pathogens before entering the cells and helps in the detection of microbial membrane components like lipids, lipoproteins, and proteins (Zückert, 2014); secondly, within the cytoplasm in endosomes or lysosomes. This distribution of TRLs allows them to detect a wide range of pathogens and non-native molecules (Kawasaki, 2014).

Compared to mammals, fish have a broader range of TRLs that allow them to face different pathogens and adapt to new environmental situations (Carlson, 2023).

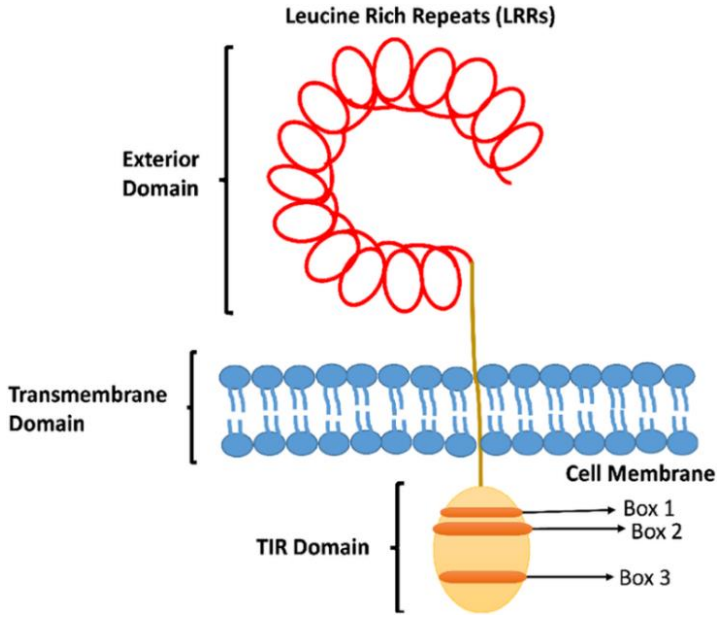


Figure 6: Toll-like receptor's typical structure *(Bhardwaj, 2024)*.

TOLL-LIKE RECEPTOR 2 (TRL2)

Toll-like receptor 2 (TRL2) participates in the early detection of pathogens and has a quick response (innate immune system). TRL2, with the help of other TRLs, detects microbial or bacterial (like peptidoglycan) components and activates signalling routes (Arancibia, 2007). This rapid action is beneficial to eliminate pathogens and prevents any further infections in the host's body (Aderem, 2000) (Supajatura, 2002). It is primarily found on the surface of immune cells, particularly macrophages, dendritic cells, and certain types of epithelial cells. Its structure includes three compartments: the extracellular, transmembrane, and intracellular TIR domains. The extracellular domain has leucine-rich repeats (LRRs) to detect unmethylated CpG motifs in bacteria and viruses. The transmembrane domain is the alpha-helix that helps bind TRL2 to the cell membrane and maintains its proper position, and the intracellular TIR activates signals in the cells (Figure 6) (Janssens, 2003).

It is critical in recognising pathogen-associated molecular patterns (PAMPs) and activates immune reactions. These PAMPs could be divided into different groups such as peptidoglycans, lipoproteins (together with TRL1 and TRL6) (Takeda, 2002), and lipoarabinomannan in the cell walls of bacteria and fungi (Takeuchi, 2002). After detecting PAMPs, TRL2 makes heterodimers with other TRLs that generate a cascade of signalling impacts to produce cytokines and other types of immune responses (Janssens, 2003).

TRL2 identifies protein molecules, and the connection between adaptor proteins and TRL2 occurs (Nilsen, 2015). This connection induces pathways at the intracellular level, which can lead to the activation or deactivation of a gene to produce immune responses (Rebl, 2010).

TOLL-LIKE RECEPTOR 9 (TRL9)

Toll-like receptor 9 (TRL9) gets involved in the innate immune system as a first line of defence and adaptive immunity at cellular levels (Iwasaki, 2004). The activation of TRL9 prompts modulating and synthesizing mitochondrial ATP, helping immune and non-immune (like neurons) cells protect themselves from damage and stresses (Shintani, 2014). They are mainly located within intracellular compartments, such as endosomes, in immune cells like B cells, dendritic cells, and macrophages. Like TRL2, in TRL9, we have three main parts in its structure: the extracellular domain, the transmembrane domain, and the intracellular Toll/Interleukin-1 receptor (TIR) domain (Figure 6) (Shirey, 2020). TRL9 can identify the foreign DNA of the pathogens and participate in the early detection of infections, which limits the time for disturbing them (Dongye, 2022).

When TRL9 bonds with CpG DNA (DNA sequences in bacteria and viruses), a cascade signalling effect is generated inside the cell (Bauer, 2001). The TIR domain interacts with adaptor proteins, stimulating downstream signal pathways to activate cells and other routes (Radhakrishnan, 2009). Then, the production of pro-inflammatory cytokines elevates inflammation, recruits immune cells at the infection sites, and initiates other elements of the immune system (Gao, 2012).

FISH SPLEEN

The spleen acts in both the immune and hematologic systems as a filter of the blood and as a reservoir for blood cells. It forms part of the clearance mechanism whereby old and damaged red blood cells are removed from the circulation, enabled by the red pulp of the spleen made up of macrophages specialized in phagocytosis (Lewis, 2019). The spleen in the fish also does immune responses with the help of white pulp. Like higher vertebrates, the fish spleen has a significant role in responding to immunological stimuli in aquatic environments (Figure 7) (Sayed, 2022). It regulates immune responses and changes in gene expression during challenging periods and has a vital role in the health status of fish. (Kohyama, 2008). It generally includes different types of immune cells, such as macrophages, lymphocytes, and dendritic cells (Wan, 2015). If the pathogens attack, the spleen produces more immune responses by producing antibodies, activating and proliferating lymphocytes, and secreting cytokines, chemokines, and antimicrobial peptides (Xu, 2016).

The spleen's role in immune defence makes this organ the centre and essential for fish immunology in both innate and adaptive immunity levels to destroy infections rapidly with quick and effective responses (Rise, 2008; Wang, 2021). For instance, immunostimulants affect the spleen's toll-like receptors (*trl*s) genes forward, up-regulation, and activation of immune mechanisms. Also, in the innate immunity level, pro-inflammatory and anti-inflammatory cytokines are expressed in the spleen under stress pressures (like what happens in the head kidney in the same condition) (Fierro-Castro, 2013). In various experiments, the role of immunoglobulins has been found, and the transcriptions of *igm, igd*, and other *ig*s

with low molecular weight on the surface of the cells show the fact of the importance of this organ and the role of B-cell receptor activities (Stenvik, 2001).

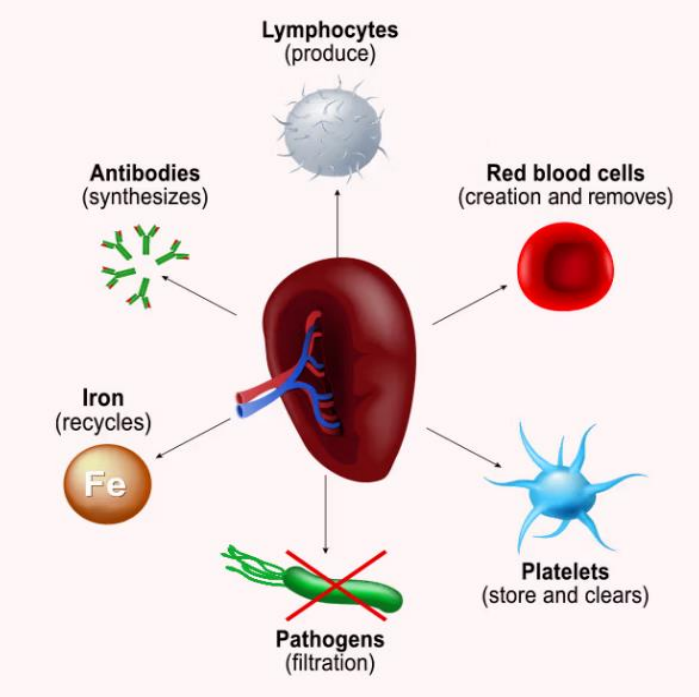


Figure 7: Functions of the spleen.

FISH LIVER

The liver is a multipurpose organ with different functions vital for the metabolism of nutrients, protein synthesis, detoxification, and immunological responses (Robinson, 2016). Its fish structure is similar to mammals; hepatocytes gather around the blood vessels, and its adaptation to aquatic areas makes it work more efficiently in defence responses (Pucci, 2003). The liver in fish acts as an immunological organ, housing intrahepatic immune cells (IHICs) that are involved in immune responses. Studies have shown that the liver in fish is actively engaged in producing acute-phase proteins, which are essential for the inflammatory response (Chaves-Pozo, 2005). Against pathogens and during infections, the fish liver regulates defence responses by producing molecules and critical proteins that contribute to the immunity system (Qi, 2021). Its immunologic function is not as specialized as the spleen.

Nevertheless, it greatly contributes to the immune defence system, emphasizing the response to environmental factors. Particularly, the liver identifies blood-borne pathogens (microorganisms like viruses and bacteria carried out by blood) that cause and cause various types of diseases and systemic infections (Causey, 2018).

Changes in gene expressions and proteins, such as toll-like receptors (*trl*s), phagocytosis, and blood coagulation cascades, are some key mechanisms that the liver puts to use in its immunological role, and all of these mechanisms work as a united complex to neutralize the pathogen's effects and terminate their activities (Qi, 2021).

2. AIM OF THE STUDY

This research aims to investigate the impact of rising seawater temperatures on the immunological responses of *T. bernacchii*, a key species in the Antarctic ecosystem. As global climate change continues to drive significant temperature increases in polar regions, understanding the physiological and immunological adaptations of native species like *T. bernacchii* becomes critical. This study explores how these fish respond at the molecular level to thermal stress, particularly how their innate and adaptive immune systems adjust to such environmental challenges.

This research will specifically examine the expression levels of crucial immune-related genes, including Immunoglobulin M (*igm*), Immunoglobulin T (*igt*), Toll-like receptor 2 (*tlr2*), and Toll-like receptor 9 (*tlr9*), in the liver and spleen of *T. bernacchii*. These genes are integral to the fish's ability to detect and respond to pathogens, and their expression levels under varying temperature conditions will provide insight into the resilience of the species' immune system. Focusing on these key immune response components, the present study seeks to elucidate how *T. bernacchii* might cope with increased temperatures occurring faster in polar regions than the global average.

The research will explore how temperature variations from the stable 0°C typical of the Southern Ocean to an increased temperature of +3°C affect the immune responses in *T. bernacchii*. These temperature regimes have been chosen to simulate both current conditions and the projected warming scenarios, thereby allowing a comprehensive analysis of the potential future impacts on the species. By comparing the gene expression profiles in fish exposed to these different temperature conditions, the study will identify potential shifts in immune function that may indicate either an adaptive response or a vulnerability to rising temperatures.

This study also aims to uncover any organ-specific responses by comparing immune gene expression in the liver and spleen, two organs central to the immune function of fish. Differences in how these organs respond to temperature-induced stress will help to build a more detailed picture of the physiological changes occurring within *T. bernacchii*. Such findings could reveal critical insights into the organ-level specialization in immunology and provide a deeper understanding of the species' overall ability to maintain health under changing environmental conditions.

The results of this research are expected to contribute significantly to the broader understanding of how Antarctic marine organisms, particularly fish species, are affected by climate change. By focusing on the immune responses, the study not only adds to the field of marine immunology but also provides essential data that could inform conservation strategies aimed at protecting Antarctic marine biodiversity, helping the aquaculture principles and supporting conservation efforts through informed management practices, and assessing the potential for disease prevention and vaccine development. Understanding the limits and capacities of these species to adapt to environmental changes is crucial for predicting the future health of Antarctic ecosystems.

Furthermore, this research has the potential to inform future studies on the impacts of climate change on polar marine life. The methodologies and findings could serve as a basis for comparative studies across different species and regions, thus broadening the scope of research on environmental stressors and biological responses in polar ecosystems. The insights gained from this study will be valuable not only for marine biologists and ecologists but also for policymakers and conservationists working to mitigate the effects of global warming on vulnerable polar species.

In summary, this research seeks to provide a detailed understanding of how *T. bernacchii*, an Antarctic fish species, responds immunologically to rising temperatures. The study will shed light on the species' adaptive capabilities and the broader implications for Antarctic marine ecosystems in a warming world by analysing gene expression in key immune organs under simulated climate change conditions.

3. MATERIAL AND METHODS

TARGETED SPECIES

T. bernacchii is an Antarctic fish species belonging to the Nototheniidae family, mostly found in shallow water (Figure 8). Its depth range can be from 0 to 700 m. It is a stenothermic species adapted to extremely low and usually stable temperatures ranging from +0.3 °C to −1.86 °C and the annual mean temperature of the Southern Ocean (Montgomery, 2000). The adult body length is between 18 to 28 cm in males and around 35 cm in females, with a brownish colour and darker spots (Bottari, 2022).

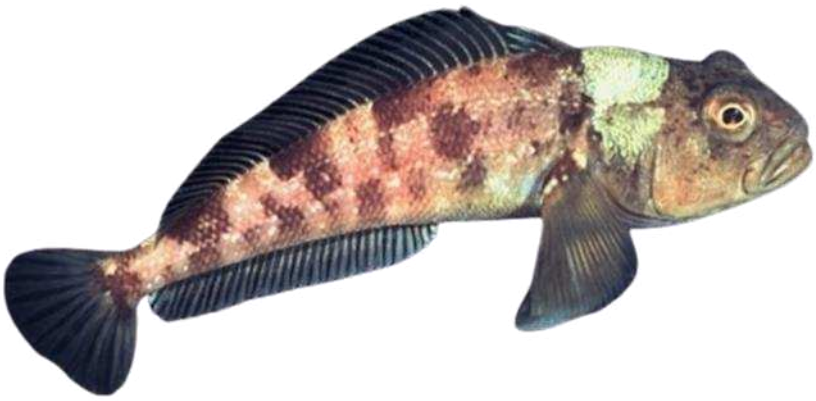


Figure 8: Photo by I R MacDonald, ©Antarctica New Zealand Pictorial Collection.

It is an important component in Antarctic marine biology, having played a critical role in the ecosystem and undergoing physiological adaptation to one of the most inhospitable environments on Earth. The species has evolved unique biochemical and molecular mechanisms for survival in these freezing temperatures by developing antifreeze glycoproteins that guard against the formation of ice crystals in blood and tissues (Shier, 1975). This species has great ecological importance, as it is both a key predator and prey for most of the Antarctic marine ecosystem, carrying out essential functions in terms of energy transfer. Based on such features, *T. bernacchii* has been used as a model organism for investigating the effects of climate change on polar species (Huth, 2013).

This research used *T. bernacchii* as a model to investigate how its immune system is affected by rising temperatures, an extrinsic modelling stressor of future climate scenarios. Research into the response of *T. bernacchii* to changes in temperature is critical for understanding the wider ecological implications of global warming on the marine ecosystems of Antarctica (Antonucci, 2019).

The sample collection and animal research conducted in this study comply with the Italian Ministry of Education, University and Research regulations concerning activities and environmental protection in Antarctica and with the Protocol on

Environmental Protection to the Antarctic Treaty, Annex II, Art. 3. All the activities on animals performed during the Italian Antarctic Expedition were under the control of a PNRA Ethics Referent, which acts on behalf of the Italian Ministry of Foreign Affairs. In particular, the required data for the project identification code PNRA16_00099 are as follows. Name of the ethics committee or institutional review board: Italian Ministry of Foreign Affairs. Name of PNRA Ethics Referent: Dr. Carla Ubaldi, ENEA Antarctica, Technical Unit (UTA). All experiments were performed under the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines; EU Directive 2010/63/EU; and Italian DL 2014/26 for animal experiments.

SAMPLING SITE AND METHODS

T. bernacchii (Boulenger, 1902) adult specimens (n= 35, length = 23,76 ± 2,99, weight = 211,92 ± 80,40) were collected at the end of October 2023, using hook and line in the Ross Sea at Baia Terra Nova (74°42′S, 164°7′E) at depths ranging from approximately 60 to 100 meters. The fish (n=35) were immediately transferred to the aquarium facility of the Italian research station Mario Zucchelli. They could recover from the sampling stress in tanks (100 L tank) with circulating seawater at 0°± 0.2 °C for 5 days. At the end of the de-stressing period, five specimens were sacrificed with a dose of (tricainemethanesulfonate, MS-222; 0.2 g l−1) and dissected (Figure 9).

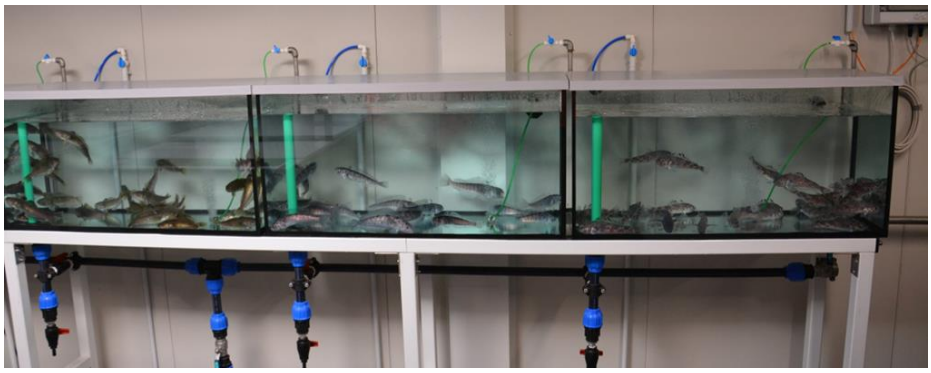


Figure 9: Control and experimental tanks of *T. bernacchii*.

The first group of five fish will represent the experiment's time zero (T0). Regarding the experimental group, the tanks were equipped with thermostats to increase the temperature inside the tank progressively. Starting from 0°C, the temperature was raised to +1°C slowly over one day with an increasing rate of (+0,1 so as not to cause heat shock, and then the specimens were kept at that temperature for 4 days. At the end of this period, 5 specimens that had just experienced the +1°C and 5 specimens from the control tank (0°C) were sampled and sacrificed. These specimens were identified as T+1 and control (CT) for 5 days. Subsequently, the temperature was increased for one day until reaching +2°C, and the specimens were kept at this temperature again for 4 days. After this period, 5 specimens were

sampled from the experimental tank and 5 from the control tank. These samples are referred to as T+2 and CT 10 days.

Finally, the last temperature step increased from +2°C to +3°C (Figure 10). At the end of the experiment, the last 5 specimens that had experienced all three different temperatures and the last 5 specimens from the control tank were sacrificed. During this period, the fish were not fed, and all the possible external sources of stress (light pollution and noise) were minimized to simulate their natural environment.

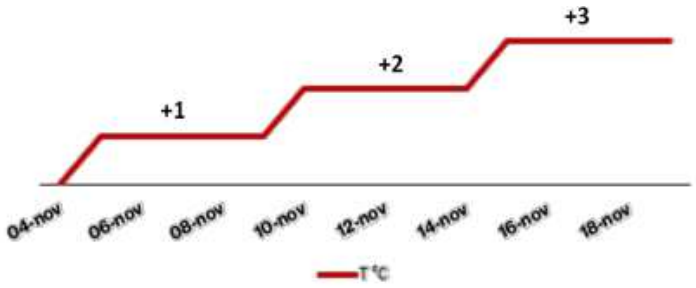


Figure 10: Timetable of the experiment.

The fish were killed at the end of the exposed period, and samples of different tissues were dissected. Tissues were immediately labelled, frozen on liquid nitrogen, stored at −80 °C, and shipped back to Italy for future analysis.

RNA EXTRACTION

Total RNA was extracted from the tissues to analyse further gene expression (*igt*, *igm*, *trl2*, *trl9*). We used the TRIzol extracting method for all 35 samples (n=5 for all the groups, T0, CT5, CT10, CT15, T+1, T+2, and T+3) for both livers and spleens. TRIzol™ Reagent is a complete, ready-to-use reagent for the isolation of high-quality total RNA or the simultaneous isolation of RNA, DNA, and protein from various biological samples. This monophasic phenol and guanidine isothiocyanate solution is designed to isolate separate fractions of RNA, DNA, and proteins from cell and tissue samples of human, animal, plant, yeast, or bacterial origin within one hour. It can permit the isolation of RNA, DNA, and protein from the same sample and offers superior lysis capability, even with difficult sample types, as well as optimized formulations and protocols for tissues, cells, serum, viruses, and bacteria (Figure 11).

About 100 mg of tissue was weighed and transferred to a sterile and clean Eppendorf tube for the extraction procedure.

1) Homogenization Step:

The first and most important step is homogenizing the sample using pastels with 1 ml of TRIzol per 100 mg of tissue. This step is important since a good homogenization improves the quality of the RNA extracted.

2) Phase Separation:

The samples were then incubated at room temperature for 5 minutes before starting the next steps. Then, 200 µL of chloroform was added per 1 mL of TRIzol. Chloroform promotes phase separation, so RNA is isolated from DNA and proteins in a biological sample. This releases the RNA from the cells and other cellular components. It promotes phase separation, so RNA is isolated from DNA and proteins in a biological sample. This releases the RNA from the cells and other cellular components.

As a non-polar solvent, chloroform can easily dissolve lipids, proteins, and other cellular components but cannot dissolve RNA. When added to the sample, chloroform forms a distinguishable layer between the aqueous phase (containing RNA) and the organic phase (containing lipids and proteins). This allows for the separation of RNA from other cellular components.

3) RNA Precipitation:

The aqueous phase (supernatant) was transferred to another clean Eppendorf tube without touching the layer below (white layer). Based on each 1 ml of TRIzol used, 500 µl of cold Isopropyl alcohol was added to the samples, which were then vortexed for a few seconds. The samples were incubated for 10 minutes at room temperature and centrifuged for 30 minutes at 13000 ×*g* at 4°C.

4) RNA Washing:

The supernatant was discharged carefully without touching the white pellet in the next step. The remaining pellet should be washed twice with 75% Ethanol, adding at least 1ml of Ethanol per 1ml of TRIzol. After every wash, the samples were put at 7500 ×*g* in a centrifuge at 4°C for 5 minutes.

5) RNA Resuspension:

The last step of the first day is discharging the supernatant and letting the pellet air-dry under the chemical hood for around 10 minutes or occasionally more. Then, an appropriate amount of RNAse-free water was added to each sample for RNA resuspension. The samples were kept for 2 hours at room temperature to improve the dissolution.

RNA PURIFICATION

This step, particularly suggested when working with Antarctic fish, provides RNA purification from glycoproteins or carbohydrates that can be present in the samples using lithium chloride (LiCl).

Samples were incubated in a warm bath at 40°C for 5 minutes. Then, they were centrifuged for 15 minutes by 13000 ×*g* in the cold room. After that, the liquid

phase had to be transferred to another sterile Eppendorf tube, and Lithium chloride (LiCl) was added (1/3 of the amount of water used in the resuspension stage). Lithium chloride is used to extract and purify RNA to specifically precipitate RNA, while DNA and proteins remain in a dissolved phase. This method aids in achieving more refined and pure RNA samples by efficiently isolating RNA from other components within the cell. After treatment with LiCl, the samples were kept overnight on ice in the fridge.

On the second extraction day, the procedure starts by centrifuging the samples for 20 minutes with 13000 at 4°C. The supernatant was discharged, and the pellet was washed twice with 200 µl Ethanol 75%, with a centrifuge for 20 minutes with 13000 at 4°C. After the washing steps, the aqueous phase was discharged, and the pellet was air-dried for 10 minutes or more under a chemical hood. Based on the pellet's size and dimension, add around 40 µl RNAase-free water and leave it to incubate at room temperature for 2 hours. In the final step, incubate samples in the warm bath at 55°C for 10 minutes and vortex a few seconds.

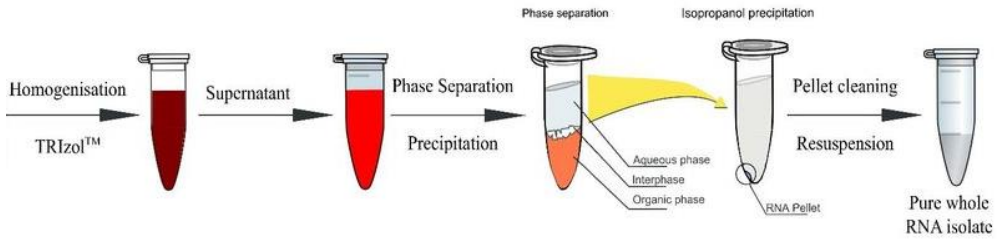


Figure 11: RNA Extraction steps.

RNA QUANTIFICATION

The extracted RNA was quantified using the Nanodrop, a Spectrophotometer to measure the concentration and purity of your DNA, RNA, or protein samples. Using only 1 µL, you can obtain your results in less than 15 seconds, from sample pipetting to wiping the pedestal clean. Every reading is done at 230, 260, and 280 nm to evaluate the A260/280 and A260/230 ratios. Aa A260/280 ratio of ~2.0 is generally accepted as "pure" for RNA. The expected A260/230 values are commonly in the range of 1.8-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants that absorb at 230 nm.

Another way to ensure the quality of extracted RNA is by running an aliquot of RNA (1000 ng/ µL) in a denaturing agarose gel electrophoresis. Intact total RNA ran on a denaturing gel, and when visualized on a UV transilluminator, it showed clear 28S and 18S rRNA bands. Technically, the 28S bands should be approximately twice as intense as the 18S rRNA bands.

PRIMER DESIGN

suitable primers were designed to analyse the selected genes (*igm*, *igt*, *trl2*, *trl9*) of *T. bernacchii*. In the first step, we had to choose the sequence for the targeted organs

(spleen and liver). The sequences of genes *T. bernacchii* are available on the GenBank server. The Blast NCBI program was used to identify these sequences.

We designed primers using the Primer3 program, Oligo Analyzer, and Beacon Designer website.

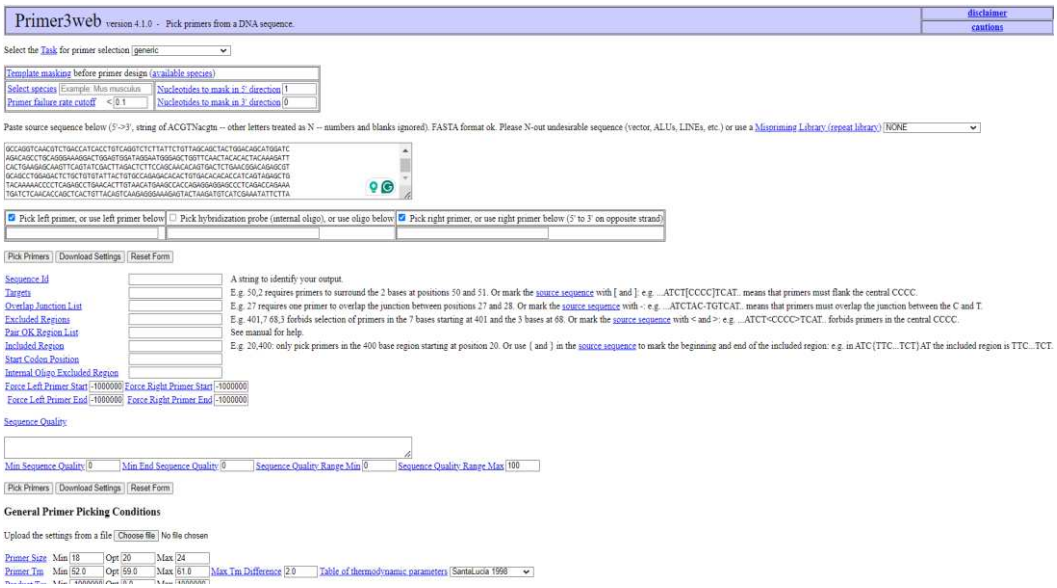


Figure 12: Primer3 program screenshot, designing primer pairs.

The sequences obtained through NCBI were later uploaded to the Primer3 program, and then some special features should be set (Figure 12). The most important ones include:

- Primer size from 18-25 and optimal 20
- Melting temperature 62-67C and optimal 65C
- Difference between Tm of the two primers less than 2C
- Product length between 100-150 and 150-200 (insert both)
- Number of returns = 50
- Maximum 3' stability = 9.0
- CG% content 50% in optimal condition
- Without any dimers or loop structures

Some primers were given as output by giving these conditions to the program. The primer pairs were further analysed using the IDT OligoAnalizer program by performing the harping analysis ($\Delta G < 3$); no bind at 3'-end is preferable (Figure 13). Of the 13 designed primers, only the primers that respect the set parameters were tested with *T. bernacchii*, as shown in Table 2. The same pathways had been done to design a primer for *gapdh* as a housekeeping gene during the PCR reactions. Housekeeping genes operated in real-time PCR as internal references to normalize data, verifying that alterations in gene activity are a consequence of experimental circumstances rather than fluctuations in sample quantity or quality.

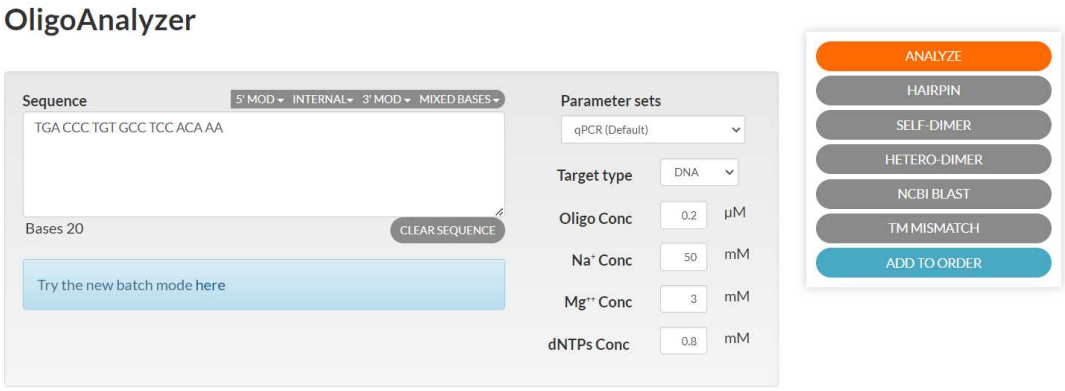


Figure 13: OligoAnalizer program screenshot.

Table 2: Primers used for qRT-PCR together with percentage of CG and melting temperature.

Primer Name	Sequence	CG %	Melting temperature
IgT-Forward-Tb	TCAACAACAACCAGGCAAGG	50%	61 °C
IgT-Reverse-Tb	GCAGAACAGCGCACATTACT	50%	61 °C
IgM -Forward-Tb	CTGACCTACGGATGGAGCAA	55%	61 °C
IgM-Reverse-Tb	CGTTCCCGCTTGATGTGTC	57.9%	61 °C
TRL2-Forward-Tb	TGACCCTGTGCCTCCACAAA	55%	66 °C
TRL2-Reverse-Tb	CACCACTCGGACTGGACGAA	60%	65 °C
TRL9-Forward-Tb	TCCCTGCCCACACAATCTCC	60%	65 °C
TRL9-Reverse-Tb	TTGGAAAAGCCCCACTGGAA	50%	64 °C

cDNA SYNTHESIS

The purified total RNA extracted from the spleen and liver of *T. bernacchii* samples is used to reverse transcribe to cDNA to use for quantifying the mRNA expression of genes involved in immunological responses (*igm, igt, trl2, trl9*), using the real-time PCR technique (qRT-PCR) (Polymerase Chain Reaction). In this synthesis, we used the Biotechrabbit cDNA Synthesis kit, which was highly efficient in synthesizing long cDNAs (≥19 kb). The kit involves RevertUP™ II Reverse Transcriptase by offering high fidelity, efficiency in synthesizing full-length cDNA, thermal stability, and the ability to work with a broad range of RNA templates. The RNase Inhibitor is also used to produce cDNA to prevent RNA from deterioration

caused by RNAses), thus preserving RNA's integrity throughout the reverse transcription procedure.

Oligo (dT) consists of a short sequence of thymine bases that are used in the process of cDNA synthesis to target and bind to the polyadenine (poly(A)) tails present in mRNA. By reverse transcription, mRNA is preferentially converted into cDNA.

The cDNA synthesis reaction was performed based on the volume as described in the table below:

Table 3: Reagents for cDNA synthesis reaction, together with enzymes.

COMPONENT	VOLUME (µl)
dNTP Mix (10 mM each)	2
RNase Inhibitor	0.5
Oligo (dT)12–18 (10 µM)	0.5
5× cDNA Synthesis Buffer	4
RevertUP™ II Reverse Transcriptase	1
RNA Template	0.1–1 µg total RNA
PCR Grade Water	Variable
Total volume	**20**

Based on these volumes (Table 3) and the number of samples, calculate the amount of each one of the materials and mix all of them, except RNA templates, in an RNase-free reaction tube with sufficient volume (1.5 ml) to collect and mix all the drops using a quick centrifuge. In the next step, 19 ml of the mix is transferred to a smaller Eppendorf tube, and at the last step, 1 ml of diluted RNA template is added to each one. Before putting the samples in the thermocycler to start the reaction, it is better to do another quick centrifuge. Each tube should be incubated into the thermocycler at 50-55°C for 60 minutes and at 99°C for 5 minutes so that enzyme inactivation can occur.

The cDNA was examined by Agarose gel electrophoresis (2%) and loading 5 µl of each sample + 1 µl of dye into the gel wells. The result of the electrophoresis run for all the genes was a single clear band, so the purification was likely starting from the PCR amplification products.

cDNA AMPLIFICATION

The cDNA obtained from the previous step and reverse transcriptase were amplified by PCR to verify the proper function of the designed primers.

Table 4: Reagents for the PCR reaction.

Components	Volume (µl)
10X PCR Buffer (grisp®)	2.5
$MgCl_2$ 25mM (grisp®)	1.5
dNTP mix (2.5 mM each)	2.0
Taq DNA Polymerase (5U/ µl)	0.5
cDNA Template	0.5
Forward primer 10 µM	1.5
Reverse primer 10 µM	1.5
RNAse-free water	Up to 25
Total volume	25

The reagents used for the PCR reaction are listed in Table 4. General cycling conditions include:

1) Initial denaturation: for 5 minutes at 95°C (complete denaturation of template DNA including removal of all secondary DNA structures like hairpin)
2) Annealing: 35-40 cycles for 20 seconds at 95°C, 30 seconds at Ta, and 30 seconds at 72°C for the extension. For each primer pairs analysis, three different annealing temperatures (T_a) were tried simultaneously. In this case, 52°C, 57°C, and 61°C were set, however, the best results were obtained at 61°C. Optimizing the annealing temperature is crucial, as a too low temperature might result in non-specific amplification. At the same time, a too high temperature might result in no amplification. The melting temperature (T_m) is the temperature at which 50% of the primer and its complementary sequence of the target DNA are present as duplex DNA.
3) Extension: 5 minutes at 72°C to ensure all amplicons are fully extended and include 3'-A-overhang.

The PCR results were then analysed after a run in a 2% agarose gel electrophoresis (the percentage of the gel was the best suggested for the DNA ladder 50bp used). Only the amplicons for which a positive result was observed underwent qRT-PCR.

GENE EXPRESSION ANALYSIS ON qRT-PCR

Quantitative real-time PCR is the most powerful technique used in molecular biology for gene expression analysis. It includes amplifying target DNA sequences using specific primers and a fluorescent dye or probe emitting fluorescence proportional to the amount of DNA amplified. This will provide real-time monitoring of the PCR process and lead to an exact quantification of the initial amount of target gene mRNA (Schmittgen, 2008). qRT-PCR is essential in gene expression evaluation, as it produces quantitative data that provides the dimension for comparing expressions in different samples or conditions. Unlike traditional PCR, which is qualitative, qRT-PCR quantifies small differences in gene expression that are very important in comprehending biological processes and the mechanisms behind certain diseases. Because of its sensitivity, it can detect even low-abundance transcripts and is very useful in studying genes involved in rare cellular processes or low-expressed genes (Valasek, 2005).

Each cDNA was tested using qRT-PCR analysis to evaluate the targeted gene expression rates in different individuals and groups. The reagents used for the qRT-PCR reaction are listed in Table 5. Comparing and normalising the tested specimen and the housekeeping gene is essential for each analysis. In this study, the *igm, igt, trl2*, and *trl9* genes from liver and spleen tissues have been compared and normalized with the *gapdh* housekeeping gene.

Table 5: Reagents for the real-time qPCR reaction.

Reagents	Volume for samples (µl)
2x qPCRBIO SyGreen Mix	5.0
Forward primer (10µM)	1.0
Reverse primer (10µM)	1.0
cDNA	1-2
RNAse-free water	Up to 10
Total volume	10

STATISTICAL ANALYSIS

A methodical statistical strategy was used to systematically evaluate the gene expression data resulting from qRT-PCR. Initially, an independent-sample t-test was applied to compare gene expression between two experimental groups, while a one-way ANOVA (Analysis of Variance) was used for multiple group comparisons. One-way ANOVA is a statistical technique for determining if there are any statistically significant differences between the means of three or more independent groups, in this case, our different experimental conditions/treatments (Kim, 2017).

The qRT-PCR measurements were taken as Ct (cycle threshold) values, which denote a round number where the fluorescence observed in a reaction crosses a fixed intensity signal level and hence is equivalent to the mRNA of target genes present in each sample. These Ct values were first normalized against a housekeeping gene, *gapdh* (glyceraldehyde-3-phosphate dehydrogenase), which demonstrated consistent expression across the samples to correct for similar RNA input or efficiency during reverse transcription. The normalization was performed using the ΔCt method, where the ΔCt value is calculated by subtracting the Ct value of the housekeeping gene from that of the target gene:

$$\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$$

The ΔΔCt method was subsequently used to compare the target gene's relative expression level in different groups of experiments. The data was compared to the two-cycle threshold values: one was of the control group, and the other was of the test group. The ΔΔCt value is derived from the difference of ΔCt of the control group and ΔCt of the experimental group:

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{control}$$

A set of genes that display specific patterns of time-varying mRNA levels, known as circadian genes, represent the relative expression levels, and the 2^(-ΔΔCt) method is utilized to calculate the fold-change in gene expression concerning the control, which is the method used to quantify it. This sorting method allows users to group the data by various treatments and repeats.

The ANOVA test showed that a p-value corresponding to the significance of the observed differences between each of the four groups was obtained. The p-value, a very important part of testing your hypothesis, shows the chance of these differences occurring out of the total observation number. Generally, the value of 0.05 is set for a p-value threshold; this value means that the null hypothesis (which states that there is no difference between the groups) is accepted. The null hypothesis is rejected if the p-value is less than 0.05 and the differences are proven statistically significant.

On the final graphs, Specific points are also pointed out with stars, which indicate p-values. These are probabilities that the observed differences in gene expression between the control and treatment groups are due to chance. The more stars, the lower the p-value, and thus, the higher the statistical significance of the result. The stars can be interpreted as:

> * (one star) generally indicates a p-value less than 0.05.
>
> ** (two stars) indicates a p-value below 0.01.
>
> *** Three stars: p-value less than 0.001.

Sometimes, points in the graph are topped with stars, indicating statistically significant differences in gene expression between treatment and control at those time points. The number of stars shows the exact level of significance.

Moreover, to probe deeply into the differences among the pairs of experimental groups, the post hoc t-tests were done. The t-test is a statistical method usually used to compare the mean of two groups, which helps show if they are significantly different. In this study, gene expression t-tests were applied after the ANOVA to locate which pairs of groups exhibited significant differences in gene expression. Several adjustments, such as the Bonferroni correction, were contemplated when the multiple comparisons were conducted to control for the increased risk of Type I errors, which happen when a true null hypothesis is wrongfully rejected.

The findings of such statistical tests were presented visually in graphs, where the differences among the groups were discerned using signs such as asterisks or letters. The graphs have been artfully drawn to illustrate the relative expression levels of the target genes in a patently clear way, with the error bars showing the variability within each group. The approach of ANOVA and t-tests, combined with appropriate data visualization, has provided a complete analysis frame to ensure the gene expression differences observed were statistically validated and precisely interpreted.

This analytical framework is well documented, and it is widely regarded for its potential to reveal variations in gene expression data, especially in the case of experimental studies that consist of multiple treatment conditions.

Data were analysed using the Primer program (Version 1.0, Stanton A. Glantz, Italy). It was used to analyse the data comprehensively in the form of each group's mean and standard deviation (Figure 14). The program can manage complex datasets and perform ANOVA with precision. At the first step of the process, the number of groups, cases per group, their means, and the standard deviation were entered into the software to ensure the accuracy of the statistical analysis (Figure 15).

Next step, a Student-Newman-Keuls test was performed on the data (Figure 16). This test was considered an important complement to the ANOVA since it allowed the detection of specific differences between experimental groups. The column on the right side shows how the program calculated the statistically significant differences between different groups.

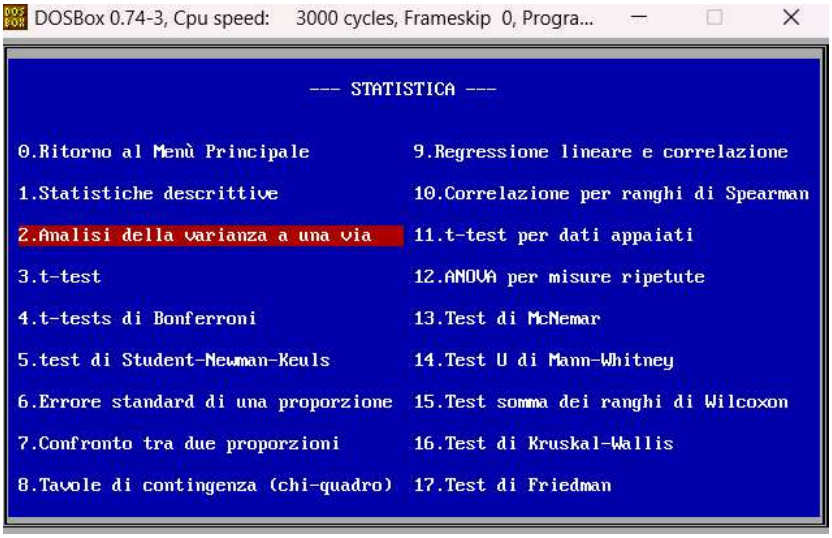


Figure 14: Primer program, the possibility for various statistical analyses.

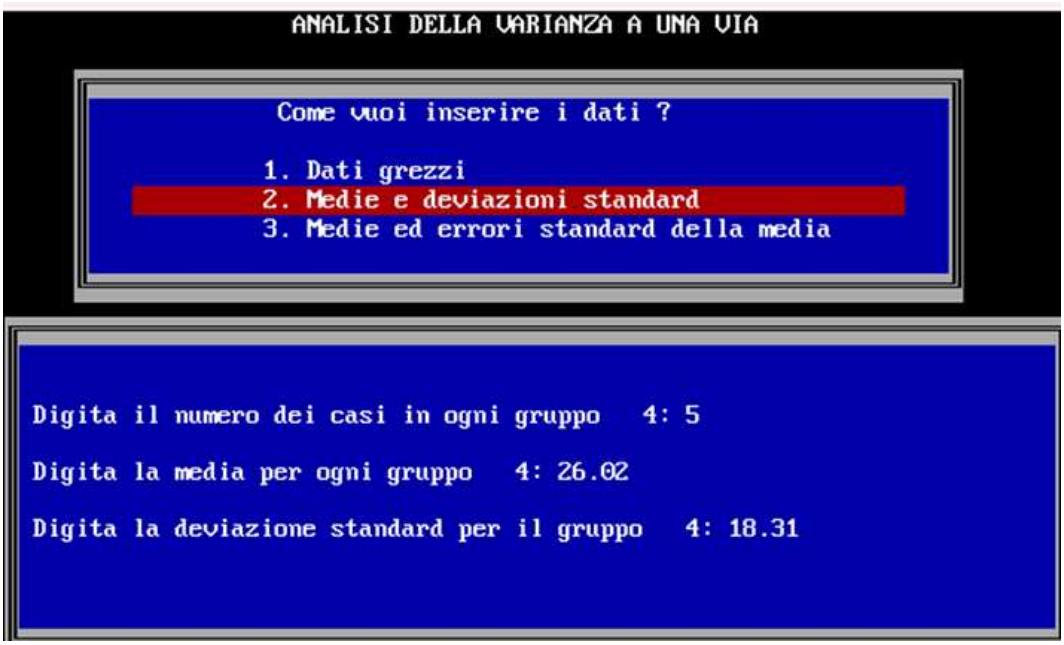


Figure 15: Primer program screenshot. Providing essential data for ANOVA analysis, including the number of groups, number of the examined cases in each group, their means, and standard deviation.

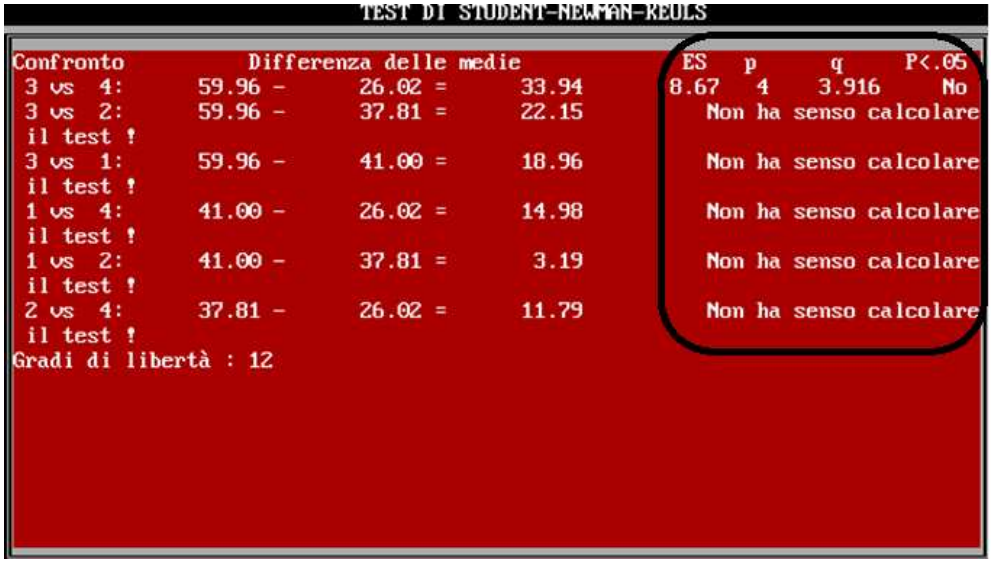


Figure 16: Example of a Student-Newman-Keuls test screenshot. The final column displays the differences between four times of *igt* expression in the spleen of *T. bernacchii*'s control group, indicating no statistically significant difference among the control groups, thereby confirming the validity of the experimental conditions.

4. RESULTS

GENE EXPRESSIONS IN THE LIVER

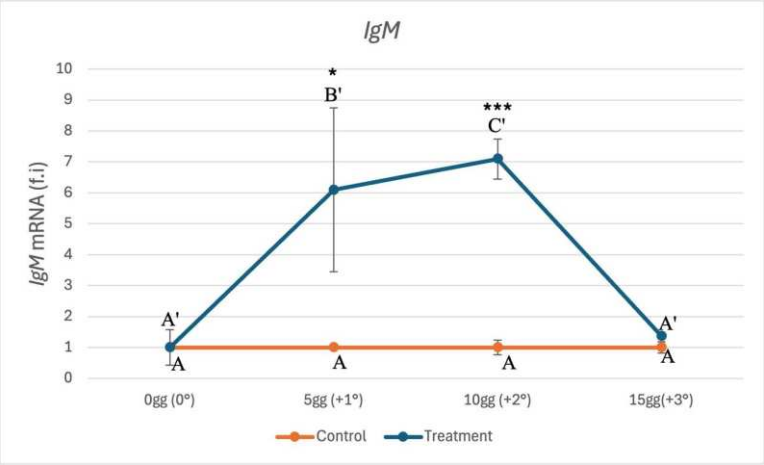
As a result of the fifteen-day experiment, the activation of genes in the liver did not change in the control groups, indicating that the only stress factor is elevated temperature since the other factors remained constant and did not interfere with the experiment.

Immunoglobulins *(igs)* Expression:

To investigate whether the dynamics of *igt* and *igm* expression were the same in response to increased temperature, we examined their respective patterns of responses across multiple time points from the first day until the end of the experiment at fifteen days. Analyses of *igm* expression showed an increase in the very initial response ($p < 0.05$), peaking at the intermediate time point (T+2; 7 times higher than controls, $p < 0.001$) before it decreased drastically by the final time point (T+3) (Figure 17A). While there is a difference between T+1 and T+2 time points, the statistically significant difference is relatively moderate, as shown by a single asterisk (*), suggesting that the large standard deviation means between these points should be interpreted more cautiously. The expression pattern suggests that *igm* is first upregulated in the liver as a component of its immediate response to thermal stress but quickly downregulated as the exposure continues.

According to this pattern, IgT might play a role in the liver's response to prolonged thermal stress, with its expression becoming more prominent as the stress continues. Its expression level temporarily increased more than 3 times with respect to controls at T+2 ($p < 0.01$) (Figure 17B).

A)



B)

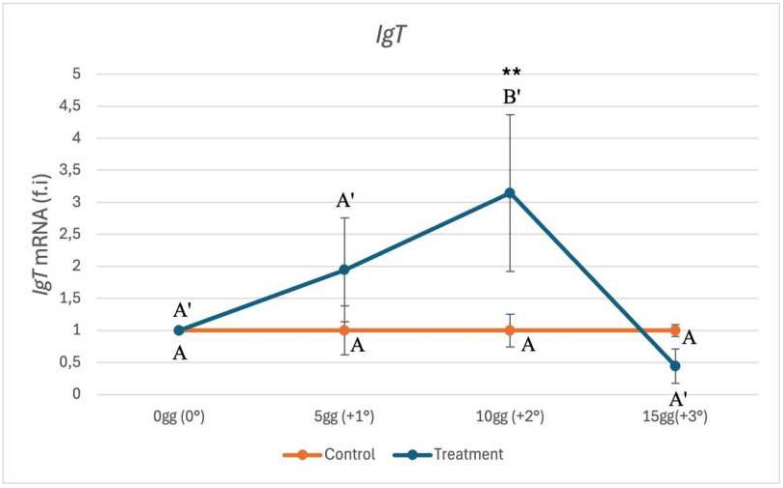


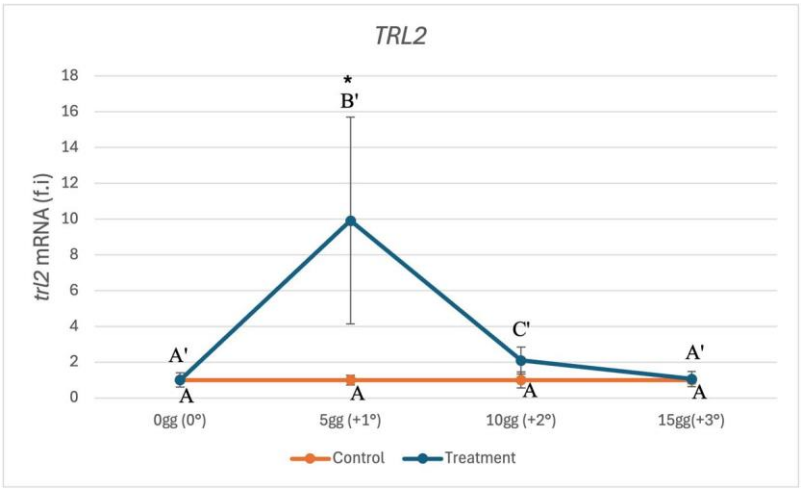
Figure 17: A) *igm* and B) *igt* gene expression levels in the liver of *T. bernacchii*.

Toll-like receptors (*trl*s) Expression:

A similar expression pattern of *tlr2* and *tlr9* was observed in the liver as for *igm*. Upon T+1 (5 days), both toll-like receptors show increased expression. *tlr2* expression demonstrated significant up-regulation early in the response, reaching its maximum at T+1 (5 days; 10 times higher than controls, $p < 0.05$) and declining right after (Figure 18A).

Similarly, *tlr9* followed the same trend, with expression peaking at T+1 (5 days; more than 12 times with respect to controls, $p < 0.01$) and declining after that (Figure 18B).

A)



B)

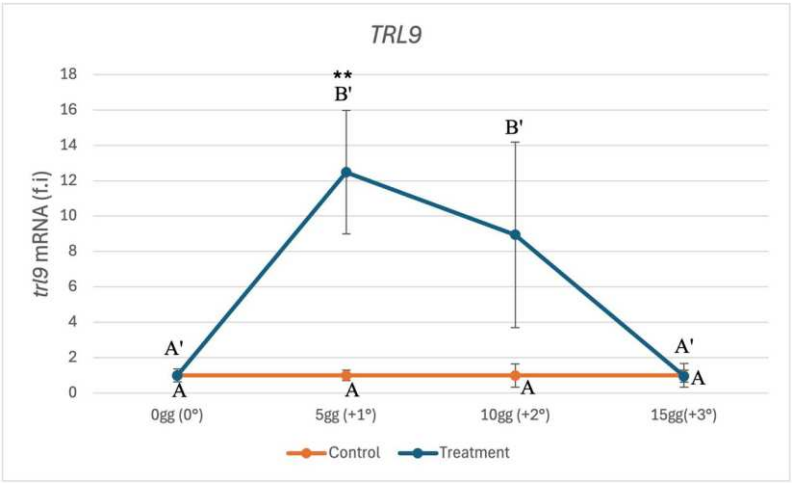


Figure 18: A) *trl2* and B) *trl9* genes expression levels in the liver of *T. bernacchii.*

In general, it has been observed that *trl*s' genes reached their peaks more quickly than *ig*s. Therefore, they experienced an early up-regulation and were activated after five days at the starting point at T+1. Later, they decreased until they reached the baseline at the end of the experiment. Reaching the peak for *ig*s took longer, from the first day to the ten days.

GENE EXPRESSIONS IN THE SPLEEN

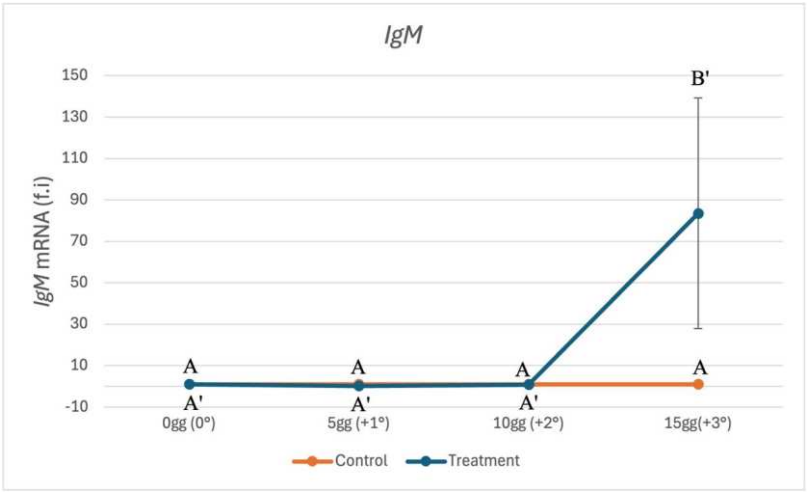
Like in the liver, gene activation in the spleen did not change in the control groups following the fifteen-day experiment. As a result, all other conditions remained constant and did not affect the outcomes, and elevated temperature remained the sole stress factor.

Immunoglobulins *(igs)* Expression:

T. bernacchii's spleen expressed *igm* and *igt* in distinct temporal patterns under increased temperature conditions. *igm* expression demonstrated an increase only between T+2 and T+3 (about 30 times higher than controls, $p < 0.05$) (Figure 19A).

In contrast, *igt* expression in the spleen showed a different pattern. It started to decrease at T+1 and T+2 ($p < 0.01$ and $p < 0.001$, respectively) and increased sharply (more than 3 times with respect to controls, $p < 0.05$) at T+3. The error bars also indicate the variability within each group, particularly at T+3, where the variation in response is most pronounced (Figure 19B).

A)



B)

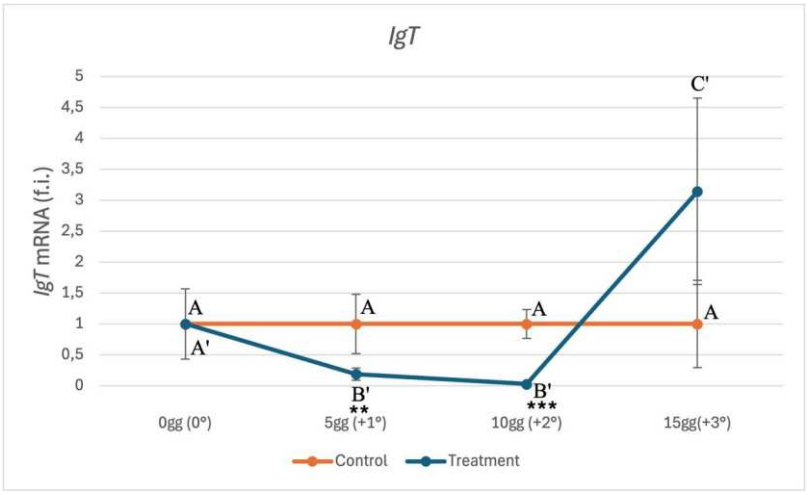


Figure 19: A) *igm* and B) *igt* genes expression levels in the spleen of *T. bernacchii.*

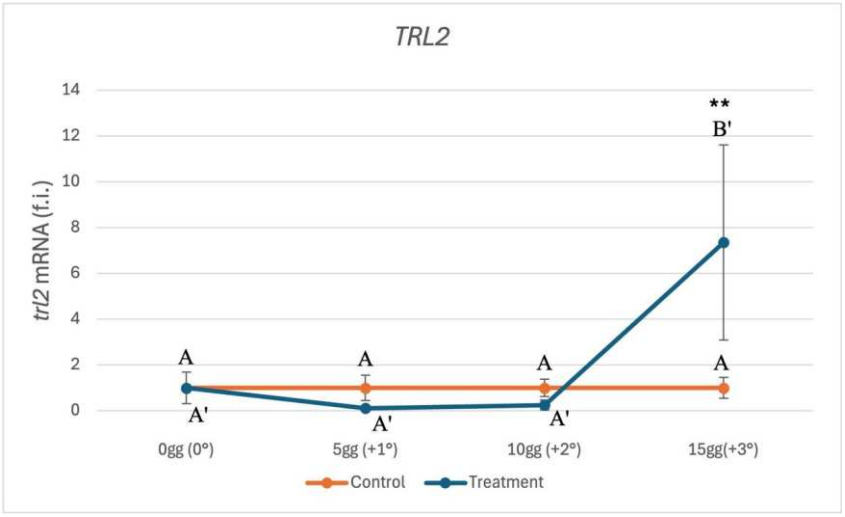
Toll-like receptors *(trl*s) Expression:

A significant up-regulation of *tlr2* was observed in the spleen of *T. bernacchii* only at T+3 (15 days) when the mRNA levels increased more with respect to controls with a statistically significant difference ($p < 0.01$) (Figure 20A).

Similarly, *tlr9* also showed a remarkable increase in expression, with significant up-regulation occurring only at T+3 ($p < 0.05$) (Figure 20B).

Even though both *tlr2* and *tlr9* expression levels were significantly higher at T+3, it is crucial to note that these changes occurred only at this final time point, with little to no change observed at earlier time points.

A)



B)

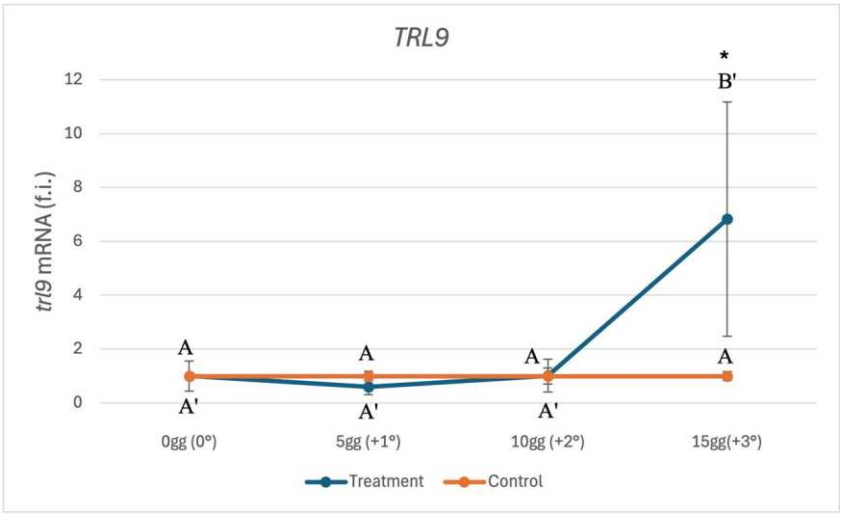


Figure 20: A) *trl2* and B) *trl9* genes expression levels in the spleen of *T. bernacchii.*

5. DISCUSSION

The present study aimed to study the expression of some immune-related genes in the liver and the spleen tissues of *T. bernacchii* exposed to an acute temperature change. It demonstrated a homogeneity in the organ-specific expression of the genes considered, as well as different timings in the functionality of the organs examined. These findings emphasize that although the liver and spleen represent both critical players in the immune response to temperature stress, their nature of functions is quite different yet complementary. Understanding their differences is key to appreciating how such organs can protect *T. bernacchii* under thermal stress.

The data obtained from the qRT-PCR analyses in the liver of *T. bernacchii* show that all four targeted genes were up-regulated from the baseline (T0) and then returned to near-baseline levels at the end of the experiment. Hence, such a trend characterizes a well-controlled, timely immune response suited to cope with the environmental stressors' immediate and short-term effects (Buckley, 2009; Pone, 2015). The instant reaction of the liver is a critical component facing this first wave of stress, producing cytokines and other signalling molecules to initiate a more general immune response. This, however, is a transient response designed to control an initial threat rapidly and avoid over-committing energetic resources (Buonocore, 2016).

Initial up-regulation of all four genes suggests immediate and robust activation of the immune system in response to temperature stress. This early activation, particularly of *trl* genes, underlines the role of the liver in primary defence through the innate immune system (Carrera-Silva, 2008). TLR2 and TLR9 are known to recognize PAMP and DAMP as part of the innate immune response and signal for a quick immune response (An, 2002; Zhan, 2015). The very early activation occurring at T+1 for both *tlr2* and *tlr9* could be explained by the fact that the liver rapidly detects thermal stress and probably recognizes it as a threat to cellular integrity.

Moreover, the transcription of *trl*s' genes elevated faster than immunoglobulin genes (*igm* and *igt*), which might be due to its original location on the surface of the immune cells, allowing it to perceive the environmental changes faster and reach the expression peak points earlier. Although we observed almost the same expression patterns for both *ig*s, their mRNA accumulation increased less than *trl*s because their receptors are inside cells, requiring pathogens or danger signals to be internalized before they can be detected (Nishiya, 2004; Sun, 2016).

Compared with IgM, IgT might have a more supporting role in the liver, as it is further specialized for mucosal surfaces rather than systemic immunity (Zhang, 2010). Its expression might increase later in response to continuing stress, particularly if the stressor targets mucosal tissues or needs a more targeted immune response.

These quick reactions of the liver to stress can be attributed to its role in detoxification and metabolism. This is because the liver is the major organ responsible for processing the toxins the body takes and maintaining its metabolic rate (Chen, 2020). It should respond quickly to any physiological stress to maintain

metabolic balance within the body. Metabolic activity is likely increased in the liver under temperature stress to react to increased energy demand and by-products of stress, including reactive oxygen species (ROS) (Spolarics, 1998). This will help support immune responses and protect hepatocytes against heat damage.

Another study on *T. bernacchii* facing the same experimental conditions revealed that heart rate increased, proving an overall increase in metabolic activity. This raised metabolic rate goes hand in hand with an increase in such enzymes as *gpx4* (a mitochondrial enzyme) and *gpx1* (an enzyme found in the cytoplasm), key regulators of cellular oxidative stress. It is presumed that the increase in the activities of these enzymes reflects that, with a temperature rise, the liver not only responds to the immediate physiological stress but also prepares for the sustained increase in oxygen demand and metabolic by-products (Piva, 2023). The increase in heart rate, together with the up-regulation of *gpx4* and *gpx1*, aligns with the role of the liver in adjusting to higher metabolic demands of the body during acute temperature stress. Accordingly, livers might coordinate multicomplex responses that balance energy production with protection from oxidative damage, giving rise to survival under adverse environmental conditions.

The aspect of antioxidant cellular defences is also interesting concerning the activation of immune defences, as they are also known to act by increasing the rate of ROS formation (Bassoy, 2021). In this sense, the up-regulation of *gpx4* and *gpx1* could also be linked to the need to keep H_2O_2 levels within the liver under homeostatic control.

Despite continuous temperature stress, the decreasing expression levels from the peak points to the baseline at the end of the experiment (T+3) may indicate that the liver had entered a different phase of the immune response. Contrary to the complete resolution of stress, this phase may stand for adaptive homeostasis (Vasadia, 2019), whereby the liver reduces the reaction to prevent excessive immune activation, which might be harmful if it were to last too long (Buckley, 2009).

Overall, physiological homeostasis must be maintained, even under continued stress. The decrease in transcription of these genes could represent a change from an acute, high-alert state to a more sustainable but controlled long-term response that avoids the adverse effects associated with chronic inflammation (Vasadia, 2019). This adaptive response would likely involve recalibrating immune activities to sustain defence mechanisms at levels at which tissues are not damaged and energy is not wasted in prolonged exposure to stress (Buckley, 2009).

This return to near-baseline levels could further underscore the engagement of feedback mechanisms that fine-tune the immune response (Buonocore, 2016). Otherwise, such mechanisms might protect the liver from devastating consequences of prolonged hyperactivation, such as tissue damage or immune exhaustion, to ensure the continued ability for immune function throughout extended periods of environmental stress (Carrera-Silva, 2008).

On the other hand, the increase in all gene expressions in this study indicates that temperature stress caused a highly coordinated response by the liver (Nawab, 2019).

Immune components, even in the absence of pathogens, such as TLRs and immunoglobulins (IgM and IgT), come together to ensure the effects of the increased temperature are handled (Engelsma, 2003). Such coordination ensures that the liver's response to counter these stresses emanating from the environment is effective, thereby offering stability to the organism.

The liver also has a primary role in systemic circulation. It receives blood from two major sources: the hepatic artery, which carries oxygenated blood, and the hepatic portal vein, which supplies nutrient-rich but deoxygenated blood from the gastrointestinal tract, spleen, and pancreas, whereby it can continually sense and react to changes within the internal environment of the body. As it circulates in the hepatic portal system, the liver senses changes in metabolic rates and stress-related signals through circulating factors in the blood and removing various toxins and metabolic byproducts (Bieghs, 2013; Jenne, 2013). This continual surveillance enables the liver to mount a prompt, localized immune response by activating resident Kupffer cells (liver macrophages that help clear pathogens and debris from the blood) and other immune pathways.

With the rise in metabolic activities, the composition of the blood might alter in return. The liver responds to these changes through various signalling pathways. For instance, temperature-induced stress may activate Heat shock proteins (HSPs) in the liver, which act as molecular chaperones, preventing denaturation of proteins and taking part in refolding damaged proteins. These HSPs may also be danger-associated molecular patterns recognized by pattern recognition receptors expressed on Kupffer cells, including TLR2 and TLR9. Activation of TLRs results in downstream signalling cascades, including activation of the NF-κB pathway with the induction in the production of TNF-α, IL-1β, and IL-6, pro-inflammatory cytokines. They can increase further the immune response by promoting the expression of other immune-related genes, including *igm* and *igt*, which are required to produce the humoral immune response (Ma, 2016).

Responses like these are important in reducing cellular damage and preventing systemic stress-induced immune suppression (Wanner, 1996).

Such rapid activation of the liver immune-related genes can also be viewed through the context of evolutionary adaptation. *T. bernacchii*, as an Antarctic fish species, is adapted to live in extremely cold environments where temperature fluctuations, although rare, can be sudden and severe (Buckley, 2004). The immediate response of the liver to temperature variation might be an evolutionary adaptation to ensure that in such a harsh and unpredictable environment, homeostasis is still maintained for survival. This could represent an evolutionary response to sudden environmental changes in which gene activation occurs rapidly to handle thermal stress without disturbing metabolic and immune balance (Bilyk, 2014).

The heat stress-related immune response in *T. bernacchii* was delayed in the spleen compared with the liver but ultimately more extreme. This time lag and eventual increased activation of immune-related genes likely reflect the unique role of the spleen as a lymphoid tissue primarily involved in generating more oriented and

stronger, slower, longer-term, and more sustained immune responses (Buonocore, 2016).

At T0, the spleen had minimal gene activation. Subsequently, from T+2 onwards, all four genes considered in this study were significantly upregulated. Compared to the liver, the spleen thus reacted more delayed, probably due to its function in the adaptive immune system. The adaptive immune system generally responds more gradually than the innate one but reacts more precisely and intensively (Romano, 1997). This slower activation could allow the spleen to act as a second line of defence, boosting the liver's rapid response. Therefore, the liver responds to the immediate physiological stress, whereas the spleen takes over when a more chronic exposure is established (Davison, 1994).

One theory behind this delayed activation is that the spleen starts to ramp up when the response in the liver begins to weaken. This hypothesis may indicate a handoff from the liver to the spleen, with the spleen increasing its immune activity as the role of the liver decreases. High up-regulation of *igm* and *tlr2*, specifically at T+2 in the spleen, might be related to recognizing more sustained stress signals (Buonocore, 2016). It was reported that the temperature increase was not a momentary environmental fluctuation but a stable change for which a robust adaptive immune response should be induced.

The spleen is also part of the adaptive immune system, which involves activating lymphocytes and eventually producing immunological memory. Whereas the liver does have a relatively rapid stress response, the spleen responses are more specific and sustained (Borghans, 1999). This specificity is key to recognition and action taken against a particular antigen, but in our laboratory experiment, temperature is used as the stress and not pathogen exposure. Another possible reason for the spleen's delay may be that it purifies the blood by removing old/damaged cells and surveying threats. It ensures that no energy is spent on pointless immune responses, but it saves energy until it realizes it needs to respond.

Furthermore, the spleen also serves as a reservoir for immune cells, thus storing them until needed. This storage function means it does not have to react to stress immediately but waits to see if other parts of the immune system can handle the initial response, such as the liver. Only when the stress persists, as seen at T+2, does the spleen begin to mobilize its resources. Furthermore, it can switch its activity toward blood cells in long-term stress, particularly during periods of strong stress when the body is strongly engaged in reconstituting or improving its immune and circulatory abilities.

The *igt* response, with its typical signature of initial depression followed by a steep increase, underlines the role of the spleen in immune response adjustment. IgT is involved in adaptive immunity and balancing the immune system's responses. This initial dip may, therefore, be a regulatory mechanism that prevents over-response to small stress, thus saving energy and not initiating a needless activation of the immune response. The increase of the *igt* expression during obvious and continuous temperature stress shows its preparedness for safeguarding such continuous environmental changes by spleen action.

The spleen response is slow yet integral, suggesting that the organ acts as a highly fine-tuned regulator, much like a thermostat. The system would thus prevent overreacting by the spleen to minor stressors, which would be wasteful in terms of energy balance and potentially harmful due to too much activity of the immune system. The delay in reaction time gives the spleen a chance to react appropriately and ensures proper need for activation, avoiding the wasting of metabolic energy. Once the relevance and persistence of a stressor have been confirmed, the spleen gets into top gear in preparing the organism for sustained contact with the stressor.

This fined-tuned regulation is the essence of a balanced immune response. By controlling the timing and intensity of its activation, the spleen assures that it does not waste the body's energy resources in response to non-threatening transient signals. Such regulation does not expose the body to the otherwise enormous damage from persistently activated immune reactions, sometimes causing chronic inflammation or, in the worst instance, autoimmunity. The spleen monitors immunity and purges the body of non-functional, physiologically damaged cells and other potential dangers that could be a source of inflammation in the circulation (Lewis, 2019). More importantly, the spleen acts as an organ responsible for immune homeostasis. Storing cells for the immune system and only providing these when needed saves that energy for more intense and prolonged challenges, especially since stress is difficult to avoid.

This fine-tuned regulation brings to the fore the spleen's role in responding to stress and maintaining immune balance. The capacity of the spleen to modulate the pace and amplitude of its response ensures that it can effectively cope with chronic stress while providing energy savings to be deployed when truly required. Proper adaptation in this response is key to survival in extreme environmental conditions where energy conservation and immune regulation are essential.

6. CONCLUSION

The current study illuminated the unique and complementary roles of the liver and spleen concerning the temperature-stress immune response in *T. bernacchii*. The assessment of expression patterns of key immune-related genes encoding IgM, IgT, TLR2, and TLR9 in both organs indicated that each organ makes a unique contribution to the immune strategy of this Antarctic species.

The liver response, acting as the body's first responder, showed early, short-lived induction of immune-related genes. This prompt response is plausible because some of the liver's main functions include metabolic detoxification and, more importantly, the rapid detection of stress signals due to its being a part of blood circulation. This may indicate that the liver is prepared to respond well to an immediate threat but avoids staying activated, leading to harmful inflammation. This rapid up-regulation of genes encoding TLRs returning to baseline might imply a finely tuned mechanism for maintaining metabolic and immune homeostasis despite continuous environmental stress.

In contrast, the spleen showed a more delayed but more potent and sustained response, mostly at later time points of up-regulation for *igm* and *tlr2*. The delayed activation underlines the role of the spleen as a secondary responder that provides a more targeted and long-term immune response. Involvement of the spleen in the adaptive immune response enables this organ to mount a more specialized and durable response once the liver's initial reaction subsides. Because of this, the observed gene expression profile in the spleen illustrates its role as an important modulator of chronic immune stimuli, saving resources until a more definitive need arises for a broad and long-lasting response.

The complementarity of these responses suggests a highly orchestrated immune strategy in *T. bernacchii,* where the liver deals with immediate threats. At the same time, the spleen provides long-term protection in a balanced manner. Division of labour such as this would not only maximize the efficiency of the output of the organism but also be much better for maintaining homeostasis in an environment that is constantly changing and harsh at the same time. More likely still, it is how these organs can cooperate, with each playing its specialized role, as a key adaptation that enables *T. bernacchii* to survive and thrive in the extreme conditions of the Antarctic.

This study indicates that in *T. bernacchii*, heat stress differentially modulates liver and spleen tissues by the specialized physiology of the two organs. The rapid, unspecific response of the liver and the late, specific, and continued activation of the spleen secure a balanced immune defence. This coordinated response system could represent one of the evolutionarily developed mechanisms by which *T. bernacchii* has been able to confront the extreme characteristics of Antarctica and which may enable them in the future to meet the challenges of an environment that is no longer as stable as it once was.

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