The effects of distinct heatwaves on the immunological and oxidative stress responses of the bivalve *Scrobicularia plana*

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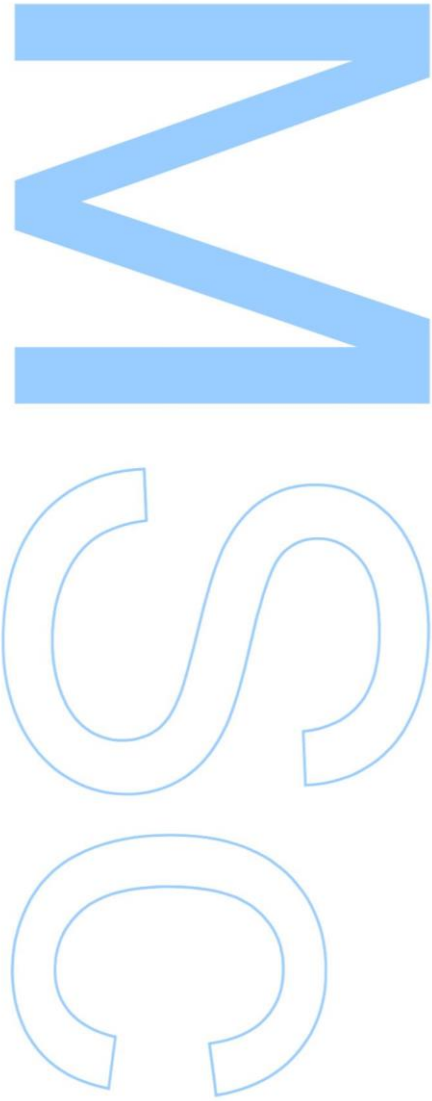

“We ourselves feel that what we are doing is just a drop in the ocean. But the ocean would be less because of that missing drop”

Mother Teresa

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Abstract

Marine heatwaves are considered extreme climatic events that have a major impact on aquatic organisms, because temperature is an important variable that controls the functioning of their biological and physiological systems. Furthermore, it has been found that these events are occurring with more intensity and frequency as global warming is increasing.

Our study consisted on the evaluation of the immunological effects and the oxidative stress responses of the bivalve *Scrobicularia plana* when exposed to two distinct heatwaves (17ºC to 25ºC) with different durations (7 days - P1 *versus* 14 days - P2) and recovery times (14 days e 7 days, respectively).

In this study, heatwaves (P1 and P2) showed a higher mortality rate when compared to the control group, although there were no statistically significant differences among treatments. The condition index increased over the experimental time without any influence of temperature, demonstrating that heatwaves do not have a huge influence on *S. plana* fitness.

Regarding the responses of the cellular immune system, it has been shown that temperature increase had a negative influence on several parameters of cell immunity (e.g. total haemocytes count, cell viability and phagocytosis). However, phagocytosis was the only parameter that demonstrated capacity to recover when exposed to shorter heatwaves (7 days) with longer recovery times (14 days). In contrast to phagocytosis, there was an upward trend in reactive oxygen species (ROS) production, particularly during longer heatwaves (i.e. at P2). As for humoral immune system responses, there were no significant differences in total protein content of plasma. Accordingly, there were also no significant differences among treatments for protease and bactericidal activity. NO production increased at rising temperatures (from T0 to T1) and then decreased again even at elevated temperatures, demonstrating a great adaptability to external conditions.

Regarding the oxidative stress response, the MDA production between P1 and P2 was not significantly different. However, there was a significant decrease in intermediate sampling times.

As for antioxidant enzymes such as SOD and CAT, which are important in removing ROS, it was shown that SOD had a similar response to the NO, with a significant increase in the first sampling times (T1 to T2) but without significant differences between treatments, followed by a decline. Catalase activity tended to

decrease over time without influence of temperature. And finally, GST activity decreased progressively with time.

In general, it has been shown that *S. plana* has a high tolerance and adaptability to the heatwaves. However, longer heatwaves with shorter recovery periods seemed to be more hazardous to the studied species than shorter heatwaves with longer recovery times.

Keywords: Climate change, Marine Heatwaves, Immune responses, Oxidative stress, *Scrobicularia plana*.

Resumo

As ondas de calor são consideradas eventos climáticos extremos que têm um grande impacto nos organismos aquáticos, pois a temperatura é uma importante variável que controla o funcionamento dos seus sistemas biológicos e fisiológicos. Além do mais, tem-se verificado que estes eventos estão a ocorrer com maior intensidade e frequência à medida que o aquecimento global aumenta.

O nosso estudo consistiu na avaliação dos efeitos ao nível imunológico e de stress oxidativo no bivalve *Scrobicularia plana*, de duas ondas de calor distintas (17ºC para 25ºC) com diferentes durações (7 dias – P1 *versus* 14 dias – P2) e diferentes períodos de recuperação (14 dias e 7 dias, respetivamente).

Neste estudo, as ondas de calor (P1 e P2) demonstraram uma maior taxa de mortalidade do que no grupo controlo, apesar de estatisticamente não existirem diferenças significativas entre tratamentos. O índice de condição aumentou ao longo do tempo sem qualquer influência da temperatura, demonstrando que as ondas de calor não têm influência na condição física da *S. plana*.

Quanto às respostas do sistema imuno celular, observou-se que a temperatura tem uma influência negativa em vários parâmetros da imunidade celular (por exemplo, na contagem total de hemócitos, viabilidade celular e fagocitose). No entanto, a fagocitose foi o único parâmetro a demonstrar capacidade de recuperação quando expostos a ondas de calor mais curtas (7 dias) com tempos de recuperação mais longos (14 dias). Em contraste à fagocitose, houve uma tendência de aumento da produção de espécies reativas de oxigénio (ROS), nomeadamente em exposições mais longas (isto é, em P2). Quanto às respostas do sistema imuno- humoral, não houve diferenças significativas na concentração total de proteína no plasma. Em conformidade, também não existiram diferenças significativas entre tratamentos nas atividades da protease e bactericida. A produção de NO aumentou aquando a subida da temperatura (T0 para T1) seguida de uma diminuição mesmo a temperaturas elevadas, demonstrando uma grande capacidade de adaptação às condições externas.

Em relação às respostas do stress oxidativo, a produção de MDA entre P1 e P2 não foi significativamente diferente. Contudo, houve uma diminuição significativa em tempos de amostragem intermédios.

Quanto às enzimas antioxidantes, como a SOD e a CAT, importantes na remoção de ROS, demonstrou-se que a SOD teve uma resposta semelhante ao óxido nítrico, com um aumento significativo nos primeiros tempos de amostragem (T1 para T2) mas sem diferenças significativas entre tratamentos, seguida de um declínio. A

atividade da catalase diminuiu tendencialmente ao longo do tempo sem influência da temperatura. E, por fim, a atividade da GST diminuiu progressivamente ao longo do tempo.

Em geral, demonstrou-se que a *S. plana* tem uma grande tolerância e capacidade de adaptação às ondas de calor. Contudo, ondas de calor mais longas com tempos de recuperação mais curtos parecem ser mais prejudiciais à espécie em estudo do que ondas de calor mais curtas com tempos de recuperação mais longos.

Palavras-chave: Alterações climáticas, Ondas de calor, Respostas imunes, Stress oxidativo, *Scrobicularia plana*.

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List of abbreviations

4-HNE	4-hydroxynonenal
BHT	Butylated Hydroxytoluene
CAT	Catalase
CDNB	1-Chloro-2, 4-dinitrobenzene
CO_2	Carbon dioxide
$CuSO_4$	Copper sulfate
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediamine Tetraacetic Acid
GHG	Greenhouse Gases
GSH	Glutathione
GST	Glutathione S-Transferase
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
H_3PO_4	Phosphoric acid
HBSS	Hanks' Balanced Salt Solution
$HO^{\cdot}$	Hydroxyl
iNOS	inducible Nitric Oxide Synthase
IPMA	Instituto português do Mar e da Atmosfera
KOH	Potassium hydroxide
LPO	Lipid peroxidation
MDA	Malondialdehyde
MHW	Marine Heatwaves
Na_2CO_3	Sodium carbonate
Na_2EDTA	Ethylenediaminetetraacetic Acid, Disodium Salt
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium chloride
NO	Nitric Oxide
O_2	Oxygen

O_2^-	Superoxide
$ONOO^-$	Peroxynitrite
PBS	Phosphate Buffered Saline
PI	Phagocytosis index
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Trichloric Acetic Acid
THC	Total Haemocyte Count
TSA	Tryptic Soy Agar

1. Introduction

1.1. Climate change

Over the past three decades, climate change has become an increasingly evident reality (IPCC, 2014) with implications for biological systems. In general, it can alter the structure and functioning of communities (Parmesan and Yohe, 2003; Rosenzweig *et al.,* 2008; Cheung *et al.,* 2009) and, consequently, constitute a threat to the biodiversity of ecosystems (Smale *et al.,* 2019). According to the Intergovernmental Panel on Climate Change (IPCC, 2014), greenhouse gases (GHG) released essentially by human activities are responsible for this phenomenon. The increase of these GHGs, mainly carbon dioxide (CO_2), results in the retention of heat in the atmosphere and leading to an increase of temperature of the planet, the global warming (Nikinmaa, 2013).

Global warming has a major impact on aquatic ecosystems, particularly marine ecosystems (MacKenzie and Schiedek, 2007; Meehl *et al.,* 2007; Richardson and Poloczanska, 2008). It is an important variable in these systems, capable to determine the global functioning of aquatic organisms (Miranda *et al.,* 2013). When abnormal water temperature increases, it can affect survival due to the arrival of invasive species competing for space and food (Jeppesen *et al.,* 2010), reproduction, immune response, growth and geographic distribution of organisms (Madeira *et al.,* 2002; Miranda *et al.,* 2013). Thus, water warming may compromise the sustainability, stability and biodiversity of the aquatic ecosystem (IPCC, 2014; Durant *et al.,* 2007).

In parallel with global warming, extreme events, such as marine heatwaves (MHW) are considered one of the climate events more devastating for the aquatic ecosystems (Oliver *et al.,* 2018; Frölicher and Laufkötter, 2018).

1.2. Marine Heatwaves

A marine heatwave (MHW) is defined as a prolonged rise of water temperature that can be described by its duration, intensity, rate of evolution and spatial extent (Hobday *et al.,* 2016). It is considered when they last at least 5 days with temperatures higher than the 90th percentile based on a historical 30-year baseline period (Hobday *et al.,* 2016).

MHWs are important extreme climate events whose knowledge of their effects on aquatic ecosystems is still scarce (Smale *et al.,* 2015). However, there is evidence that these extreme events alter the functioning and structure of all aquatic ecosystems (Garrabou *et al.,* 2009; Wernberg *et al.,* 2013) and affect resources and assets with socioeconomic implications (i.e. extinctions or change in the geographical distribution of

important species in fisheries) (Oliver *et al.,* 2018). Over the past 90 years, the frequency and duration of a heatwave has increased by an average of 34% and 17%, respectively, with the annual average increase of 54% (Oliver *et al.,* 2018), being in conformity with the IPCC, (2012), which describes an increase of frequency and intensity too.

In the last two decades there have been several heatwaves with negative impacts on ecosystems. In the summer 2003, an atmospheric heatwave hit northern Europe and led to the thermal stratification of the Mediterranean Sea with an increase of 2 to 3ºC of surface water (García-Herrera *et al.,* 2010). Consequently, a high mortality rate, changes in species distributions and declines in local biodiversity have been observed (Garrabou *et al.,* 2009; Marba and Duarte, 2010). In 2011, a heatwave raised the Indian Sea temperature between 2 and 4°C with a duration of over 10 weeks by about 2000 km along the coast of western Australia leading to widespread mortality of benthic organisms (Wernberg *et al.,* 2013) with serious consequences for local fisheries (Caputi *et al.,* 2015). Another MHW, known as “The Blob”, caused a 6°C rise in the northeast Pacific Ocean (Frölicher and Laufkötter, 2018). And more recently, the “Lucifer” heatwave that hit more than 12 European countries in 2017 with devastating effects on the entire human population, economy and biodiversity (Smale *et al.,* 2019). Briefly, Oliver *et al.,* (2018) reported as the main consequences of MHWs, the “sustained loss of seaweed forests, coral bleaching and mass mortality of marine invertebrates due to thermal stress”. In addition, harmful algal blooms, associated with human poisoning by paralyzing molluscs, have been observed (Walsh *et al.,* 2018), which certainly affected fishery. Such impacts demonstrate the influence of MHWs on the structure and sustainability of aquatic communities and ecosystems, including estuarine environments. This particular ecosystem is of extreme concern to the scientific community due to the highly productive system (Savage *et al.,* 2012) that are being affected by climate change, either by degradation of water quality (Wetz and Yoskowitz; 2013) or by high mortality (i.e. Verdelhos *et al.,* 2015).

Despite all the negative effects, this extreme climate event is still not well understood. However, it is known that, for example, Australia's MHW in 2011 was caused by the prevailing conditions of La Niña in 2010/2011, that led to a warm current to the Australian coast (Frölicher and Laufkötter, 2018) and MHW of the Pacific Ocean was caused by the abnormal rise in sea level pressure followed by a decreased ocean heat loss, which led to an increase of ocean temperature (Frölicher and Laufkötter, 2018).

Subsequent to these episodes, awareness of the importance of these extreme events increased. However, there is still much to do to understand the responses of organisms to MHWs. This is because they may respond differently depending on the

geographical distribution and location of their habitat. According to Smale *et al.,* (2019), species from tropical climates are extremely vulnerable to temperature increase because they live very close to their upper thermal limit (Deutsch *et al.,* 2008). The same applies to cold climates species with short thermal limits (Peck *et al.,* 2004) and, therefore, are more vulnerable to any change in temperature. Moreover, unlike tropical animals, they cannot escape to colder places (Cheung *et al.,* 2009). Finally, for temperate climates, prediction is more complicated to do because of "diversity of life history patterns, trophic relationships and habitat variability" (Roessig *et al.,* 2004). For example, in the study of Smale *et al.,* (2015), there were no significant differences when various species of sessile organisms were exposed to a short-term temperature increase. This response was explained by the fact that invertebrates used in the experiment are from a temperate climate and, moreover, intertidal zones. Stillman and Somero (1996) explain that organisms living in tidal fluctuating zones (intertidal zones including estuaries) have greater tolerance to temperature variations than species living in the subtidal zone. This is because they are adapted to these fluctuations daily. However, there is evidence that organisms at these zones are at risk to the MHW due to the proximity of the upper thermal limit (Somero, 2010). However, the response of organisms can still be influenced by sex, diet, age, O_2 concentration, photoperiod and history of thermal exposure (Abstract de Hutchison, 1976).

In assessing the effects of MHW, it is also important to consider the time of exposure, since this was positively related to the decrease of ecological health (Smale *et al.,* 2019). But not all effects are negative. In the study by Smale *et al.,* (2015) some invertebrate species exhibited a positive response to warming with increasing abundance throughout the experiment. Therefore, in assessing the effects of MHW on aquatic organisms is necessary to consider the species under study, since the answer is species-specific (Sorte *et al.,* 2010).

1.3. Biological responses

During organism's life, they are exposed to various environmental factors, including the temperature rise caused by global warming and more frequent and intense MHW. This exposure can alter biological mechanisms such as the immune and oxidative stress responses of organisms and, consequently affect their normal functioning. A group of aquatic organisms most affected by these adverse conditions are the bivalves because of their sessile characteristics and therefore, the next chapters focus on them.

1.3.1. Immunology

The immune system of bivalve molluscs is divided into two groups: cellular and humoral immunity (Soudant *et al.,* 2013). The immune cells of these molluscs are known as haemocytes that move freely among all the cells of the organism (Hégaret *et al.,* 2003; Soudant *et al,* 2013).

Haemocytes, particularly granulocytes (Hine, 1999), are responsible for several mechanisms, such as phagocytosis, encapsulation and production of reactive oxygen species, in response to external agents (Soudant *et al.,* 2013). In more detail, phagocytosis involves 4 steps: chemotaxis that aims to attract pathogens, recognition and attachment (lectin-mediated) (Chu, 2000), internalization to form a phagosome, and, finally, intracellular degradation of the pathogen by hydrolytic enzymes from lysosomes (Soudant *et al.,* 2013). When the pathogen is large, the granulocytes cannot internalize it. For this reason, they encapsulate it through secretion of a polypeptide and then, through lysosomal enzymes, there is extracellular degradation of the encapsulated external agent (mechanisms shown in blue in figure 1) (Soudant *et al.,* 2013).

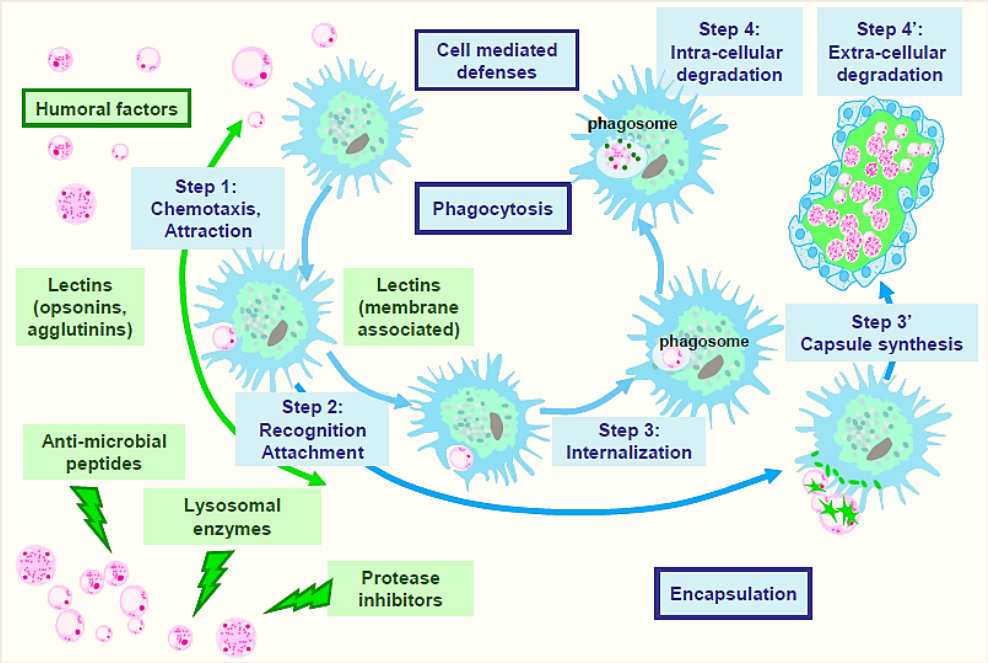

Figure 1: Representative scheme of pathogenic phagocytosis and encapsulation through haemocytes (Blue) and production of humoral factors by haemocytes (Green). From Soudant *et al*., (2008).

Furthermore, bivalve granulocytes produce reactive oxygen species (ROS) (Cajaraville *et al.,* 1995) during NADPH-oxidase complex activity on the plasma membrane. ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl ($HO^\bullet$) can be triggered by increased oxygen (O_2) absorption (respiratory burst) by external

stimulation or phagocytosis (Cajaraville *et al.,* 1995). These ROS can help in the destruction of invading microorganisms (Klebanoff *et al.,* 1983; Adema, 1991). However, it can be irrelevant if pathogens produce insufficient amounts of antioxidants against ROS.

In parallel to the production of these ROS, bivalves are also capable of producing nitric oxide (NO) in response to immune stimulation (Tafalla *et al.,* 2003). This is produced by inducible nitric oxide synthase (iNOS) from arginine, O_2 and NADPH, that when combined with the superoxide ion generates peroxynitrite ($ONOO^-$); radical with high oxidative power constituting a complementary method in the destruction of pathogens in bivalves, more specifically in clams (Taffala *et al.,* 2003). Thus, phagocytosis, ROS and NO are synergistic mechanisms of bivalves in host defense (Figure 2).

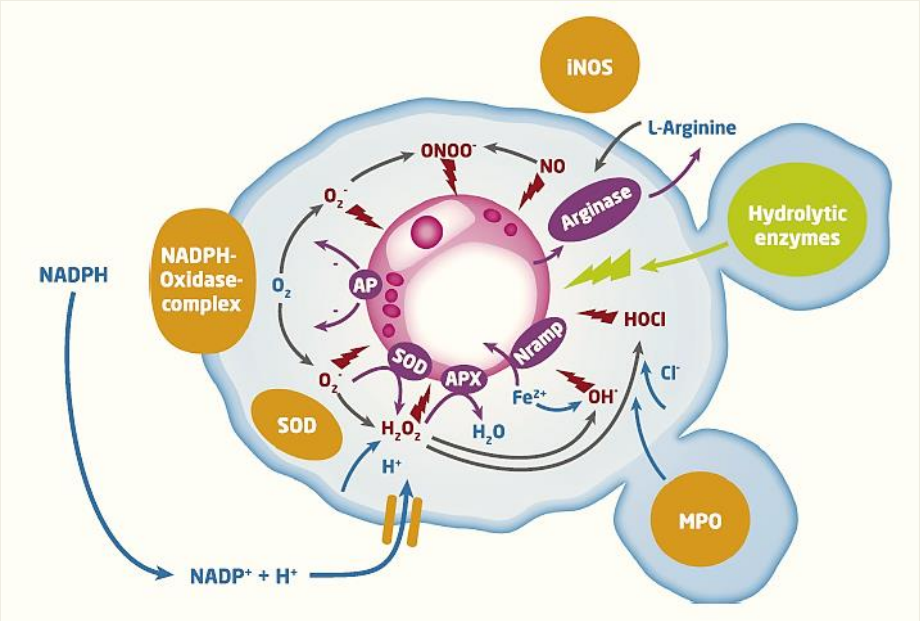

Figure 2: Representative scheme of a pathogenic (pink) destruction in granulocyte (blue) through (1) the production of reactive oxygen species (ROS) in the NADPH oxidase complex (2) production of NO by inducible nitric oxide synthase (iNOS) from arginine (3) and the action of hydrolytic enzymes from lysosomes. From Soudant *et al.* (2013).

In addition, haemocytes are also responsible for the secretion of various other humoral factors (figure 1), such as lectins, major plasma proteins, antimicrobial peptides and protease inhibitors (Soudant *et al.,* 2013) and are equally important in the defence of the organism (Fernandez-Boo *et al.,* 2018). These are usually found in haemolymph plasma of aquatic organisms, including bivalves (Soudant *et al.,* 2013).

Proteases are a humoral factor that plays an important role in the defense mechanisms of bivalves (Anderson and Beaven, 2001) because they prevent the proliferation of bacteria due to their peptidase activity (Fernandez-Boo *et al.,* 2018).

Briefly, this substance interacts with plasma membranes by forming openings in cell membranes causing their lysis (Canicatii, 1991 cited from Fernandez-Boo *et al.,* 2018). With this, bactericidal activity decreases, reducing the susceptibility of the bivalve to an infection.

However, the defence mechanisms of organisms can be affected by various biotic or abiotic factors. Water temperature is one of the most important factors in bivalve's immune system (Abele *et al.,* 2002; Gagnaire *et al.,* 2006). For example, an increase in temperature (20°C to 30°C) in clam *Chamelea gallina* led to an increase in total haemocyte count (TCH) and a decrease in phagocytosis by 50% (Monari *et al.,* 2007). The same results were observed with clam *Mactra veneriformis* and clam *Ruditapes philippinarum* (Yu *et al.,* 2009; Paillard *et al.,* 2004). These studies demonstrate that there is a correlation between temperature and the immune system of bivalves, including clams.

1.3.2. Oxidative stress

Oxygen (O_2) is an important chemical element for energy production in aerobic organisms (Mailloux and Harper, 2011). However, when there is a reduction of this molecular O_2, in the electron transport chain to the formation of water (H_2O), naturally produce reactive intermediates, called reactive oxygen species (ROS) (Mailloux and Harper, 2011). These include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$) (Figure 3), which are produced by various body tissues (Lesser, 2006). They are highly harmful to cells that can cause damage to proteins, DNA and lipids (Lesser, 2006). This last one (lipids) is considered the most common damage in the cells (Abele *et al.,* 2011). Lipid peroxidation (LPO) is a set of reactions occurring in lipid initiated by ROS, especially $HO^\bullet$ (Lesser, 2006). In general, ROS capture electrons from lipids, most often from polyunsaturated ones, causing their change and, consequently, alters the function of cell membranes (Lesser, 2006). This process involves three well-defined steps: initiation, propagation, and termination (Gutteridge, 1995). In the last step, LPO molecules are degraded into by-products such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and other dialdehydes (Repetto *et al.,* 2012).

To prevent these harmful cell damage, organisms have developed defense mechanisms to reduce or even neutralize the action of ROS, especially O_2^- and H_2O_2 so that the most harmful of ROS ($HO^\bullet$) is not produced (Barreiros *et al.,* 2006). To this end, cells produce antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and Glutathione-S-Transferase (GST) (Lesser, 2006).

SOD is the first line of defense, responsible for converting O_2^- into H_2O_2 and O_2 (Lesser, 2006). However, the cell is not yet fully detoxified. H_2O_2 has a large diffusion capacity across biological membranes and can enter numerous other reactions (Lesser, 2006; Mailloux and Harper, 2011) and can directly damage DNA and proteins or even lead to cell apoptosis (Lesser, 2006). To degrade this molecule acts the CAT, transforming it into water and oxygen (Figure 3) (Lesser, 2006). Regarding GST, it transforms xenobiotics into other conjugates (Lesser, 2006).

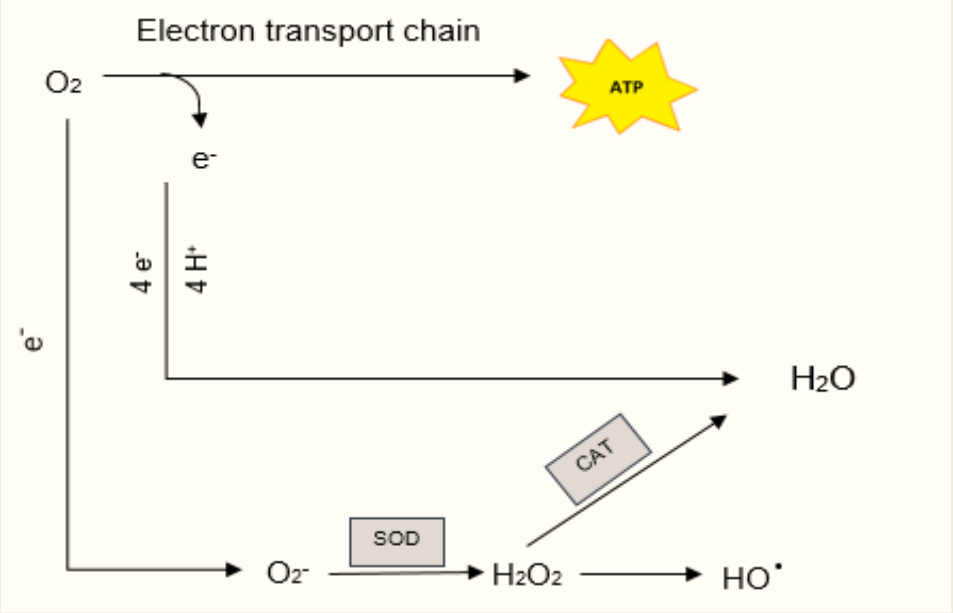

Figure 3: Routes of oxygen (O_2) metabolism in marine organisms and reactive oxygen species (ROS) formation. Electron (e^-) release during an O_2 reduction to produce adenosine triphosphate (ATP). Formation of ROS as superoxide (O_2^-), hydrogen peroxide (H_2O_2) by SOD activity, radical hydroxyl ($HO^•$), and water as the end product by CAT activity. O_2 reduction by four electrons to form H_2O. Adapted from Mailloux and Harper, (2011).

The formation of ROS and antioxidants occurs in a balanced manner to avoid oxidative stress on cells. However, organisms are exposed daily to a number of external factors, either chemical or non-chemical (i.e., temperature) that cause a cell imbalance. Consequently, the ROS production rate exceeds the degradation rate by establishing oxidative stress on cells (Sies, 1993; Sies, 1997).

Thermal stress is one of the environmental factors responsible for the abnormal production of ROS and, consequently, the induction of antioxidant enzymes. According to Abele *et al.,* (2011), temperature increases ROS production in ectothermic animals by accelerating mitochondrial respiration. There are several studies in the literature showing that temperature has a strong influence on oxidative stress in organisms, particularly bivalves. For example, the clam *Mya arenaria* when exposed to an increase of 10°C

(15°C to 25°C), a significant increase in ROS content was observed (Abele *et al.,* 2002). Another example from Anacleto *et al.,* (2014) demonstrated that the activity of antioxidants (CAT, GST and SOD) was correlated with temperature increase in two clams (*Ruditapes decussatus* and *Ruditapes philippinarum*). However, in that same study, it was observed that around LT_{50}, in general, their activity was gradually decreasing with temperature. Regarding LPO, the response of bivalves is in agreement with that of antioxidants, with significant lipid damage (Anacleto *et al.,* 2014). Moreover, the induction of enzyme antioxidant activity in many other invertebrates was observed when exposed to high temperatures (Abele *et al.,* 1998; Heise *et al.,* 2003; Abele *et al.,* 2011). Therefore, with the analysis of these biomarkers, it is possible to understand if organisms are under stress when exposed to temperature increase and subsequently recover from it.

1.4. *Scrobicularia plana*

Scrobicularia plana (da Costa, 1778) belongs to the class Bivalvia and Family Semelidae (World Register of Marine Species). This clam has a yellowish, flat, thin and rounded shell (Santos *et al.,* 2011). This mollusc is found from the northeast Atlantic to the Mediterranean coast (Santos *et al.,* 2011). *S. plana* is typical of estuarine environments and is usually found in muddy sediments at 5 to 20 cm depth (Santos *et al,* 2011). In Portugal, it is easily found all over the country, from the Lima river estuary, to Sado river estuary and Guadiana river estuary (Coelho *et al.,* 2014).

S. plana is a dioecious species. It has two separate sexes and they release the gametes to the water, where occurs fertilization, embryonic and larval development (Santos *et al.,* 2011). The spawning time differs between locations. In Portugal, two spawning periods are observed, one in the winter and the other in the spring (Santos *et al.,* 2011).

As for food, this species feeds on particulate matter suspended in the water column through the siphons (Essink *et al,* 1991 cited from Santos *et al.,* 2011) or when it emerges collects food by diverting sediment with theirs siphons (Santos *et al.,* 2011). On the other hand, *S. plana* is an important food resource mainly for avifauna and ichthyofauna (Santos *et al.,* 2011). Furthermore, it is important for the economy, particularly in Portugal, due to its high commercialization (Santos *et al.,* 2011).

Finally, these bivalves are great bioindicators for assessing the effects of climate change on estuaries as they are considered temperature sensitive (Sorte *et al.,* 2011) and have been used several times for several studies to evaluate MHW responses (Verdelhos *et al.,* 2014; Verdelhos *et al.,* 2015).

1.5. Objectives

The main goal of this study was to evaluate the effects of two distinct heatwaves (i.e. with different duration and recovery periods) on the immunological and oxidative stress responses of the bivalve *Scrobicularia plana.*

For that, the immunological response was studied based on: (1) cell viability and total haemocytes count, phagocytosis and respiratory burst on haemocytes and (2) total protein content, protease activity, nitric oxide and bactericidal activity on plasma. On the other hand, the oxidative stress response was also studied based on the lipid peroxidation and antioxidant enzymes activity (i.e. superoxide dismutase, catalase and glutathione-S-Transferase) in order to understand the mechanisms induced by different patterns of temperature increase during heatwave and later, during the recovery period.

2. Materials and methods

2.1. Organisms collection and acclimation

Scrobicularia plana adults were collected by hand from the southern arm of the Mondego estuary (40º08'N, 8º50'E) during low tide in November of 2018. This estuary is considered a reference site and is often used for the collection of several species, namely *S. plana* for experimental purposes (see, Dolbeth *et al.,* 2011, Verdelhos *et al.,* 2015).

The organisms, measuring between 3-4 cm, were transported to the Marine and Environmental Interdisciplinary Research Centre (CIIMAR) in a cool box with water from the original site. In the facilities, they were divided into four tanks and acclimated for four days with seawater (salinity 35, 17°C, pH 8±0.3), continuous aeration and 16:8h photoperiod to simulate the natural conditions.

After the acclimation period, three individuals of *S. plana* from the same size class (3+), according to Verdelhos *et al.,* (2005), were distributed for each of the 48 (4 replicates x 3 treatments x 4 times) cylindrical glass flasks (2L each). Each flask had 10 cm of sand, previously washed and burnt for 4h at 500ºC, to eliminate any tracers, and it was added 1L of seawater under the same conditions as above. Over the next four days, the temperature was gradually increased to 25ºC (2ºC/day).

2.2. Experimental set-up

Clams were exposed to different experimental conditions for 25 days: 1) control (C); 2) Heatwave Pattern 1 (P1) and 3) Heatwave Pattern 2 (P2). Each treatment had its own water reservoir that circulated between tanks (Figure 4). Thus, the temperature of the flasks of the same treatment was equal. This temperature was controlled with a temperature control system (Aqua Medic® AT Control-SW, version 9.0).

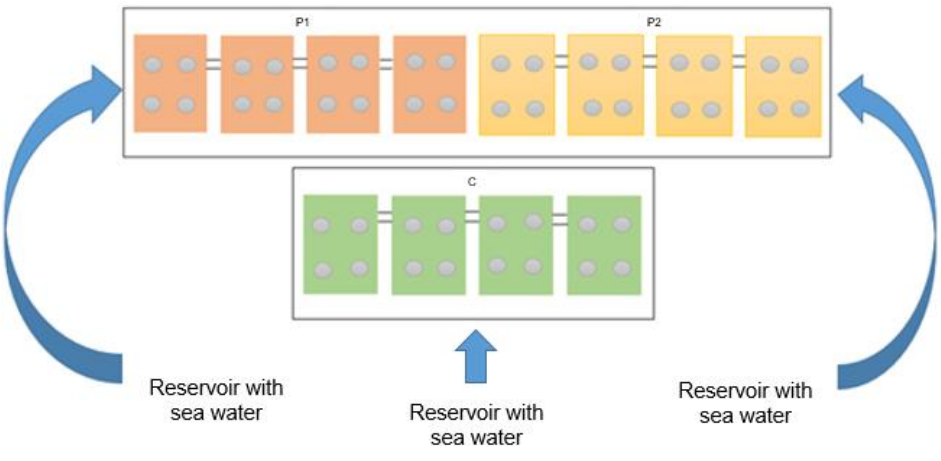

Figure 4: Representative scheme of the experimental system

The organisms corresponding to P1 were exposed to a temperature of 25°C±0.1 for 7 days and P2 were exposed to the same temperature (25ºC±0.3) for 14 days. Thus, organisms of P1 were exposed to a short heatwave and a longer recovery time (14 days) compared to P2, with a longer heatwave and a shorter recovery time (7 days). As for control, *S. plana* was exposed to natural habitat temperature (17ºC±0.2) during the 25 experimental days (figure 5).

The water in the flasks was renewed twice a week to guarantee the water quality of the system. In addition, analyses to nitrates and ammonia were made regularly to guarantee that quality. The clams were fed with a concentrated phytoplankton solution (Liquizell, Hoby) 3 times a week. The flasks were checked daily for mortality.

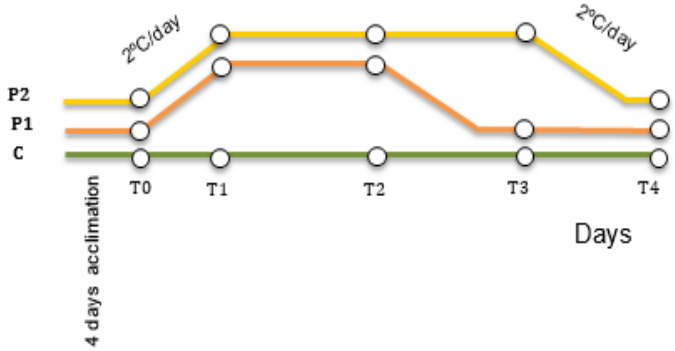

Figure 5: Graphical representation of the different experimental conditions (C, P1 and P2)

Table 1: Schematic representation of the different experimental conditions (C, P1 and P2)

Samples / Treatments	T0 Day 0	T1 Day 4	T2 Day 11	T3 Day 18	T4 Day 25
Control	17ºC	17ºC	17ºC	17ºC	17ºC
P1 (Short heatwave, longer recovery)	17ºC	25ºC	25ºC	17ºC	17ºC
P2 (long heatwave, short recovery)	17ºC	25ºC	25ºC	25ºC	17ºC

Sampling was divided in five sampling times: Time 0 (T0) - Day 0, Time 1 (T1) - Day 4, Time 2 (T2) - Day 11, Time 3 (T3) - Day 18 and Time 4 (T4) - day 25, as shown in table 1. T0 was performed after 4 days of acclimation before temperature rise, T1 corresponds to time when P1 and P2 reached 25ºC, T2 corresponds to the first 7 d exposure to 25ºC for both P1 and P2, T3 corresponds to the first 7 d of recovery for P1 and 15 d of exposure to 25ºC for P2; and, finally, T4 corresponds to 15 d of recovery for P1 and 7d of recovery period for P2.

In general, at each sampling time, for each clam it was done: 1) measurement of biometric parameters (i.e. total weight and soft tissue weight) 2) haemolymph extraction for evaluation of immunological response; 3) tissues freezing at -80ºC for oxidative stress analyses.

2.3. Condition index (CI)

At each sampling day, each organism (with shell and without shell) was weighed to determine possible differences in growth and development between treatments. For this, according to Cruzeiro *et al.,* (2016), the following formula was applied:

$$\mathrm{CI} = \left(\frac{\text{Fresh weight}}{\text{Shell weight}}\right) * 100$$

2.4. Biochemical endpoints

Biochemical analyses were divided in two groups: immune responses (i.e. cell viability and total haemocytes count, phagocytosis and respiratory burst on haemocytes and total protein content, protease activity, nitric oxide and bactericidal activity on plasma) and oxidative stress analyses (e.g. lipid peroxidation and antioxidant activity, such as superoxide dismutase, catalase and glutathione-S-Transferase).

2.4.1. Immune responses

To analyse the immune responses, an average of 700 µl of haemolymph was carefully removed from the organisms, using a 26-gauge needle attached to a 1 mL syringe, by insertion between the valves near to the posterior adductor muscle and kept on ice immediately. Prior to extraction, the syringe was washed with antiaggregant (27mM sodium citrate, 385mM sodium chloride and 115mM glucose dissolved in distilled water) to avoid coagulation of the cells. The haemolymph was used to analyse the cell viability and total haemocytes count, phagocytosis and oxidative burst activity. The remainder was centrifuged at 4500 xg for 10 minutes at 4ºC and the supernatant (CS) was stored at −80°C. The plasma was used for other analyses such as total protein content, protease activity, nitric oxide and bactericidal activity.

2.4.1.1. Cell Viability and Total Haemocytes Count

Haemocytes viability was assessed microscopically using the trypan blue exclusion test (0.1% in PBS) and cells counted in a Neubauer haemocytometer.

For the total haemocyte count, it was mixed 50 µl of haemolymph extracted from each sample with 50 µl of the marine antiaggregant solution and 5 µl of Tripan Blue (0.1% PBS) and all mixed in a vortex and kept on ice in a microtube. The number of viable and non-viable haemocytes per mL of haemolymph in each individual was determined using a Neubauer chamber and counted in duplicate under the light

microscope (Olympus BX40) at a magnification of 400x. To obtain cell density, the following formula was performed:

$$Cell\ density\ = \frac{\text{Total viable cells}}{\text{number of squares counted}}\ \text{x dilution x}\ 10^4$$

2.4.1.2. Phagocytosis

This parameter required 100 µl of haemolymph and the use of fluorescent beads. Initially, it was made the beads solution with 1 µl of beads (10^9 µg/mL) and 100 µl of Hanks' Balanced Salt Solution (HBSS). To this mixture was added 100 µl of haemolymph making a total of 200 µl and kept in the dark for one hour. Then, a cytospin was done (5G, 3 minutes) using half of the sample that was distributed in two sections per slide. After drying, the sample are fixed with formaldehyde/ethanol (50:50), stained with hematoxylin-eosin (for 2 min each) and kept at 4°C. Later, the slides were observed under light microscope (Olympus BX40 using immersion oil at a magnification of 1000x and 200 cells were analysed per sample. Phagocytosis was expressed as a phagocytic index (PI) according to Mahapatra *et al.,* (2017), where:

$$PI\ = (\frac{engulfed\ particles}{phagocytic\ cells}) \times (\frac{phagocytic\ cells}{total\ cells}) \times 100$$

Finally, to observe the fluorescence of beads in cells, the nuclei were labeled with DAPI (5µl/mg PBS) and photographs were taken to demonstrate phagocytosis.

2.4.1.3. Oxidative Burst Activity

Oxidative burst activity was measured according to the method of Fujiki and Yano, (1997) with some adaptations. Diluted and deactivated 50 µl of *Vibrio* spp. (10^6cell/mL) was added to triplicates of 100 µl haemolymph for each sample in a microplate and stored in the dark for 2h at room temperature. A new 30 minutes incubation was done after the addition of 100 µl of NBT (0.1% HBSS). Then, the plate was centrifuged 5 minutes at 1000G and the pellet was washed with 70% methanol. After centrifugation and carefully removal of the supernatant, the pellet was dried for 20 minutes at 40ºC. Finally, 125µl of 1M KOH and 125 µl DMSO were added to each sample in plate and sonicated for 10 minutes. The microplate was read in spectrophotometer (BioTek, USA) at 630nm.

2.4.1.4. Total protein content

Total protein concentration of all plasma samples was determined using the Pierce BCA protein assay kit (Thermo Scientific). This analysis is based on the Lowry method (Lowry *et al.,* 1951). In a 96-well flat-bottom microplate, 25 µl of plasma was added. Then, it was necessary to prepare a final solution with 200 µl of solution A (0.20M Na_2CO_3 and 0.70mM Sodium Potassium Tartrate in 0.1M NaOH) and 4 µl of solution B (drop of H_2SO_4 and 0.2M $CuSO_4$ in 100 mL deionized water). From this final solution, were added 200 µl to each well and incubated for 1 hour at 25°C. Subsequently, the plate was read at 562 nm. Protein concentration (µg/mg) was calculated from the generalized standard curves of known albumin concentration.

2.4.1.5. Protease activity

Protease activity was quantified in plasma using the azocasein hydrolysis assay according to Machado *et al.,* (2015) with some modifications. To each microtube was added 50 µl of plasma, 60 µl of 100nM ammonium bicarbonate buffer ($NaHCO_3$) and 125 µl azocasein in 2% NaHCO3. The mixture was incubated for 24 hours with agitation at room temperature. The reaction was stopped upon the addition of 250 µl of 10% trichloric acetic acid (TCA). Then, this mixture was centrifuged at 6000xg for 5 minutes. Finally, 100 µl of the supernatant plus 100µl of 1N NaOH were added to a flat bottom 96-well plate. The absorbance of the samples was read on a spectrophotometer (BioTek, USA) at 450nm. In the positive control, plasma was replaced by trypsin (100% protease activity) and in the negative control was replaced by ammonium buffer (0% protease activity).

2.4.1.6. Nitric oxide

The Nitric Oxide (NO) production in the plasma was based on Tafalla *et al.,* (2003). Briefly, 25 µl of plasma sample, 100 µl of 1% sulfanylamide solution (Sigma) in 2.5% of phosphoric acid (H_3PO_4), 100 µl of 0.1% N-naphthylethylenediamine solution (Sigma) in 2.5% of phosphoric acid (H_3PO_4) were added in a microtube. After 10 minutes, it was incubated at room temperature. Finally, the absorbance of the samples at 540 nm was read. The concentration of nitrite (µM/mL) in the sample was determined from standard curves generated using known concentrations of sodium nitrite.

2.4.1.7. Bactericidal activity

Bactericidal activity of the plasma was determined following the method of Graham *et al.,* (1988) with modifications. First, *Vibrio anguillarum* (strain PC696.1) was

cultured in tryptic soy agar (TSA) (Difco Laboratories) at 22°C with NaCl to a final concentration of 1%. Bacteria were suspended in sterile HBSS and the concentration adjusted to 10^6 colony-formin units (cfu) mL^{-1}. Briefly, 50 µl plasma and 20 µl of bacteria (10^6) were added in duplicate to a round bottom 96 well microplate and incubated at 25°C for 3 hours. As a positive control, Hank's balanced salt solution (HBSS) was added to wells instead of plasma (100% viability). After 3 hours, 25 µl of 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (1 mg/mL; Sigma) was added and incubated for 10 minutes at 25°C. Subsequently, the plates were centrifuged at 2000xg for 10 minutes. The supernatant was removed and 200 µl of DMSO was added to the precipitate and transferred to a 96-well flat-bottom microplate. Finally, the absorbance was recorded at 560 nm on the spectrophotometer (BioTek, USA). Bactericidal activity is expressed as a percentage, calculated from the difference between surviving bacteria compared to the number of bacteria from positive controls.

2.4.2. Oxidative stress

2.4.2.1. Sample Homogeneity

All samples were initially homogenized with phosphate buffer (50 mM buffer, pH 7.4, 2mM EDTA) in a proportion of 100 mg of tissue to 1 mL of HB buffer (100g/L). After this process, 2 mL was centrifuged at 15000G for 20 minutes at 4°C. After collecting the supernatant, 5 µl of sample and 95 µl of distilled water were pipetted into a 96-well flat bottom plate. Then, 80 µl of solution A (0.20M Na_2CO_3 and 0.70mM Sodium Potassium Tartrate in 0.1M NaOH) and solution B (drop of H_2SO_4 and 0.2M $CuSO_4$ in 100 mL deionized water) were added to the microplate and kept 10 minutes at room temperature. Finally, Folin's reagent (1:1) was added and incubated in the dark, with shaking, for 30 minutes and read at 690 nm. The determination of protein content (mg protein/mL) was done by the Lowry method (Lowry *et al.,* 1951). For that, standard solutions with successive dilutions of Serum Bovine Albumin (BSA) in 0.9% NaCl, 0.1 NaN_3 (0 to 250 µg protein) were used.

2.4.2.2. Determination of Malondialdehyde (MDA) concentration

Lipid peroxidation was determined by malondialdehyde (MDA) quantification, a method described by Ferreira *et al.,* (2008). For this, thiobarbituric acid reactive

substances (TBARS) were used, which reacts with MDA, increasing fluorescence according to concentration.

Briefly, 16.8 µl of TCA (100%) was added to 150 µl of diluted sample vortexed 2 times for 1 minute and centrifuged (5000rpm, 20 min, 4ºC) to allow removal of compounds that may interfere with the process. Later, 120 µl of supernatant, 120 µl of EDTA and 720 µl TBA (1%, 0.05m NaOH, 0.025% BHT) was collected into a microtube previously pierced with a needle. Afterwards, the samples were boiled in the dark for 30 minutes and, finally, they were placed in a 96 well microplate in duplicate and read at 532 nm. The results were expressed in nmol MDA/ mg protein.

2.4.2.3. Antioxidant enzymes

Superoxide dismutase (SOD) activity was determined by an indirect method involving the inhibition of cytochrome c reduction (Ferreira *et al.,* 2008). In this method SOD competes with cytochrome c for the superoxide anion generated by the xanthine and xanthine oxidase reaction. SOD activity was determined in the mitochondrial fraction as the degree of inhibition of cytochrome c reduction at 550 nm (Ferreira *et al.,* 2008). Briefly, 25 µl of diluted sample was pipetted into a triplicate 98-well plate. Then, it was added 25 µl of sodium phosphate buffer (50 mM, 0.1mM Na_2EDTA, pH 7.8), 200 µl of xanthine 0.7 mM and 0.3 g/L of cytochrome c. Finally, 50 µl of xanthine oxidase 0.4U/mL was added. Standard solutions with successive dilution of SOD stock solution (300U/mL) were required to determine SOD activity (1 to 40 U/mL). The activity is given in SOD units (1 SOD unit = 50% inhibition of xanthine oxidase reaction) per mg of protein.

The determination of catalase activity was obtained by reacting it with hydrogen peroxide (H_2O_2) and transformed it into oxygen and water, as described in Ferreira *et al.,* (2007). Briefly, 15 µl of diluted sample and 135 µl of HB buffer was added to the 96-well UV plate and then, 150 µl of the reaction mixture (24.8 µl 30% H_2O_2 in 10 mL HB buffer) was added. Microplate reading in the plate reader was taken at 240 nm for 2 minutes recording absorbance every 15 seconds and results were expressed in µmol/min/mg protein.

Regarding GST activity based on the method of Habig *et al.,* (1974) changed to a microplate, the conjugation of CDNB (1-Chloro-2, 4-dinitrobenzene) with the Glutathione thiol group decreases absorbance allowing enzymatic activity to be determined. Briefly, 25 µl of diluted sample and 75 µl of GST buffer (0.1M, pH 6.5) were added. Finally, 200µl of reaction mixture (0.1M GST buffer, pH 6.5; 10mM GSH and 60mM CDNB) were added and read at 340 nm for 5 minutes at 20 second intervals and the results were expressed in nmol/min/mg protein.

2.5. Statistical Analysis

First, data from both immune response and oxidative stress were checked for normality of data and homogeneity of variances using the Kolmogorov-Smirnov test and Levene's test, respectively. When the assumptions were not accepted, the data were transformed into ranks and nonparametric tests were applied. Then, a Two-way ANOVA or equivalent non-parametric test (Two-way ANOVA on ranks) were used to test the effects of temperature and time. The survivorship of individuals was compared between paired treatments by log-rank tests (Cardoso *et al.,* 2017). Significant differences were set as $p < 0.05$. All the statistical analyses were performed in STATISTICA7 (StatSoft, USA).

3. Results

3.1 Survival

Regarding survival, for the first two sampling times no mortality was observed in any of the three treatments. Concerning the control, there was a slight decline in survival rate in the last sampling times. For the other treatments, P1 showed a lower survival rate (75%) than P2 (80-90%) and control (90-100%) for T2 and T3, while in the last sampling time, P2 presented a lower survival rate (80%) than P1 (90%) and control (90%). However, statistically, there were no significant differences among treatments (log-rank tests, $p > 0.05$).

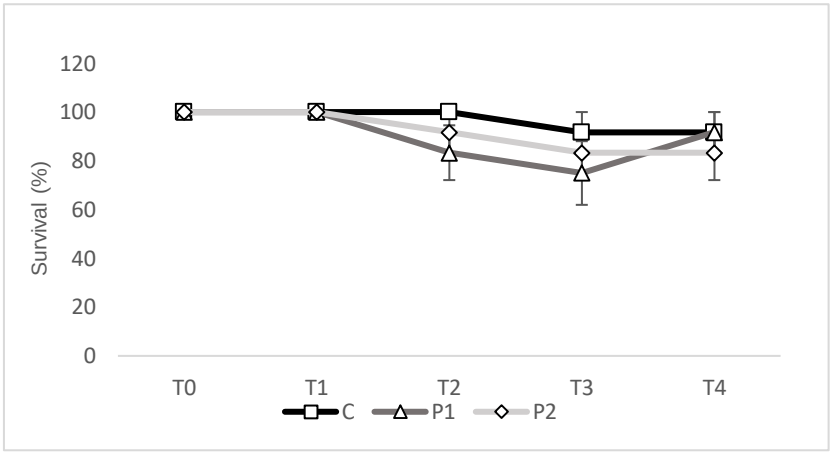

Figure 6: Survival (%) of *S. plana* exposed to the different treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E.

3.2. Condition Index

Regarding the condition Index (CI), there were significant differences among sampling times (two-way ANOVA on ranks, $F_{(4,163)} = 16.29$; $p < 0.05$). According to figure 7, it is explicit that CI increased significantly over time. On the other hand, no significant differences among treatments were observed ($p > 0.05$).

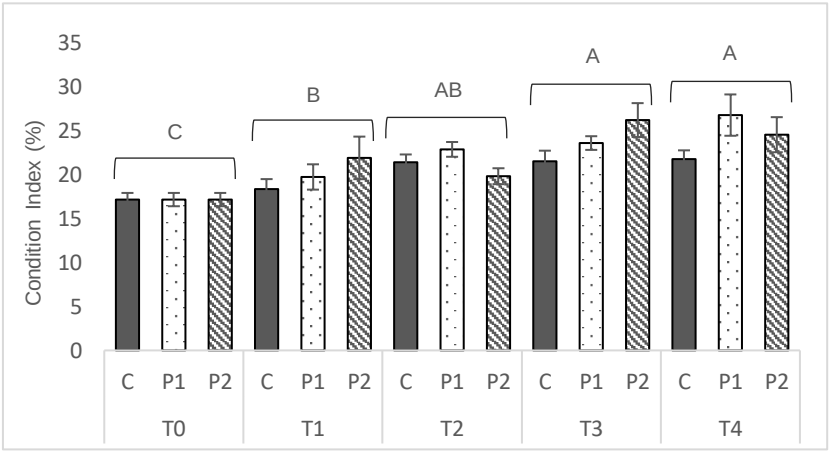

Figure 7: Condition Index (%) of *S. plana* exposed to the different treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E. Different letters represent significant differences among sampling times.

3.3. Immunity responses

3.3.1. Haemocytes viability

According to figure 8, the control group always had higher viability rates (an average of 94%) than P1 (87%) and P2 (80%). It was also observed a significant interaction between both factors (Two-way ANOVA on ranks, $F_{(8,148)} = 4.45$; $p < 0.05$). In general, the viability of haemocytes exposed to both heatwaves were significantly lower than in control and also declined from T1 to T4 compared to T0 (Figure 8).

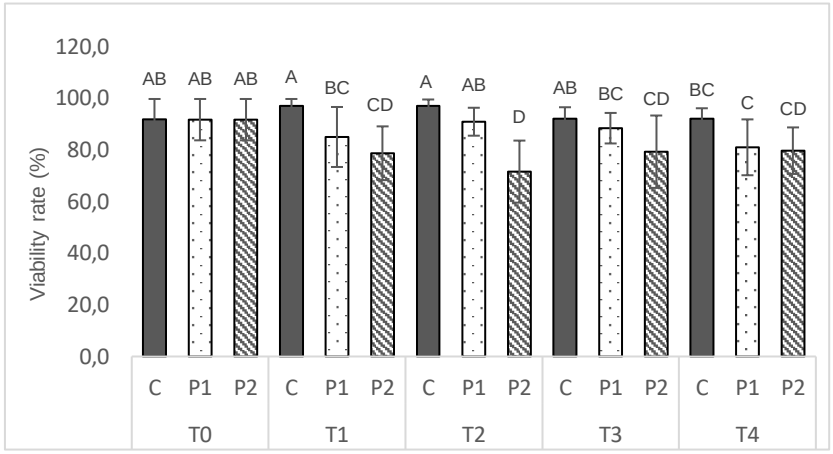

Figure 8: Haemocytes viability (%) of *S. plana* for the different treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E. Different letters represent significant differences among groups.

3.3.2. Total Haemocytes Count (THC)

Concerning THC, a significant interaction between temperature and time was observed (Two-way ANOVA, $F_{(8,225)} = 2.99$; $p < 0.05$). In general, there was a clear decline in THC with a longer exposure to temperature (i.e. from control to P2), particularly from T2 to T4. On the other hand, there was a significant increase in THC among sampling times, namely between the first two sampling times (T0, T1) and T2 and T3.

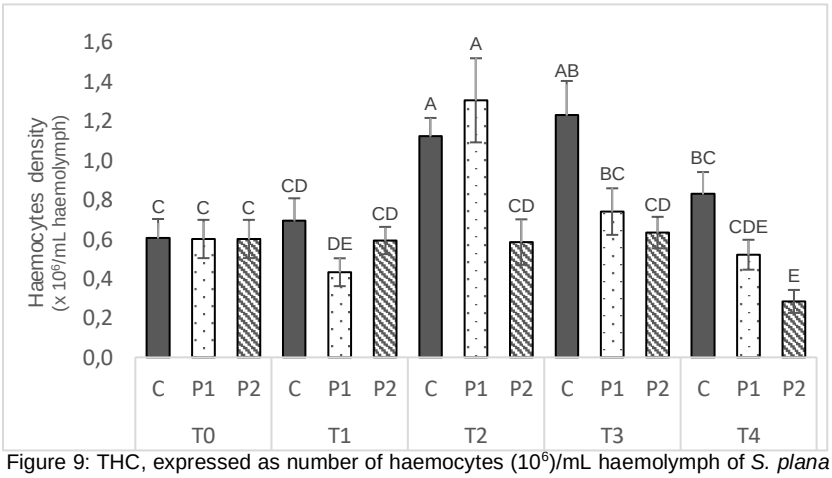

Figure 9: THC, expressed as number of haemocytes (10^6)/mL haemolymph of *S. plana* in the different treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E. Different letters represent significant differences among groups.

3.3.3. Phagocytosis

Regarding the phagocytosis, a significant interaction between temperature and time was also observed (Two-way ANOVA, $F_{(8,179)} = 6.70$; $p < 0.05$) (figure 10). Concerning the temperature treatments, there was a clear decline in the phagocytic activity with exposure to temperature compared to control. However, no significant differences were observed between P1 and P2, except in T4. Regarding sampling times, there was a clear decrease in phagocytosis between T0 and the remaining times ($p < 0.05$). It was also observed that T1 significantly varied from T2 and T3 ($p < 0.05$).

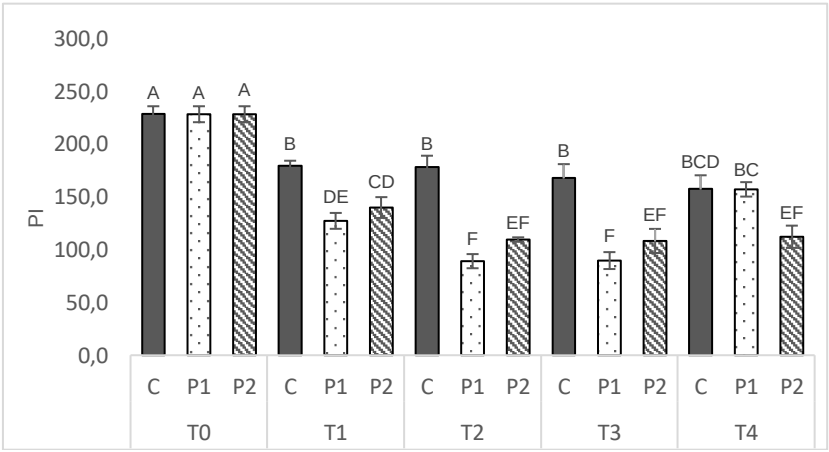

Figure 10: Phagocytosis Index (PI) of *S. plana* haemocytes in the different treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E. Different letters represent significant differences among groups.

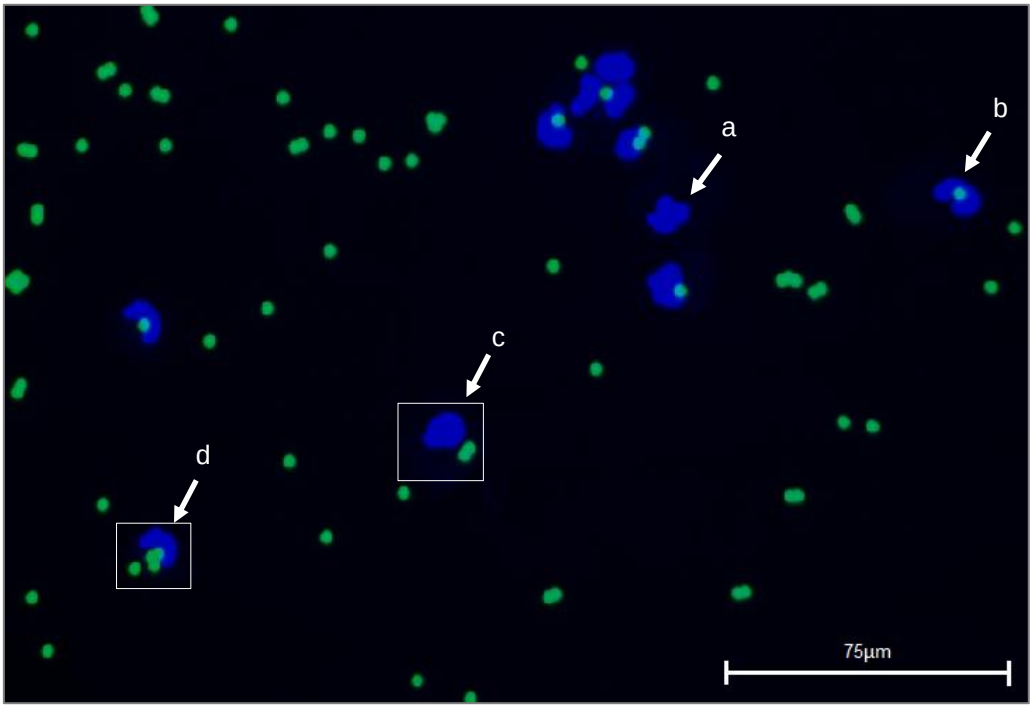

Figure 11: Figure representing fluorescent beads (green) phagocytosis (arrows). a) 0 phagocytic fluorescent beads; b) 1 phagocytic fluorescent beads; c) 2 phagocytic fluorescent beads; d) 4 phagocytic fluorescent beads. Nuclei marked with DAPI (blue). Squares represent limits of the cell.

3.3.4. Respiratory Burst

As for the respiratory burst, there was a significant interaction between the two studied factors (Two-way ANOVA on ranks, $F_{(8,137)} = 2.98$; $p < 0.05$). In general, according to figure 12, it is clear an increase in the formation of reactive oxygen species (ROS) with exposure to longer periods of higher temperature (i.e. from control to P2), contrarily to the phagocytosis response. This increment was evident from T0 to the remaining sampling times.

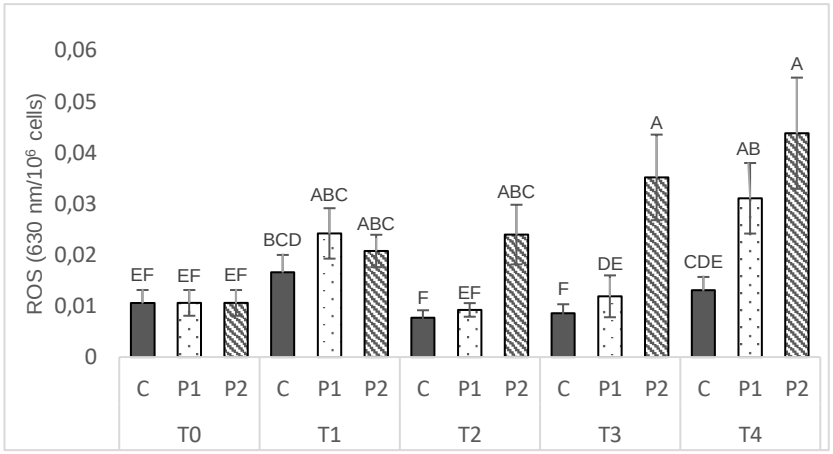

Figure 12: Respiratory burst of *S. plana* haemocytes in the different treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E. Different letters represent significant differences among groups.

3.3.5. Total protein content

Regarding total protein content in plasma, no significant differences among temperature treatments and sampling times were detected, being the response very homogeneous among groups ($p > 0.05$).

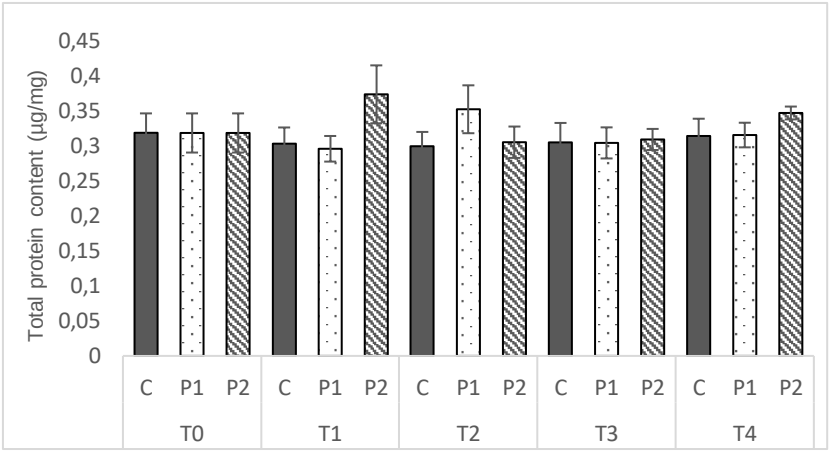

Figure 13: Total protein content (µg/mg) of *S. plana* plasma in the different treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E.

3.3.6. Protease activity

For protease activity, significant differences were observed among sampling times (Two-way ANOVA on ranks, $F_{(4, 128)} = 5.91$, $p < 0.05$) with a tendency to decrease with time. The temperature had no influence on protease response ($p > 0.05$).

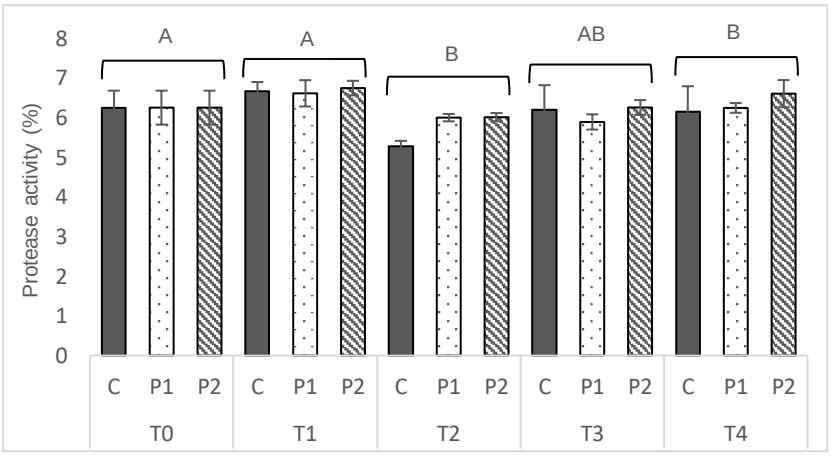

Figure 14: Protease activity (%) of *S. plana* plasma in the different treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E. Different letters represent significant differences among times.

3.3.7 Nitric Oxide

Regarding nitric oxide (NO) concentration on plasma, a significant interaction between temperature and time (Two-way ANOVA on ranks, $F_{(8,140)} = 3.14$; $p < 0.05$) was observed. As shown in figure 15, there was an increase in NO concentration on time T1 but declined again in the following times.

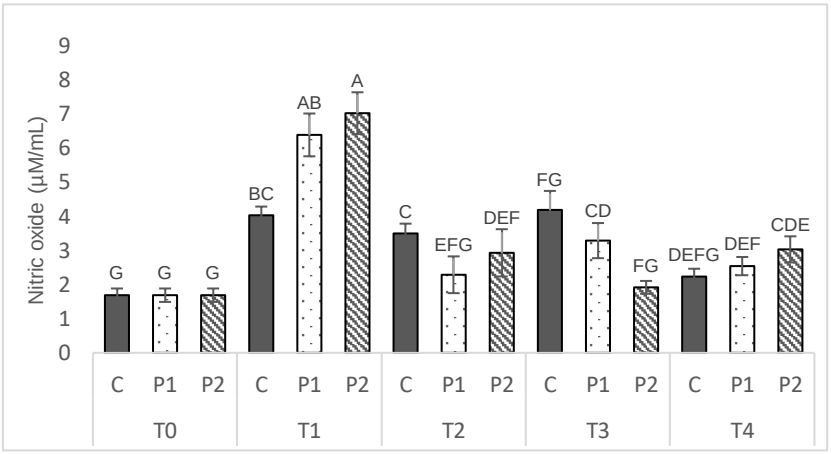

Figure 15: Nitric Oxide (µM/mL) of *S. plana* plasma in the different treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E. Different letters represent significant differences among groups.

3.3.8. Bactericidal activity

Regarding bactericidal activity in plasma, no significant differences among temperature treatments and sampling times were observed, being the response very homogeneous among groups ($p > 0.05$)

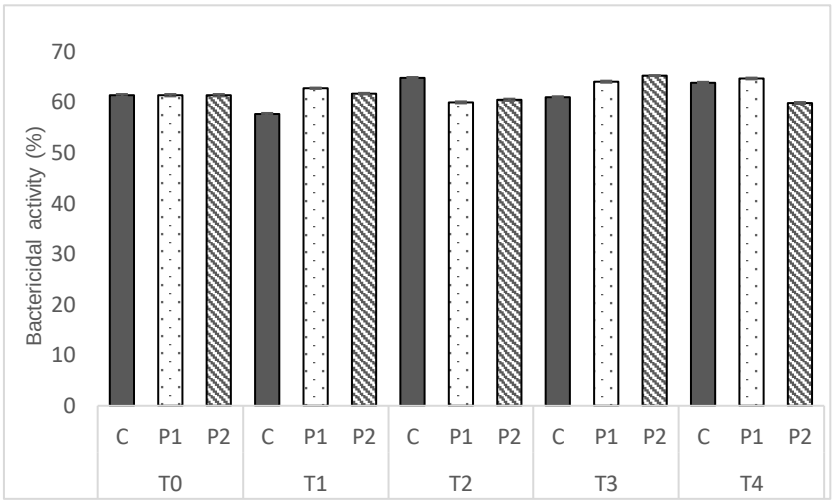

Figure 16: Bactericidal activity (%) of *S. plana* plasma in the different treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E.

3.4. Oxidative stress analyses

3.4.1. MDA concentration

Considering MDA production, there were significant differences among temperature treatments (Two-way ANOVA on ranks, $F_{(2,150)} = 3.25$; $p < 0.05$) and sampling times ($F_{(4,150)} = 9.16$; $p < 0.05$). No significant interaction between factors was observed ($p > 0.05$). Concerning temperature treatments, the MDA production was slightly lower in P1 than control but equal to P2. Regarding sampling times, there was a clear increase of cellular damage at T4 compared to the remaining ones (i.e. T1 to T3).

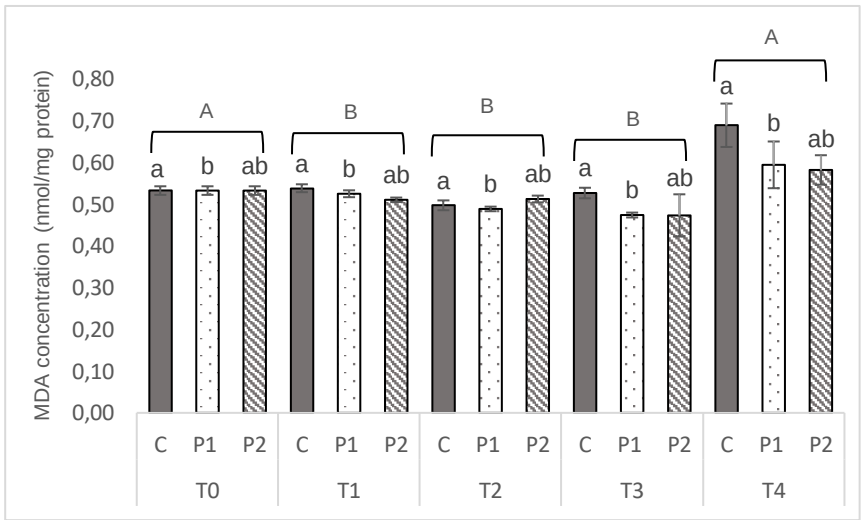

Figure 17: MDA concentrations (nmol/mg protein) on *S. plana* haemocytes exposed to different treatments. Values represent mean ± S.E. Different capital letters represent significant differences among sampling times. And different small letters represent significant differences among treatments.

3.4.2. Antioxidant enzymes

Superoxide dismutase (SOD) activity also showed a significant interaction between treatments and sampling times (Two-way ANOVA on ranks$F_{(8,138)} = 3.10$; $p < 0.05$). There was a clear increase in SOD activity from T0 to T2 and then a decline (figure 18).

Regarding catalase (CAT) activity (Figure 19), there was a significant difference among sampling times (Two-way ANOVA on ranks, $F_{(4,138)} = 15.42$; $p < 0.05$). No significant differences among temperature treatments were observed ($p > 0.05$).

Glutathione-S-Transferase (GST) activity showed a significant interaction between treatments and time (Two-Way ANOVA, $F_{(8,153)} = 2.17$; $P < 0.05$). According to figure 20, there was an evident decrease in GST activity with time. In general, a significant decrease among T0 and the remaining sampling times was observed. However, no significant differences among treatments were observed ($p > 0.05$).

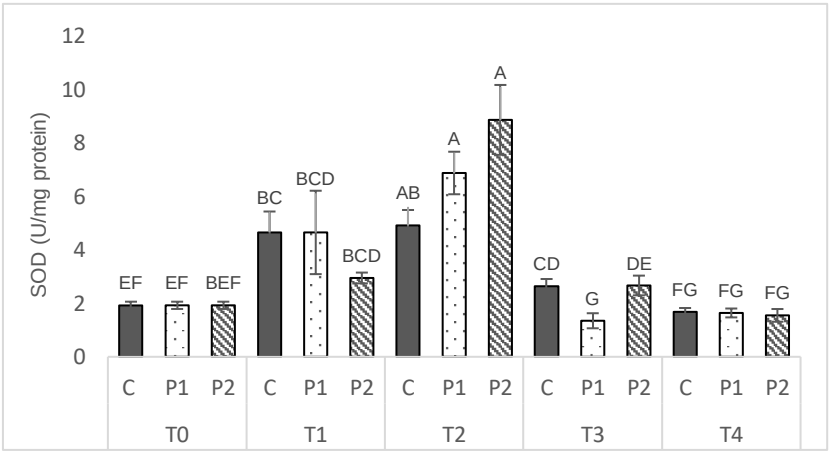

Figure 18: SOD activity (U/mg protein) of *S. plana* exposed to different temperature treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E. Different letters represent significant differences among groups.

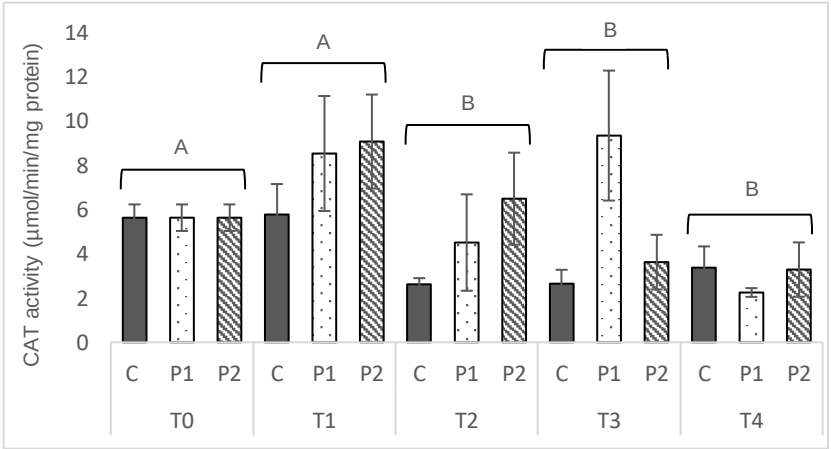

Figure 19: CAT activity (µmol/min/mg protein) of *S. plana* exposed to different temperature treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E. Different letters represent significant differences among sampling times.

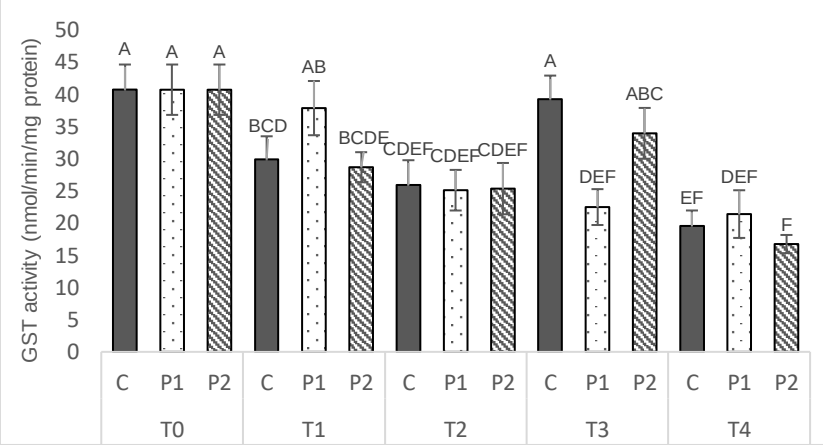

Figure 20: GST activity (nmol/min/mg protein) of *S. plana* exposed to different temperature treatments (C, P1 and P2) at different sampling times (T0, T1, T2, T3 and T4). Values represent mean ± S.E. Different letters represent significant differences among groups.

4. Discussion

In estuarine environments, abnormal temperature increases may affect immune systems and alter the chemical and enzymatic reactions of organisms, particularly in bivalves (for example, Rahman *et al.,* 2019; Matoo *et al.,* 2013). To better understand this effect, we evaluated the immunological and oxidative stress responses of *S. plana*, to distinct heatwaves with different exposure and recovery times.

Our data revealed that bivalve's survival was affected over time. In both control and heatwaves treatments, there was a decline in survival at intermediate sampling times (i.e. T2 and T3), due to factors not related to temperature, but probably due to the laboratory conditions that they were subject to (e.g. limited space in the flasks, handling). However, at T4 it appears that bivalves adapted to those conditions, with stabilization (C and P2) or even increased survival rate in P1. These low mortality values can be explained by *S. plana*'s high thermal window and therefore, greater tolerance to temperature variations. According to Verdelhos *et al.,* (2015), this bivalve, also collected in the Mondego estuary, has an optimal temperature between 15ºC and 23ºC with a LT_{50} of approximately 29ºC. Moreover, Verdelhos *et al.,* (2014) indicated that this species, in the Mondego Estuary, supports temperature oscillations that can vary between 10ºC and 25ºC in years without incidence of a heatwave.

The condition index (CI) results are in agreement with the survival rate, since we concluded that temperature had no influence on *S. plana* fitness. However, the CI increased during the experimental time. The same was observed in oysters, *Crassostrea virginica*, when exposed to temperatures between 20ºC and 24ºC (Lannig *et al.,* 2006; Chu and La Peyre, 1993). However, with higher temperatures (28ºC), the CI decreases significantly (Lannig *et al.,* 2006). This may indicate that the organisms were probably within optimal thermal values at 24ºC, as in our study.

Thus, these data suggest that *S. plana* tolerates quite well the exposure to the studied heatwaves.

4.1. Immune response

Bivalves, including *S. plana*, when exposed to a pathogen initiate certain defence mechanisms. However, there is evidence that temperature has a strong impact on bivalve's immune response (for example, Rahman *et al.,* 2019). The first line of defence are the haemocytes (Soudant *et al.,* 2013) and it was observed, in this study, that temperature influenced the viability of haemocytes with a significant decrease in

temperature treatments compared to controls, with no signs of recovery. The same results were obtained in Hégaret *et al.,* (2003) and Cherkasov *et al.,* (2007) with an increase in the mortality rate of haemocytes in the oyster *Crassostrea virginica* after a sudden rise of 8ºC (20ºC to 28ºC). The same results were observed in clams and mussels. For example, in *Ruditapes philippinarum* (Paillard *et al.,* 2004), *Mercenaria mercenaria* (Perrigault *et al.,* 2011) and the mussel *Mytilus coruscus* (Wu *et al.,* 2016). This decrease in viability is due to apoptotic processes (Goedken and De Guise, 2004). According to Hégaret *et al.,* (2003), it is normal to have a low percentage of unviable cells (below 5%), that are usually recycled by other haemocytes, reducing their accumulation in the haemolymph. However, temperature may result in the death of more haemocytes, as in the present study, and therefore compromise their removal. On the other hand, if the ROS production/antioxidant ratio was high, it may lead to haemocyte apoptosis (Wu *et al.,* 2016). All this shows that temperature can influence the viability of haemocytes and therefore, compromise the host defence of pathogens. However, no differences were observed between P1 and P2.

Total haemocytes count (THC) may be affected by several factors such as gender, age, reproductive period (for example, Husmann *et al.,* 2014; Dang *et al.,* 2012; Taskinen *et al.,* 1995) and, also by salinity, temperature or contaminants (for example, Matozzo *et al.,* 2007; Matozzo and Marin, 2011). According to Matozzo *et al.,* (2012), low or high THC values on haemolymph may be due to displacement of haemocytes to cells or haemolymph, respectively. In the present study, THC was significantly affected by temperature during the experiment with a tendency to decrease mainly in the last sampling times (i.e. T2 to T4) showing no recovery after exposure, with no significant differences between P1 and P2. Similar results were observed in other marine organisms; for example, in the clam *Chamelea gallina* with an increase of temperature of 20ºC to 25ºC, (Monari *et al.,* 2007) and in the mussel *Mytilus coruscus* with an increase of 25ºC to 30ºC (Wu *et al.,* 2016). It was also demonstrated by Lin *et al.,* (2012) cited by Wu *et al.,* (2016) that values may vary seasonally, with higher THC values observed during favourable temperatures and higher food availability and lower values in summer and early autumn (period with higher water temperatures). However, there are studies that indicate the opposite, with an increase on THC values with temperature rising. For example, for the clam *Chamelea gallina*, when exposed to a temperature increase of 6°C (22ºC to 28°C) (Matozzo *et al.,* 2012) or 5°C (25ºC to 30°C) (Monari *et al.,* 2007) was reported an increase of haemocytes.

Phagocytosis is the first line of defence of bivalves against external agents (Soudant *et al.,* 2013). In the present study, the temperature decreased the phagocytic

activity, always being lower than in the control. No significant differences were reported between P1 and P2, except in the last sampling time that demonstrates a significant recovery in P1, which is not observed in P2 (exposure to a longer heatwave and only 7 days of recovery) with a decrease of phagocytic activity. The decrease of phagocytosis with elevated temperature was observed by Wu *et al.,* (2016), which obtained a negative correlation between phagocytosis and temperature, with an increase of 5ºC (20ºC to 25º) over 14 days in the mussel *Mytilus coruscus.* Similarly, Hégaret *et al.* (2003) reported a decrease in phagocytosis activity with temperature increase of 8ºC (20ºC to 28ºC) for one weak in the eastern oyster *Crassostrea virginica* (20ºC to 28ºC) and also, Monari *et al.* (2007) observed a continuous decrease when temperature increased from 20ºC to 25ºC and from 25ºC to 30ºC in the clam *Chamelea gallina.* According to Alverez *et al.,* (1989) cited by Monari *et al.,* (2007), these results are due to thermal stress in haemocytes resulting in less responsive phagocytosis. On the other hand, there are other studies indicating that temperature positively influences phagocytosis (for example, abstract of Foley and Cheng, 1975; Gagnaire *et al.,* 2006). However, our study indicated that temperature had a strong negative influence on phagocytic activity. But, as already mentioned, *S. plana* appears to be able to recover by increasing its phagocytic response following exposure to a short-term heatwave and longer recovery.

Finally, in this study, it was observed a tendency to an increase of ROS production over time, principally in P2 compared to control. Such results were observed in study of Rahman *et al.,* (2019) with the oyster *Crassostrea gigas*, the mussel *Mytilus galloprovincialis* and the mud cockle *Katelysia rhytiphor.* All species had a continuous increase of ROS production with temperature rise from 15ºC to 20ºC and to 25°C (Rahman *et al.,* 2019). Another study from Wu *et al.,* (2016), obtained similar results for the *Mytilus coruscus*, with a significant increase of ROS only after 1 day, maintaining the same results for the next 14 days, with a temperature rise from 25ºC to 30ºC. These results can be explained by the improvement of some enzymatic activities, important in the production of ROS, with the increase of temperature.

Furthermore, there is often a relationship between phagocytosis and ROS in order to improve the effectiveness of pathogen destruction (Cajaraville *et al.,* 1995); which is verified, for example, in the study of Rahman *et al.,* (2019). In the present study, this relationship is not verified. In here, when phagocytic capacity decreases, there is a tendency to ROS increase over time, mainly comparing control with P2. However, despite the decrease of phagocytic capacity, the increase of ROS may help host defence against pathogen. On the other hand, ROS can be harmful to the haemocytes because if the cell does not produce sufficient amount of antioxidants enzymes to protect against

these reactive species, it may be damaged or even occur cell apoptosis (Wu *et al.,* 2006; Torreilles *et al.,* 1996).

Humoral factors present in plasma are also an important host defence against pathogens (Soudant *et al.,* 2013; Fernandez-Boo *et al.,* 2018). In the present study, it was evaluated the amount of total protein on *S. plana* plasma. There were no significant differences among treatments or sampling times. However, protease activity was significantly different over time, with a tendency to decrease. Protease is an important enzymatic protein capable to destroy the membrane of pathogens leading to cellular apoptosis (Canicatii, 1991 cited from Fernandez-Boo *et al.,* 2018) and, in this way, decreasing bactericidal activity. In our work, temperature had no influence on protease activity meaning that the organism can reduce the susceptibility to an infection. In the literature, information on the effects on these parameters is scarce. However, our results can be explained by the fact that the temperature does not exceed the optimal thermal limit of *S. plana* (Verdelhos *et al.,* 2014). Bactericidal activity of *S. plana* plasma is in agreement with these results. No significant differences were found between treatments with a very homogeneous response over time.

As for nitric oxide (NO), it was observed an increase at T1 with a subsequent decrease in the remaining sampling times, showing high ability to adapt quickly even at high temperatures. As reported in the introduction, NO is produced and released by bivalve haemocytes and it is an important factor for the annihilation of pathogens (Tafalla *et al.,* 2003). Similar to the previous parameters, there is not much information about the effects of temperature on this parameter. However, according to Novas *et al.,* (2007), the release of NO in summer is higher compared to the winter in *Mytilus galloprovincialis*, indicating probably that temperature may have a positive correlation with NO production.

These results help to understand the influence of distinct heatwaves with different recovery times on certain immune responses of *S. plana*, especially in cellular immunity.

4.2. Oxidative stress

In this study, exposure of *S. plana* to temperature variations over a short or longer period of time, did not cause significant changes in MDA concentration. However, significant differences among sampling times were reported, with a decrease in intermediates sampling times and an increase in the last one. A study that obtained the same results was with *Crassostrea virginica* (Matoo *et al.,* 2013), which showed no significant differences with temperature increase but, exactly like ours, there was an increase in MDA during the experimental time (Matoo *et al.,* 2013). These results may be due to some stress that organisms were exposed to during the experiment,

associated to handling or host conditions. On the other hand, there are several studies that indicate that temperature influences lipid peroxidation. For example, Matoo *et al.,* (2013) demonstrated that in the clam *Mercenaria mercenaria* after 15 days of exposure to higher temperatures (28°C), there was an increase in MDA concentration and, consequently, perhaps cellular damage. The same results were obtained by Verlecar *et al.,* (2007) in the digestive gland and in gills with an increase of 6ºC (26ºC to 32ºC) in *Perna viridis* and by Matozzo *et al.,* (2013), with temperature increase from 22ºC to 28ºC in the clam *Chamelea gallina*.

Regarding SOD and CAT, which are responsible for the removal of ROS, they can help to understand better the results of MDA concentration because they are mainly responsible for the damage of cell membranes.

For SOD activity, the first line of defence, it was found a significant increase from T0 to T1, which can be explained by the temperature rise, which continues to increase significantly until T2. From this sampling time, there is a decline in SOD activity. Explicitly, regardless of the time of a heatwave (7 or 14 days), *S. plana* can adapt to new conditions in 7 days. In Verlecar *et al.,* (2007), the same results were observed (study explain above). In *Perna viridis*, SOD activity increased significantly after 7 days of exposure to high temperature. However, after 14 days of the beginning of the experiment its activity decreased in both tissues analysed (gills and digestive gland) (Verlecar *et al.,* 2007). On the other hand, in the study from Matozzo *et al.,* (2013), the temperature had no influence on SOD activity, either in clam *Chamelea gallina* or in mussel *Mytilus galloprovincialis*.

In general, in the present study, results seem to demonstrate an adaptation of the organisms to the external conditions, in line with some results observed for the immune responses.

CAT is also an important antioxidant enzyme whose function is to remove H_2O_2, product of SOD activity, and one of some factors responsible for cellular damage (Lesser, 2006). In the present study, CAT activity varied among sampling times without significant differences among temperature treatments. In general, it appears to occur a decline in catalase activity from T1, in accordance with some of our results. However, several studies indicate that temperature increases its activity. In Khessiba *et al.,* (2010) study, there was a significant rise at 25°C in *Mytilus galloprovincialis*. The study consisted of increasing the temperature from 10ºC to 15ºC and 25ºC. Further studies demonstrate that temperature has the ability to increase catalase activity (Velercar *et al.,* 2007; Matozzo *et al.,* 2013; Wang *et al.,* 2018).

Finally, regarding GST activity, there was an evident decrease over time. In our results, it was observed a significant decrease among T0 and the remaining sampling

times. As a result of this decrease, it may lead to increased cell damage. The indifference to temperature increase is in agreement with the study by Matozzo *et al.,* (2013), where there were no significant differences in any of the analysed tissues (gills and digestive gland). On the other hand, Verlecar *et al.,* (2007) demonstrated that the activity of this antioxidant enzyme increased significantly after 14 days of exposure in *Perna viridis*.

5. Conclusion

Stochastic events, like heatwaves, are becoming more and more frequent and intense on Earth, however their effects have been insufficiently studied. The present study breakthrough the state of the art and allowed us to better understand the response of the marine bivalve *S. plana* during and after two distinct heatwaves, which permitted us to have some insights about their impact on marine ecosystems.

Our study has shown that temperature can play an important role on this species, especially in cellular immune system. In general, although in most endpoints no differences were observed between the two studied heatwaves, as regards, for example, phagocytosis, it was observed that in P2 the recovery capacity was lower than P1. Thus, it seems to occur a tendency for longer heatwaves with shorter recovery times to be more harmful to organisms, such as bivalves, than shorter ones.

In the future, it is important to continue this work, considering other heatwaves patterns, slightly exceeding the upper thermal optimum limit of *S. plana*, to better understand the effects of temperature variations on the immune system and oxidative stress responses. Afterwards, studies should focus on the effects of long-term heatwaves (more than two weeks), because the tendency is to increase the duration and frequency and also explore these studies with other species too.

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7. Attachments

Table 2: Results of two-way ANOVA evaluating the effects of temperature treatment (C, P1 and P2) during time (T0, T1, T2, T3, T4) on the Condition index (CI), immune and oxidative stress responses of the *S. plana*

	Df	MS	F	p
CI (Two-way ANOVA on ranks)				
Treatment (T)	2	4513	2.37	0.97
Time (t)	4	31006	16.19	0.00
T x t	8	3535	1.86	0.07
Error	163	1903		
Viability of haemocytes (Two-way ANOVA on ranks)				
Treatment (T)	2	43290	33.50	0.00
Time (t)	4	8257	6.39	0.00
T x t	8	5750	4.45	0.00
Error	148	1293		
THC (Two-way ANOVA)				
Treatment (T)	2	1.69	10.76	0.00
Time (t)	4	1.26	7.98	0.00
T x t	8	0.55	3.52	0.00
Error	225	0,16		
PI (Two-way ANOVA)				
Treatment (T)	2	30952	35.38	0.00
Time (t)	4	88421	101.07	0.00
T x t	8	6815	7.79	0.00
Error	179			
Respiratory burst (Two-way ANOVA on ranks)				
Treatment (T)	2	2164.3	17.84	0.00
Time (t)	4	15060.4	12.41	0.00
T x t	8	3617.9	2.98	0.00
Error	137	1213.8		
Total protein content (Two-way ANOVA)				
Treatment (T)	2	0.006	0.934	0.40
Time (t)	4	0.002	0,253	0.90
T x t	8	0.006	0,901	0.52
Error	148	0.007		
Protease activity (Two-way ANOVA on ranks)				
Treatment (T)	2	952.6	0.63	0.54
Time (t)	4	8961.9	5.91	0.00
T x t	8	1172	0.77	0.63
Error	128	1516.2		

Two-way ANOVA on ranks

	Df	MS	F	p
NO				
Treatment (T)	2	255.7	0.26	0.77
Time (t)	4	26251.9	37.09	0.00
T x t	8	3081.8	3.14	0.00
Error	140	980,1		

Two-way ANOVA

Bactericidal activity				
Treatment (T)	2	568	0.28	0.76
Time (t)	4	2411	1.19	0.31
T x t	8	3264	1.61	0.13
Error	143	2033		

Two-way ANOVA on ranks

LPO				
Treatment (T)	2	5914	3.25	0.04
Time (t)	4	16615	9.13	0.00
T x t	8	3100	1.70	0.10
Error	150	1821		
SOD				
Treatment (T)	2	1132.6	1,72	0.18
Time (t)	4	43406.6	56.11	0.00
T x t	8	1294.5	3.10	0.00
Error	138	773.6		
CAT				
Treatment (T)	2	2865	2.12	0.12
Time (t)	4	20739	15.42	0.00
T x t	8	2119.7	1.58	0.14
Error	138	1345		

Two-way ANOVA

GST				
Treatment (T)	2	78	0.60	0.55
Time (t)	4	2213.9	17.06	0.00
T x t	8	281.5	2.17	0.03
Error	153	129.8		