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Ano 2019

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Thermal tolerance and acclimation capacity of the ragworm *Hediste diversicolor* under global change scenarios

Tolerância térmica e capacidade de aclimatação da serradela *Hediste diversicolor* às alterações globais

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica da Doutora Diana Sofia Gusmão Coito Madeira, Investigadora em Pós-doutoramento do Departamento de Biologia da Universidade de Aveiro, e do co-orientador Doutor Ricardo Jorge Guerra Calado, Investigador Principal do Departamento de Biologia da Universidade de Aveiro.

Dedico este trabalho à minha pequenina Letícia.
Da titi que te adora.

o júri

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palavras-chave Múltiplos fatores de stress, poliqueta, limites térmicos, fisiologia, biomarcadores, alocação de energia

resumo

As projeções climáticas preveem mudanças significativas na química do oceano, variações de salinidade, assim como um aumento na sua temperatura até 2100. Isso levou a um interesse substancial no estudo da ecofisiologia térmica, pois a temperatura é um fator importante para moldar as comunidades de organismos marinhos. Considerando que a maioria das espécies marinhas são ectotérmicas e, portanto, não podem regular a temperatura do seu corpo, poderão ocorrer alterações bioquímicas com as variações de temperatura no ambiente. Além disso, as variações da salinidade também podem induzir alterações fisiológicas e metabólicas nos organismos marinhos. Neste estudo, avaliamos experimentalmente as respostas fisiológicas e moleculares do poliqueta *Hediste diversicolor* sob cenários previstos de alterações globais. Os organismos foram amostrados da zona intertidal da Ria de Aveiro (Portugal) e submetidos a um ensaio experimental em condições de controlo (24 °C), e dois cenários de tratamento (aquecimento do oceano +3 ºC - 27 °C e onda de calor +6 ºC - 30 °C) previsto pelo Painel Intergovernamental para as Alterações Climáticas, combinado com variações de salinidade (20 e 30) de forma fatorial. Os dados ambientais (temperatura, salinidade e pH) foram monitorizados durante 60 dias. Os organismos foram submetidos a um ensaio experimental de aclimatação durante 29 dias e, após 14 e 28 dias de aclimatação (D14 e D28, respetivamente), os indivíduos foram amostrados, para quantificação de: biomarcadores moleculares (proteína de choque térmico 70kDa, ubiquitina, atividade de catálase, glutationa-S-transferase, e superóxido dismutase, capacidade antioxidante total, peroxidação lipídica e glicose), perfis de ácidos gordos e as suas respetivas classes e reservas energéticas (proteína total, lípido total e conteúdo de glicogénio). No dia 30, foram medidos os limites térmicos (máximo térmico crítico - CT_{Max}), margens de segurança térmica (TSM) e capacidade de aclimatação. Os dados ambientais *in situ* revelaram que a espécie *H. diversicolor* está sujeita a variações de temperatura amplas, sendo que a salinidade não apresentou variações consideráveis durante o período de amostragem. As temperaturas mais altas de aclimatação promoveram limites de tolerância térmica mais altos, confirmando que *H. diversicolor* possui alguma plasticidade fisiológica, capacidade de aclimatação e uma margem de segurança térmica positiva (CT_{Max} > temperatura máxima do habitat - MHT). Esta plasticidade ajuda os organismos a serem mais tolerantes a uma mudança repentina na temperatura da água. Para os biomarcadores, a interação significativa dos três fatores (temperatura, salinidade e dia) indica que os efeitos de cada fator estão dependentes dos níveis dos outros fatores. Particularmente, as interações significativas entre temperatura, salinidade e dia foram registadas para a Hsp70, ubiquitina e catálase. Todos os biomarcadores, exceto a ubiquitina, foram significativos no fator dia. Os biomarcadores que mais contribuíram para a separação dos tratamentos experimentais foram a glicose, glutationa S-transferase, ubiquitina, Hsp70 e catálase, sendo que as diferenças entre temperaturas foram mais evidentes e prolongadas quando combinadas com baixa salinidade. Este resultado sugere que a glicose foi mobilizada para produzir energia para as defesas celulares, sendo que as enzimas antioxidantes, a ubiquitina e proteína de choque térmico desempenharam um papel importante durante o stress térmico e hipo-osmótico, protegendo as células dos efeitos tóxicos das espécies reativas de oxigénio (ROS) e, controlando a integridade das proteínas, permitindo um adequado funcionamento celular. Relativamente aos perfis de ácidos gordos, o mais abundante dos saturados (SFA) foi o ácido palmítico (16:0), nos monoinsaturados (MUFA) foi o ácido vacênico (18:1*n*-7), nos polinsaturados (PUFA) o ácido linoléico (18:2*n*-6) e o ácido eicosapentaenóico (20:5*n*-3) nos altamente insaturados (HUFA). Observou-se um aumento nas várias classes de ácidos gordos, assim como nos ∑*n*-3 PUFA, ∑*n*-6 PUFA e ácidos gordos essenciais ao longo do tempo nos poliquetas expostos a salinidade 30, ao contrário do que se observou na salinidade 20. Estes resultados sugerem que a salinidade 30 representa uma condição ambiental preferencial para a espécie *H. diversicolor*, favorecendo a sua condição fisiológica. A temperatura também influenciou significativamente o perfil de ácidos gordos, interagindo com a salinidade. Aos 14 dias de exposição, em condições preferenciais de salinidade (30), verificou-se um aumento nos HUFA dos 24 ºC para os 27 ºC, seguido de um decréscimo aos 30 ºC. Os ácidos gordos ómega 3 e 6 mantiveram-se estáveis a 24 ºC e 27 ºC, sendo detetado um decréscimo a 30 ºC. Estas diminuições poderão estar relacionadas com alterações na composição lipídica das membranas celulares, de forma a manter

a homeostasia. Aos 28 dias não se verificaram diferenças, sugerindo que os poliquetas terão conseguido aclimatar-se às temperaturas elevadas quando estão em condições preferenciais de salinidade. Um aumento de temperatura em condições de baixa salinidade (20) promove alterações mais pronunciadas no perfil de ácidos gordos, tendo sido registado um aumento em todas as classes destas moléculas após 14 dias de exposição a 27 ºC e 30 ºC, quando comparado com o controlo (24 ºC). Aos 28 dias, esta diferença é apenas percetível nos HUFA. No geral, quando os poliquetas são cultivados a baixa salinidade, um aumento moderado de temperatura leva a que os ácidos gordos ∑*n*-3 PUFA, ∑*n*-6 PUFA, ARA, EPA e DHA aumentem. No entanto, se o aumento de temperatura for mais extremo (30 ºC), as concentrações destes ácidos gordos diminuem, seguindo o padrão esperado. Estes resultados confirmam a importância dos ácidos gordos na provisão de energia (especialmente os saturados), assim como no balanço osmótico e fluidez da membrana (especialmente os insaturados). As reservas de energia, nomeadamente os lípidos e o glicogénio não foram significativamente afetadas pelo tempo de exposição aos diferentes tratamentos experimentais. A disponibilidade de glicose para obtenção de energia, evitou a degradação do hidrato de carbono, sendo assim possível os poliquetas manterem as suas reservas energéticas. No entanto, foi verificado um aumento de proteína total ao longo do tempo nos tratamentos a 27 ºC, em ambas as salinidades, sugerindo um crescimento dos poliquetas nessa temperatura durante o mês do ensaio experimental. Verificou-se também uma diminuição dos lípidos totais a 27 ºC, potencialmente devido a uma mobilização dos lípidos para providenciar energia para a síntese de proteínas. Em conclusão, pode afirmar-se que a poliqueta *H. diversicolor* pode aclimatar-se facilmente ao aumento da temperatura da água e a variações de salinidade. Mesmo quando os organismos estão adaptados para tolerar esses ambientes extremos e as populações são capazes de persistir, a típica variabilidade ambiental dos estuários é potencialmente stressante para os animais que habitam essas condições, levando a que certos mecanismos moleculares sejam ativados para manter a homeostasia. Futuramente será necessário desenvolver ensaios experimentais mais longos que, incluam todas as etapas do ciclo de vida deste poliqueta e, contemplem igualmente os aspetos transgeracionais, assim como é necessária a integração dos aspetos fisiológicos na modelação ecológica de modo a permitir detetar as fases de desenvolvimento mais vulneráveis, assim como alterações em níveis mais altos de complexidade biológica e numa escala evolutiva.

keywords Multiple stressors, polychaete, thermal limits, physiology, biomarkers, energy allocation

abstract

Climate projections predict significant changes in ocean chemistry, salinity variations and the increase in ocean temperature by 2100. This has led to a substantial interest in the study of thermal ecophysiology, as temperature is a major factor shaping marine communities. Considering that most marine species are ectotherms, and thus cannot regulate body temperature, biochemical changes can occur with temperature variations. Moreover, salinity fluctuations can also induce physiological and metabolic changes in marine organisms. In this study, we experimentally evaluated the physiological and molecular responses of the ragworm *Hediste diversicolor* under predicted global change scenarios. Organisms were collected from the intertidal zone in Ria de Aveiro (Portugal) and subjected to an experimental trial under control (24 °C), and two treatment scenarios (ocean warming +3 ºC - 27°C and heat wave +6 ºC - 30°C) predicted by the Intergovernmental Panel on Climate Change, combined with salinity variations (20 and 30) in full factorial design. Environmental data (temperature, salinity and pH were collected during 60 days in the summer. Organisms were subjected an experimental acclimation trial during 29 days, and after 14 and 28 days of acclimation (D14 and D28 respectively), individuals were sampled (*per* time point) for molecular biomarkers analyses (heat shock protein 70kDa, ubiquitin, catalase, glutathione-S-transferase, superoxide dismutase activity, total antioxidant capacity, lipid peroxidation and glucose), fatty acid profiles and energy reserves (total protein, total lipid and glycogen content). At day 30, upper thermal limits (Critical Thermal Maximum - CT_{Max}), thermal safety margins (TSM) and acclimation capacity were measured. *In situ* data collection, showed that *H. diversicolor* experiences wide temperature variations, but salinity showed no considerable variations during the sampling period. Higher acclimation temperatures led to higher thermal tolerance limits, confirming that *H. diversicolor* has some physiological plasticity, acclimation capacity and a positive thermal safety margin (CT_{Max} > maximum habitat temperature – MHT). For biomarkers, the significant interaction of the three factors (temperature, salinity and day) indicates that the effect of one factor depends on the levels of the others factors. Particularly, significant temperature-salinity-time interactions were recorded for Hsp70, Ub and CAT, and all biomarkers except Ub were significant in factor day. The biomarkers that contributed most to the separation of the experimental treatments were glucose, glutathione S-transferase, ubiquitin, Hsp70 and catalase, and the differences between temperatures were more evident and prolonged when combined with low salinity. This suggests that glucose was mobilized to produce energy for cellular defenses, and antioxidant enzymes, ubiquitin and heat shock protein played an important role during hypo-osmotic and thermal stress, protecting cells from the toxic effects of reactive oxygen species (ROS) and managing the integrity of the protein pool, thereby allowing the proper management of cellular functioning. For the fatty acids (FA), the major saturated fatty acid (SFA) was palmitic acid (16:0), the dominant monounsaturated fatty acid (MUFA) was vaccenic acid (18:1*n*-7), in the polyunsaturated fatty acids (PUFA) the most abundant FA was linoleic acid (18:2*n*-6) and eicosapentaenoic acid (EPA) (20:5*n*-3) was the FA with the highest value between all of the highly unsaturated fatty acids (HUFA). An increase in the different fatty acid classes was observed, as well as in ∑*n*-3 PUFA, ∑*n*-6 PUFA and in essential fatty acids over time in polychaetes exposed to salinity 30, contrary to what was observed in salinity 20. These results suggest that salinity 30 is an optimal condition for *H. diversicolor*, favoring its physiological condition. Temperature also significantly influenced fatty acids, interacting with salinity. At 14 days of exposure, under optimal salinity conditions (30), there was an increase in HUFA from 24 °C to 27 °C, followed by a decrease at 30 °C. Omega 3 and 6 fatty acids remained stable at 24 ºC and 27 ºC, with a decrease detected at 30 ºC. These decreases may relate to changes in the lipid composition of cell membranes in order to maintain homeostasis. At 28 days there were no differences, suggesting that polychaetes can acclimate to elevated temperatures when they are in optimal salinity conditions. If the temperature increase occurs under low salinity conditions (20), changes in fatty acids are more pronounced, with an increase in all fatty acid classes after 14 days of exposure to 27 °C and 30 °C, comparing with the control (24 °C). At 28 days, the difference remains only in HUFA. In general, when polychaetes are grown at low salinity, a moderate increase in temperature causes ∑*n*-3 PUFA, ∑*n*-6 PUFA, ARA, EPA and DHA fatty acids to increase. However, if the temperature increase is more extreme (30 °C), the concentrations of these fatty acids decrease, following the expected pattern. These results indicate that fatty acids may be important in providing energy

(especially saturated ones) and may play an important role in osmotic balance and membrane fluidity (especially unsaturated ones).

Energy reserves, namely lipids and glycogen were not significantly affected by the time of exposure to treatments. Since the organisms had glucose available for energy, they did not need to degrade the carbohydrate, keeping their energetic reserves. However, there was an increase of total protein over time in the treatments at 27 ºC, in both salinities, suggesting some growth of the polychaetes under this temperature during the month of the experimental trial. There was also a decrease in total lipids at 27 °C, potentially due to lipid mobilization to provide energy for protein synthesis. The main conclusion is that *H. diversicolor* can easily acclimate to increased water temperature and salinity fluctuations. Even when organisms are adapted to tolerate these extreme environments and populations are able to persist, the environmental variability characteristic of estuaries is potentially stressful to animals inhabiting these conditions, leading to certain molecular mechanisms being activated to maintain homeostasis. Future studies on the current topic are therefore needed in order to evaluate the physiological plasticity of organisms to new global change conditions. Moreover, longer experimental trials are necessary, including all life-cycle stages, as well as trans-generational experiments and the integration of physiology into ecological modelling, in order to identify vulnerable life stages, and detect changes at higher levels of biological complexity and on an evolutionary scale.

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List of Abbreviations

A

ARR, acclimation response ratio

Abs, absorbance

ADP, adenosine diphosphate

ANOSIM, analysis of similarities

AOX, anti-oxidant

ATP, adenosine triphosphate

B

BSA, bovine serum albumin

C

CV, coefficient of variation

CAT, catalase

CDNB, chloro-2,4-dinitrobenzene

CI, confidence interval

CO_2, carbon dioxide

CSR, cellular stress response

CT_{Max}, Critical Thermal Maximum

D

DNA, deoxyribonucleic acid

DNPH, 2,4-dinitrophenylhydrazine

D14, 14 days of exposure

D28, 28 days of exposure

E

EU, European union

EC, Enzyme Nomenclature

EDTA, ethylenediaminetetraacetic acid

ELISA, Enzyme-Linked Immunosorbent Assay

Eq, equation

F

FA, fatty acid

FAME, fatty acid methyl ester

FAO, Food and Agriculture Organization of the United Nations

G

GST, glutathione-S-transferase

GC-FID, Gas Chromotography with Flame-Ionization Detection

H

HCl, hydrochloric acid

Hsp70, heat shock protein 70 kDa

Hsps, heat shock proteins

HUFA, highlyunsaturated fatty acid

I

IgG, immunoglobulin G

IPCC, Intergovernmental Panel on Climate Change

IUCN, International Union for Conservation of Nature and Natural Resources

K

kDa, kilodalton

KH_2PO_4, monopotassium phosphate

L

L:D, light:dark

LPO, lipid peroxidation

M

MHT, maximum habitat temperature

MALDI, matrix-assisted laser desorption/ionization

MDA, malondialdehyde bis(dimethylacetal)

MW, molecular weight

MUFA, monounsaturated fatty acid

N

Na_2HPO_4, disodium phosphate

NaCl, sodium chloride

NaOH, sodium hydroxide

NBT, nitroblue tetrazolium

P

p, p-value

PBS, phosphate-buffered saline

PERMANOVA, permutational analysis of variance

PCA, Principal Component Analysis

ppm, parts per million

PUFA, polyunsaturated fatty acid

R

ROS, Reactive Oxygen Species

S

S20, salinity value of 20

S30, salinity value of 30

SST, sea surface temperature

SD, standard deviation

SIMPER, similarity percentage analysis

SDS, sodium dodecyl sulphate

SOD, superoxide dismutase

SFA, saturated fatty acid

T

T24, temperature of 24 ºC

T27, temperature of 27 ºC

T30, temperature of 30 ºC

Tmin, minimum temperature

Tmean, mean temperature

Tmax, maximum temperature

Tcritical, critical temperature

$T_{end\text{-}point}$, temperature at which the end-point is reached

TAG, triacyglycerol

TSM, thermal safety margin

TAC, total antioxidant capacity

TCA, trichloroacetic acid

TBARS, thiobarbituric acid reactive substances

U

Ub, total ubiquitin

X

XOD, xanthine oxidase

W

WHO, World Health Organization

1. Introduction

1.1 Global changes – multiple environmental stressors

Climate projections predict significant changes in ocean chemistry, salinity variations and ocean temperature of the world's oceans by 2100 (Sala et al., 2000; IPCC, 2014; IPCC, 2019). Threats to marine habitats are increasing due to anthropogenic activities, with pronounced ecological consequences, such as shifts in species' abundances and geographical ranges (Sunday et al., 2012; Donelson et al., 2019). Additionally, changes in the strength and types of ecological interactions are expected, and in the worst case scenarios, populations' collapse and species extinctions (McCauley et al., 2015; Calosi et al., 2019).

One of the major obstacles to develop robust predictions about the consequences of climate change in marine habitats is the multifarious nature of environmental change (Côté et al., 2016; Gunderson et al., 2016). Depending on the location, organisms experience different changes, such as the increase of water temperatures (Harley et al., 2006) decrease of pH (Calosi et al., 2017), the increase of pollutants, e.g. heavy metals, changes in sea surface salinity (SSS) caused by altered freshwater inputs and decrease of oxygen availability (Pörtner, 2010). Until now, most scientific studies tested, under controlled laboratory experiments, how changes in a single environmental variable could affect organisms performances under changing conditions (Todgham and Stillman, 2013). However, the scientific community realized that single-stressor's experiments may not properly assess the effects of climate change in marine habitats, and the recent studies start to incorporate two or more environmental factors into their experimental designs, with the hope of generating better predictions about the effects of global change in marine communities. In fact, multiple stressor interactions are now considered a key topic for marine conservation and ecosystem management planning (Côté et al., 2016). For example, Richardson et al. (2019) tested how cyanobacteria and phytoplankton react to the combined effect of ocean warming, rainfall and nutrient loading and Moreno-Marín et al. (2018) tested how ocean warming, low light and nitrogen availability affect seagrasses.

Organisms exposed to multiple stressors can exhibit one out of three types of responses: additive, antagonistic, or synergistic (Byrne and Przeslawski, 2013;

Todgham and Stillman, 2013). An additive effect occurs when the combined effect of multiple stressors equals the sum of the effects of each stressor in isolation, whereas an antagonistic effect occurs when the combined effect of multiple stressors is less than the expected additive effect in isolation. A synergistic effect occurs when the combined effect of multiple stressors is greater than the expected additive effect of the stressors in isolation (Gunderson et al., 2016).

Indeed, interactive effects among different stressors are common, suggesting that the extrapolation of multi-stressor's effects from single-stressor's studies, will often lead to wrong conclusions (Alsterberg et al., 2014). Furthermore, synergistic responses appear to be the most common interactive effect observed in multi-stressor studies, suggesting that the exposure to different stressors at the same time will be extremely destructive to marine organisms under changing climatic conditions (Solan and Whiteley, 2016). In this context, multifunctional approaches are also important, integrating climate change impacts ate several levels of biological organization, from molecules to ecosystems (Madeira et al., 2018).

1.2 Temperature

The Intergovernmental Panel on Climate Change (IPCC), predicts that the sea surface temperature (SST) is increasing at a rate of 0.2 ºC per decade, leading to an increase of 2 to 4 ºC by the end of this century, depending on RCP (Representative Concentration Pathway) scenario (IPCC, 2014). Furthermore, extreme events such as heat waves, are predicted to increase in frequency, duration and intensity and are likely to have a bigger impact on ecosystems than gradual changes, as they can act as strong selective pressures (Grant et al., 2017; Pansch et al., 2018; Vinagre et al., 2018; Stillman, 2019). However, different effects in the ecosystems are expected depending on the spatial and seasonal context, i.e. location and season (Matthews et al., 2016).

Thermal stress during specific periods of the year associated with thermally sensitive life-history stages may have greater impacts than changes in mean or maximum temperatures (Vasseur et al., 2014).

Most marine organisms are ectothermic, which means that they cannot regulate their body temperature. Thus, the temperature of an organism – a quantitative measure of the kinetic energy of its molecules – constrains the rates of chemical reactions, namely its biochemical reactions (Wieser, 1973). Indeed, changes in the organism temperature influence its enzymatic activity, metabolic rate, and individual performance (e.g. body size, growth rate, reproductive output, immune competence) (Anestis et al., 2007; Pörtner, 2008; Madeira et al., 2014a; Aljbour et al., 2019; Johnstone et al., 2019). Furthermore, the organism's temperature can influence its associated ecological processes, e.g. biological interactions, influencing the population, and even the species dynamics, e.g. species distribution (Portner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner, 2010; Solan and Whiteley, 2016). For example, warming increased growth rates of juvenile cod *Gadhus morhua* on the Norwegian coast in spring but had the opposite effect in summer when thermal optima were exceeded (Rogers et al., 2011). Changes in species distribution may occur on different scales: (i) small scale, when organisms move to thermal refugia: within its own habitat, mostly happens in environments with steep temperature gradients, e.g. intertidal (Somero, 2011; Sunday et al., 2012; Donelson et al., 2019); (ii) large scale, when these distribution changes occur on a worldwide scale e.g. poleward movements in oceanic species (Harley et al., 2006; Kordas et al., 2011; Vasseur et al., 2014). These spatial or temporal distribution shifts will produce novel combinations of species in marine habitats or cause species drop-outs with consequences for community structure and functioning (Miller et al., 2018), including food-web topology (Zhang et al., 2017; Ullah et al., 2018). Indeed, Kordas et al. (2011) showed that marine organisms' distribution, abundance, and function depends not only on the direct effects of temperature but also on indirect effects, e.g. changes in the community by the immigration of new competitors, mutualists, predators, prey, or pathogens (Laffoley and Baxter, 2016). Moreover, if these changes affect important groups, such as keystone species or ecosystem engineers, the structure and functioning of the ecosystem can be deeply compromised (Solan and Whiteley, 2016), with potential impacts in economic activities such as fisheries and aquaculture, and consequently food-security (FAO, 2018).

1.2.1 Thermal tolerance

The thermal tolerance window is the favourable range of temperature or performance breadth of a given species (Madeira et al., 2012a; Velasco-Blanco et al., 2019). In fact, when the temperature is above or below this range the performance is negatively affected and species survival is at risk (Huey and Stevenson, 1979; Pörtner et al., 2017). Climate change increased the current interest in understanding the species' thermal limits, enabling to predict how species will react if this limit is overcome (Angilletta et al., 2002; Peck et al., 2014; Vinagre et al., 2016; Sunday et al., 2019).

Different experimental approaches are commonly used to determine the thermal tolerance of a species: 1) the determination of Lethal Temperature, i.e. the temperature that causes the mortality of 50% of the individuals in a sample, (Somero, 2011); and 2) the determination of the Critical Thermal Maximum (CT_{Max}), i.e. temperature that triggers the loss of motor function, e.g. loss of response and muscle spasms, and it is determined by gradually increasing the temperature until a critical point is reached (Mora and Ospína, 2001; Madeira et al., 2012a; Massamba-N'Siala et al., 2012). The second method is more used because it is easier to apply, requires smaller samples sizes, and is faster (Morgan et al., 2018). Furthermore, among ectothermic vertebrates and invertebrates, it is generally accepted that CT_{Max} is the most efficient index of upper thermal tolerance (Lutterschmidt and Hutchison, 1997). The CT_{Max} and the critical thermal minimum (CT_{Min}) are the upper and lower limits, respectively, of a species thermal tolerance (Fig.1.1). When the critical point is reached, CT_{Max} is comprehensively defined as: "The critical thermal maximum (or minimum) is the arithmetic mean of the collective thermal points at which locomotory activity becomes disorganized and the animal loses its ability to escape from conditions that will promptly lead to its death when heated from a previous acclimation temperature at a constant rate just fast enough to allow deep body temperatures to follow environmental temperatures without a significant time lag" (Scott, 1987).

Species from warmer (e.g. upper intertidal) or more stable (e.g. tropical) environments live closer to the upper acute thermal tolerance limit, being more susceptible to global warming (Tewksbury et al., 2008; Madeira et al., 2012a; Vinagre et al., 2016). Nevertheless, a considerable lack of knowledge about the thermal limits of marine organisms remain, especially in temperate species.

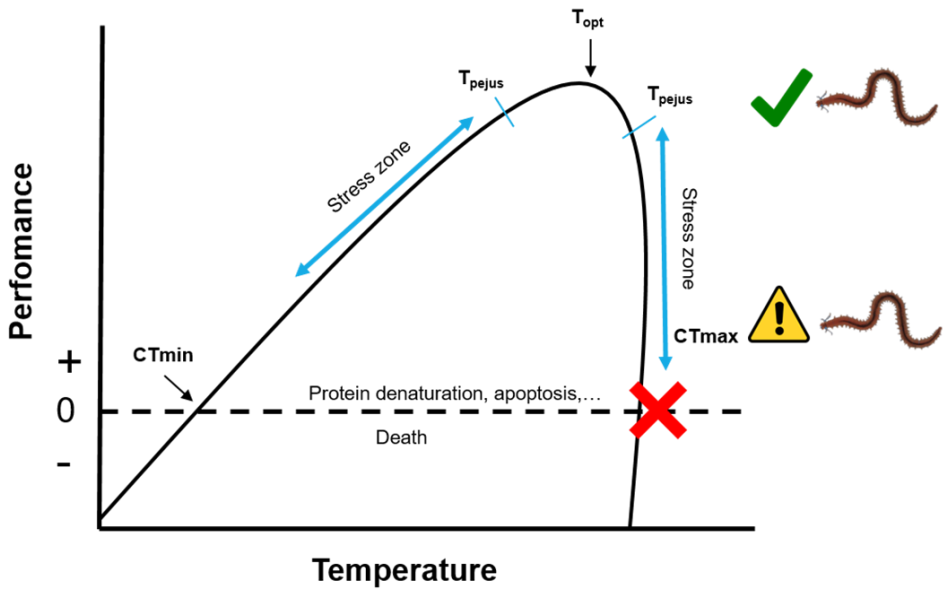

Figure 1.1 - Thermal window for animals: Critical thermal limits and acclimation under stress condition. Adapted from Pörtner (2012).

1.2.2 Thermal acclimation

Understanding if organisms can tolerate new thermal variations in their habitat and environment is only a small part of global change research. Assessing the capacity of organisms to acclimate and/or adapt to new temperatures, e.g. warmer waters, is crucial to understand the response of populations and communities to global warming.

Organisms can have different responses to the increasing temperatures: disperse towards favourable habitats, tolerate the new conditions through phenotypic and physiological plasticity, or adapt to the new conditions through genetic change via the process of evolution (Hofmann, 2005; Gibbin et al., 2017). Such adaptations can modify the organisms' behavioural, physiological or

morphological characteristics in response to increased environmental temperature. An important mechanism to cope with a changing environment is phenotypic plasticity. Regarding thermal acclimation, this phenotypic plasticity can be any phenotypic alteration in physiology in response to environmental temperature that alters organisms' performance, possibly improving their fitness (Chown et al., 2009; Massamba-N'Siala et al., 2014). These alterations can be quick or gradual, being reversible, and the organism can recover its homeostasis (Fig. 1.2). On the opposite, some phenotypic plasticity responses are non-reversible and remain throughout the organism's life cycle leading to carry over effects that determine fitness outcomes (Slotsbo et al., 2016; Moore and Martin, *in press*). However, since acclimation imposes energy costs in terms of survivorship or reproduction to an organism (Angilletta, 2009), this should be taken into consideration in species vulnerability assessments as it ultimately determines the success and viability of populations under global change. As oceans warm and heat waves expand (affect bigger areas at the same time) and become more frequent, intense and long-lasting (IPCC, 2014), temperate organisms will be facing both chronic and acute thermal stress. The potential effects on organismal performance can escalate if additional stressors, e.g. salinity variations, interact with temperature, potentially threatening the future of marine communities.

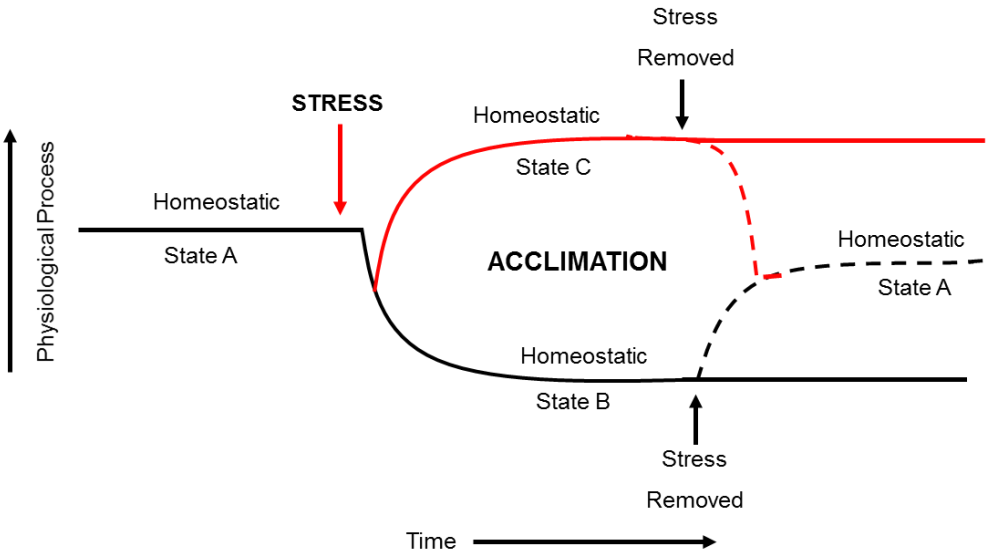

Figure 1.2 – Acclimation and adaptation ability of an organism when a stress is induced. Relation to physiological process and time. Adapted from (Whitman, 2009).

1.3 Salinity variations

Marine waters are commonly reported to have salinity values around 35, but it can vary from 10 to 70 as result of evaporation or precipitation/freshwater influxes (González et al., 2015). Osmotic stress most often resulting from fluctuations in water salinity exerts a considerable oxidative stress in organisms inhabiting intertidal areas and estuarine habitats. Indeed, salinity changes trigger a variety of physiological responses in organisms such as the release of stress-related hormones to plasma, the stimulation of metabolic activity and changes of electrolyte equilibrium (Choi et al., 2008).

The salinity gradient along estuaries can be determinant for organisms' growth and distribution, as Ruiz-Delgado et al. (2019) showed with the isopod *Synidotea laticauda* that had higher growth yield and survival rate in a specific salinity. Stress induced by salinity changes has been associated with enhanced reactive oxygen species (ROS) generation, causing oxidative damage, as shown by Lu et al. (2006) in the macroalgae *Ulva fasciata* exposed to salinity stress. Long-term exposure to hypo-saline and hyper-saline conditions inhibited growth rate and enhanced not only the availability of antioxidants, but also the activity of antioxidant enzymes to cope with the generated oxidative stress (Lu et al., 2006). However, the same salinity stress in the shrimp *Litopenaeus vannamei* had a deleterious effect due to a reduction in the activity of antioxidant enzymes and the establishment of oxidative stress (Liu et al., 2007).

A close negative relationship between salinity variations and DNA susceptibility to oxidative damage was found in the gills of the mussel *M. galloprovincialis* during the summer and winter, presumably indicating an imbalance of pro-oxidant/antioxidant status depending on the magnitude of the hypo-saline stress (Hamer et al., 2008). Thus, changes of the environmental salinity factor generate alterations in the organisms' oxidative metabolism, including the antioxidant defence systems and oxidative damage.

1.4 Effects of temperature coupled with salinity

The interaction between two, or more, stressors may lead to additive, antagonistic or synergistic responses by organisms (Section 1.1). In aquatic species, stress caused by sub or supra salty environments, interacting with warmer waters which, by itself, increase organisms' metabolic rate, may request larger amounts of metabolic energy to re-establish the organism's homeostasis than salinity or temperature individually (Spanopoulos-Hernández et al., 2005). Different metabolic strategies to deal with stressful events and the associated bioenergetic costs of maintaining homeostasis have been proposed, namely (i) the metabolic compensation strategy, when the intensity, duration or number of stressors is low and (ii) the metabolic conservation strategy when the intensity, duration or number of stressors is high (Petitjean et al., 2019). The metabolic compensation strategy involves a re-allocation of energy towards the implementation of a stress response from the molecule to the organism level, to maintain cellular functioning and survival by shifting metabolism, immune and antioxidant defences. Nonetheless, a decrease in energy reserves, growth and reproduction is expected. The metabolic conservation strategy involves a metabolic shutdown (only basal metabolism is sustained) as the costs of maintaining activity are too high and aerobic metabolism is disrupted. Anaerobic metabolism may be induced and shifts in behaviour are expected as well as halted growth and reproduction. Deleterious effects on tissues are likely to occur, which can translate into high mortality levels (Petitjean et al., 2019). For example, Norin et al. (2016) and Miller et al. (2014) showed that in the fish *Lates calcarifer* and in the marine invasive clam *Potamocorbula amurensis,* metabolism can switch from aerobic to anaerobic depending on the salinity and temperature conditions found in its estuarine habitat. Moreover, individual growth, species distribution and photosynthetic activity (in the case of primary producers, (e.g. *Zostera marina*) can also be affected by the interaction of both these factors (Denton and Burdon-Jones, 1981; Harrison and Whitfield, 2006; Nejrup and Pedersen, 2008).

1.5 Physiology under climate change

1.5.1 Regulation of cell integrity and function

Environmental stressors can have a pervasive influence on organism physiology. Several important cellular properties and physiological traits are temperature sensitive, including the composition of membranes, enzymes' activity, muscle function, digestion, and aerobic respiration (Somero, 2011). Moreover, since most of the marine fauna is ectothermic, it cannot regulate body temperature. Thus, body temperature closely tracks environmental temperature conditions (Bicego et al., 2007), with consequent changes in biochemical properties and whole-organism measures. For example, increased mortality has been reported to occur in fish due to variations in the surrounding temperature (Madeira et al., 2014b).

Organisms respond to fluctuations in temperature by modulating gene expression and protein levels (Logan and Somero, 2011). Usually, the main molecular pathways shaped by fluctuations in the thermal regime include the cellular stress response (CSR), energy related pathways, cytoskeleton dynamics and cell signaling (Jayasundara et al., 2015; Madeira et al., 2017b). Specifically, the CSR is translated into the production of molecules involved in different cellular functions such as the heat shock response, proteolysis, antioxidant and immune function, enzyme flexibility and lipid metabolism (Angilletta et al., 2002; Angilletta, 2009). Proteins involved in the CSR have been used as biomarkers to assess stress levels in field and laboratory studies of aquatic fauna (Hook et al., 2014; Quintero and Zafra, 2016; Madeira et al., 2019b).

Some approaches have been developed in order to detect stress signals, acting as biomarkers of stress in organisms. According to the World Health Organization (WHO) a biomarker is defined as "any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical, at the cellular level, or a molecular interaction". Biomarkers can be divided into three subclasses: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility. Biomarkers of exposure

include exogenous substances, their metabolites or the interaction between a substance and a target molecule within an organism; biomarkers of effect are measurable alterations within an organism, including biochemical, physiological and behavioral changes that indicate health impairment; biomarkers of susceptibility are indicators of the ability of the organism to respond to environmental challenges (Organization, 1993). According to Bucheli and Fent (1995) biomarkers are early warning signals that reflect the injurious biological responses towards some environmental factor, providing information on ecosystem's health.

Initially the CSR results in the induction of heat shock proteins (Hsps). These molecular chaperones e.g. heat shock protein 70 kDa (Hsp70) maintain the integrity of proteins during environmental stress by refolding denatured proteins and preventing the aggregation of non-native proteins through degradation (Whitley et al., 1999). Hsps are highly sensitive detectors of environmental stress, namely temperature, preventing the cell damage and increasing, temporarily, physiological tolerance (Feder and Hofmann, 1999; Kültz, 2005).

Another component of the CSR is proteolysis modulation. Persistent cellular stress caused by environmental stressors induces an alteration in pathways involved in protein degradation and metabolism (Hofmann, 2005) Accordingly, Ubiquitin (Ub), which is a regulatory protein found in most eukaryotic organisms, has been used as biomarker of irreversible protein damage (Hofmann and Somero, 1995) as, it targets irreversibly damaged proteins for proteasome degradation (Tang et al., 2014).

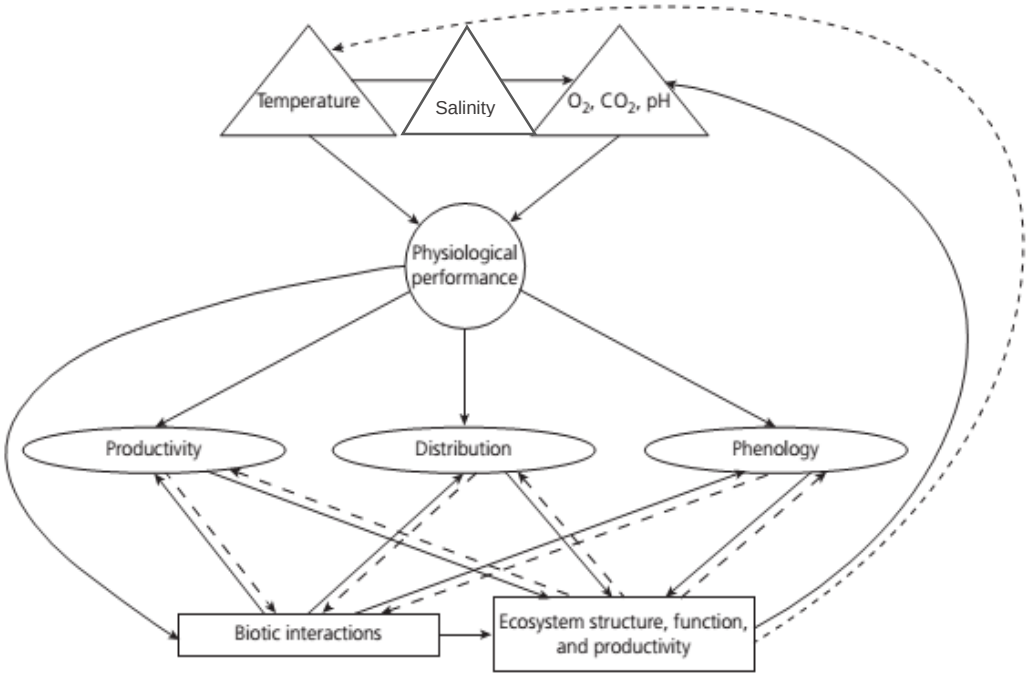

Figure 1.3 - Schematic diagram representing the direct and indirect effects of stressors on marine ecosystems. Triangles represent abiotic factors, other shapes represent hierarchical biological organizational levels. Solid arrows represent direct consequences; dashed arrows represent feedbacks (Hollowed et al., 2013).

In addition, when organisms are exposed to changes in temperature, oxidative stress is expected to occur (Madeira et al., 2013). Oxidative stress results from the excessive production of reactive oxygen species (ROS), when the buffering effect of antioxidant agents is not enough to prevent the damaging effects of ROS, leading to a disturbance in cell homeostasis, DNA degradation, lipid peroxidation, protein carbonylation and, as ultimate effect, cell death (Abele and Puntarulo, 2004; Blier, 2014). There are two types of antioxidant agents in cells, namely enzymatic (catalase, superoxide dismutase and peroxidase) and non-enzymatic ones (vitamins, carotenoids, tocopherol, glutathione – which can be measured via de total antioxidant capacity method (TAC) (Kambayashi et al., 2009; González et al., 2015; Chainy et al., 2016). Oxidative stress is known to shape physiological processes and life-history strategies of marine organisms (Birnie-Gauvin et al., 2017). Consequently, oxidative stress biomarkers have been used to assess the metabolic status and health of organisms, being an important tool for species management and conservation (Beaulieu and Costantini, 2014).

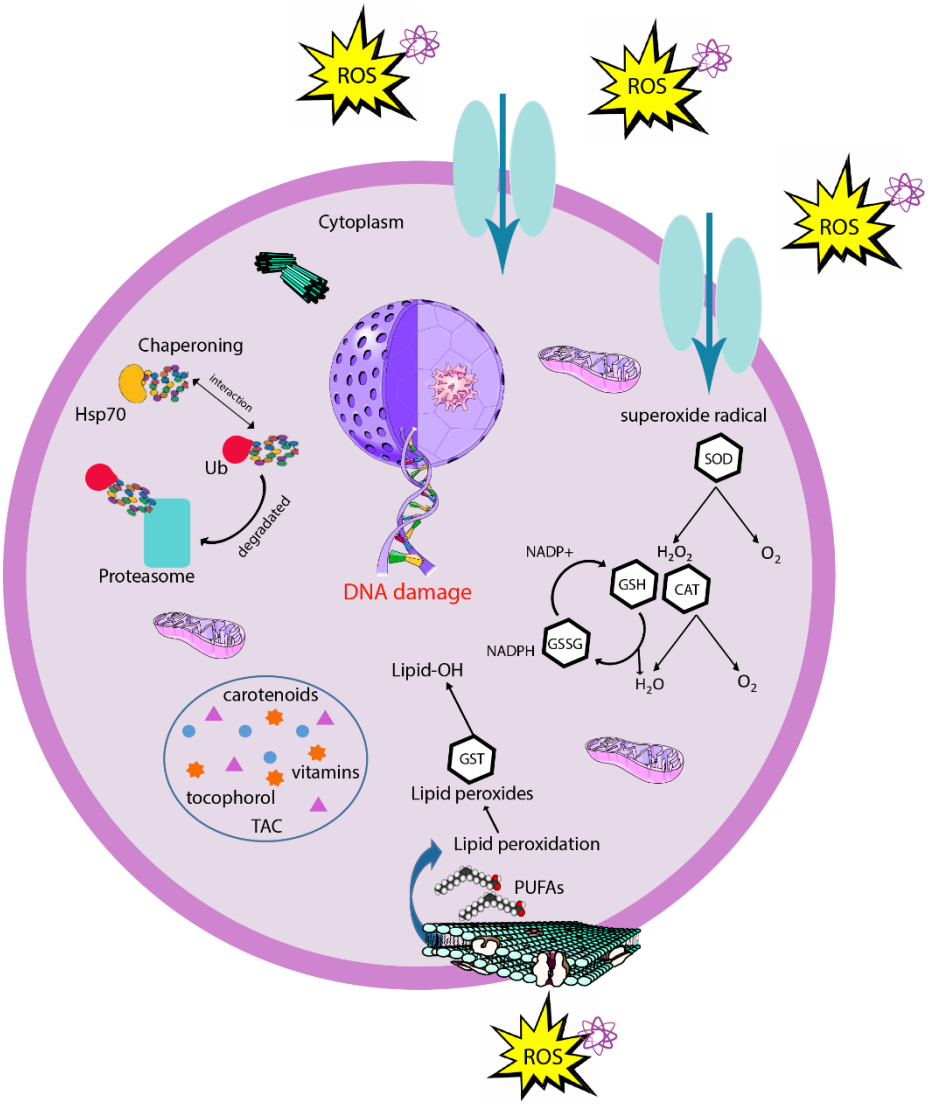

Figure 1.4 - Scheme of cellular oxidative damage and anti-oxidant enzymes induced by Reactive Oxygen Species (ROS) under stressful conditions. Such enzymes (superoxide dismutase – SOD; catalase – CAT; glutathione-S-transferase – GST) quench ROS by catalysing the reactions that transform toxic ROS into non-toxic products. Lipid peroxidation occurs when ROS attack polyunsaturated fatty acids (PUFAs) (constructed using Adobe® Ilustrator CC 2015 tools), adapted from Madeira (2016).

1.5.2 Energy allocation

In order to deal with stress, organisms undergo metabolic changes that result in a redistribution of energy to critical functions, with such responses determining the physiological tolerance of an organism to current and future stress events (Calosi et al., 2013; Kühnhold et al., 2017). In fact, a bioenergetic framework has been proposed to explain the effects of multiple stressors on aquatic organisms,

based on an “energy-limited tolerance to stress”, as defined by Sokolova (2013). In detail, this theory proposes that organisms need enough energy supply to sustain basal metabolism and other performance and fitness-related activities such as foraging, growth and reproduction, which are inherently dependent on the balance between energy input and expenditure. However, this bioenergetics balance is limited by physiological constrains (assimilation rate, conversion of food to ATP) and energy allocation trade-offs to different functions, ultimately defining metabolic strategies (compensation *vs* conservation) and tolerance limits of aquatic species exposed to multiple stressors (Sokolova et al., 2012; Sokolova, 2013). For example, when organisms are exposed to pathogenic agents combined with other stressors, the demand for energy increases, resulting in the use of glucose and glycogen as energy sources, possible resulting in higher costs to other physiological systems, such as the immune system (Turner et al., 2016). Several bioenergetic markers of physiological stress have been proposed within the described framework including the expression of heat shock proteins, induction of oxidative stress and depletion of energy reserves.

Energy reserves can be stored in three forms, namely lipids (mostly triacylglycerol, TAG), carbohydrates (mostly glycogen) and proteins, that can be used to fuel cell metabolism (which is an interconnected network of pathways, as defined by Zerfaß et al. (2019). Lipids are usually used for long-term energy storage as they yield large amounts of energy when compared to carbohydrates and proteins (Sibly et al., 2013). Overall, when the energy demand increases due to stress, carbohydrates are the first energy reserve to be mobilized, usually followed by lipids and lastly proteins, as these produce less energy and are needed for somatic growth (Kühnhold et al., 2017). Under stressful conditions, glycogen is primarily used for the maintenance of the organism’s condition (Goh and Lai, 2014). The breakdown of glycogen into glucose units fuels glycolysis, which produces pyruvate to be further converted into acetyl-CoA that can be used in the Krebs cycle to maintain basal metabolism and mitochondrial respiration.

When glycogen reserves are depleted, the organism will have to (i) hydrolyse TAGs to generate fatty acids for beta oxidation, in order to produce acetyl-CoA to fuel the Krebs cycle and (ii) hydrolyse proteins into amino acids to generate glucose

or intermediate compounds of the Krebs cycle (see Alberts et al. (2002). Moreover, the resulting fatty acids can also be used in the synthesis of phospholipids for the cellular membrane or cell signalling processes. Lipid profiles and contents have been shown to change according to habitats or regions and temperature regimes (Dalsgaard et al., 2003; Ricardo et al., 2015) and salinity (Fuhrmann et al., 2018). At higher temperatures, an increase of saturated or shorter-chain fatty acids is expected in order to maintain membrane fluidity (Török et al., 2014; Malekar et al., 2018). Moreover, lipids are known to play a role in osmoregulation in aquatic invertebrates and fish (Tocher et al., 1995; Chen et al., 2019) and are important signaling and mitigation molecules under stressful conditions in animals and plants (Kültz, 2005; Vígh et al., 2007; Okazaki and Saito, 2014; Manna et al., 2019)

Two groups of polyunsaturated fatty acids (PUFA), i.e. omega-3 (*n*-3) and omega-6 (*n*-6), are essential for all vertebrates and, indirectly for most of invertebrates. The absolute concentrations of both PUFA are important, as is the appropriate ratio between them. The optimal ratio of *n*-3/*n*-6 is not known for most organisms but is suggested to be species-specific. Moreover, *n*-3 and *n*-6 play an important role as regulators of anti- and pro-inflammatory responses, respectively, and therefore, an imbalance in this ratio can be detrimental to the immune response and cardiovascular health of vertebrates (Husted and Bouzinova, 2016). Moreover, PUFA have a high nutritional value and are prime quality fatty acids in commercial species. Several studies reported that ocean warming decreases not only total lipid content, but also the content of PUFA including eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in marine organisms, with potential consequences in the organisms' physiological homeostasis, commercial value of species and availability of essential fatty acids to human consumption (Anacleto et al., 2014; Colombo et al., 2019). Nevertheless, Kattner and Hagen (2009) suggested that climate change effects can vary between species, thus changes in lipid profiles may not be easily predictable across marine taxa.

In addition to temperature variations, changes in salinity are also known to induce lipid content and compositional alterations, associated with an energetic and membrane structural remodeling to adjust permeability and fluidity (Subramanian, 1975; Luvizotto-Santos et al., 2003; Bhoite and Roy, 2013). Interestingly, *n*-3 fatty

acids are known to modulate ion channel activity in cellular membranes (Diaz and Retamal, 2019), playing a major role in osmoregulation (Diaz et al., 2016) For instance, an increase in the ratio of *n*-3/*n*-6 PUFA and the percentage of *n*-3 PUFA was found under hyperosmotic stress in the fish *Poecilia reticulata* and *Chanos chanos*, respectively (Daikoku et al., 1982; Borlongan and Benitez, 1992). Increased *n*-3 PUFA have been associated with increased salt tolerance in freshwater and anadromous fish species (Tocher et al., 1995). Moreover, acclimation of the Pacific oyster to salinity fluctuations also involved the remodeling of membrane fatty acids, with a decrease of *n*-6 PUFA at lower salinity, especially 20:4*n*-6 (Fuhrmann et al., 2018). The authors state that the reduction in this fatty acid may be due to its mobilization to prostaglandin synthesis, as this compound is involved in osmotic cell volume regulation (Fuhrmann et al., 2018). The same trend in *n*-6 PUFA was found in the Chinese mitten crab, as higher levels were found at higher salinity (Long et al., 2017). In phytoplankton, an increase in the proportion of PUFA/SFA was observed when the community was exposed to either high temperature or low salinity leading to higher oxidative damage to lipids, but the combined effect of both stressors did not induce such change, resulting in a stable PUFA/SFA ratio and no oxidative damage (Hernando et al., 2018). Another study in euryhaline rotifers highlighted that increased salinity leads to a rise in the proportion of saturated fatty acids and a decrease of polyunsaturated fatty acids of polar and neutral lipids (Frolov et al., 1991). The authors highlight that these changes in polar lipids may reduce membrane permeability and a high percentage of saturated fatty acids in neutral lipids reflect their function as energetic reserve, as their catabolism yields more energy when compared to the catabolism of polyunsaturated fatty acids of the same chain length. Lipid mobilization for energy production seems to be especially prevalent during hypoosmotic shock, but not during hyperosmotic shock in sea-dependent species (Luvizotto-Santos et al., 2003). Still, physiological and molecular responses to fluctuating salinity are species-specific (Havird et al., 2019).

Overall, organisms have different strategies to obtain the energy that they need to overcome environmental stress, however several gaps must be fulfilled to understand how marine organisms will adapt and overthrow climate change.

1.6 Objectives

The present work investigated the performance, physiological and molecular responses of *Hediste diversicolor* under different global change scenarios. For that purpose, we maintained individuals under experimental controlled conditions of temperature and salinity, evaluating their vulnerability to global changes. We followed a physiological and molecular approach do detect thermal limits and metabolic changes under the tested environmental stressors.

More specifically, the thesis objectives (obj.) were:

Obj. 1 - Estimate the upper thermal limits, acclimation capacity, thermal safety margins of *Hediste diversicolor* between different treatments after 30 days of acclimation;

Within this objective, the null hypotheses tested were:

H_0 – Ocean warming and salinity variations do not affect the survival of *Hediste diversicolor*;

H_0 - Thermal tolerance limits of *Hediste diversicolor* do not change with acclimation temperature combined with salinity.

Obj. 2 - Quantify selected biomarkers that show *Hediste diversicolor's* stress responses' to the different tested scenarios over exposure time;

Within this objective, the null hypothesis tested was:

H_0 - Global change scenarios do not affect the cellular stress response and macromolecular damage of *Hediste diversicolor.*

Obj. 3 - Identify changes in fatty-acid profiles of *Hediste diversicolor* under the different tested scenarios over exposure time of acclimation;

Within this objective, the null hypotheses tested were:

H_0 - Fatty-acid profiles of *Hediste diversicolor* do not change with global change scenarios;

Obj. 4 - Compare energy reserves of *Hediste diversicolor* between the different tested scenarios over time of acclimation.

Within this objective, the null hypothesis tested was:

H_0 - Energy reserves of *Hediste diversicolor* are not affected by ocean warming and salinity variations.

1.7 Scientific and societal importance of this study

Given the objectives of this study, it is important to emphasize its importance to the scientific community and society.

A climate-smart conservation can be defined as: “The intentional and deliberate consideration of climate change in natural resource management, realized through adopting forward-looking goals and explicitly linking strategies to key climate impacts and vulnerabilities.” (Stein et al., 2014). In order to achieve and develop climate-smart conservation strategies, multiple steps are needed as: 1) Define planning purpose and scope; 2) Assess climate impacts and vulnerabilities; 3) Review/revise conservation goals and objectives; 4) Identify possible adaptation options; 5) Evaluate and select adaptation actions; 6) Implement priority adaptation actions; 7) Track action effectiveness and ecological response. For that, global change research developed by the scientific community has an important role in providing answers and knowledge transfer to relevant stakeholders and political organizations is crucial for them to take action and implement efficient conservation strategies. Specifically, many planners, policy-makers, and managers want to know what they could be doing differently to prepare for and respond to existing and projected climate impacts, and which of the implemented management plans continue to make sense in light of climate change impacts on marine biodiversity.

Therefore, this study tries to fill some of the lack of knowledge about climate change impacts on marine invertebrates that play major ecological roles, to realize the potential cascading effects on estuarine ecosystems. Moreover, upper thermal limits and thermal safety margins are important metrics to understand distribution changes of species over larger areas and also to distinguish an adapted species from a naturally thermally resistant species (Vinagre et al., 2019). Additionally, these

type of studies are important to help to construct risk assessment maps, predicting the occurrence of heat waves and on what scale (Galli et al., 2017). Mapping shifts in species distributions, identifying and predicting critical shifts in ecological states, assessing the capacity for adaptation to novel environmental conditions, current and emerging tools and methods to address climate change in conservation prioritization, and understanding physiological and molecular mechanisms are some key interlinked scientific requirements that are relevant topics in order to clarify or answer some questions for a good management on adaptive marine conservation planning (Rilov et al., 2019).

In addition to the ecological issues, some species have a commercial value for fishing and aquaculture, as is the case with the ragworm *Hediste diversicolor*. Under global changes, aquaculture has new requirements and issues to pay attention, such as: species thermal biology, selective breeding and shifting species, shifting production periods and implementing alternative methods and shifting production sites. Aquaculture systems where environmental parameters are not controlled e.g. pH, temperature and salinity, may be greatly impacted by climate change (Clements and Chopin, 2017). Risk assessment and adaptation strategies need to be implemented into aquaculture practices, as climate change can endanger food quality and security (FAO, 2018). Moreover, there are species with high importance in the recycling of organic matter in aquaculture systems, as the species of this study, *Hediste diversicolor*. This species has the important function of reusing the unused organic matter in fish production, playing a key role in integrated multitrophic aquaculture (IMTA) systems (Marques et al., 2017) and land-based salmon smolt aquaculture production (Wang et al., 2019). Furthermore, there is a growing demand for species and/or strains resilient to new global changes, in order to make aquaculture systems become more profitable.

2. Materials and Methods

2.1 Ethical statement

All procedures complied with EU legislation for animal experimentation (Directive 2010/63/EU) and followed international guidelines e.g. the 3 Rs principle of animal welfare (Replace, Reduce and Refine).

2.2 Sampling location

Ria de Aveiro (40°38′N, 08°45′W) is a shallow coastal lagoon located in the north-west coast of Portugal (Figure 2.1) and it is connected to the Atlantic Ocean through a single inlet (1.3 km in length, 350 m wide and 20 m deep) (Dias and Lopes, 2006). It is part of Vouga River basin, and it is approximately 45 km long (NNE-SSW) and 10 km wide (Dias et al., 2000). This lagoon forms a unique mesotidal wetland area and it has five main channels with several branches that form islands, inner basins and mudflats. The five channels are, from South to North, the Mira and Ílhavo channels with 25 km and 15 km respectively, the Espinheiro channel with 17 km and the S. Jacinto and Ovar channel with 29 Km long (Azevedo et al., 2013; Lillebø et al., 2015).

Ria de Aveiro presents a temperate maritime climate with a warm period between July and September and a cold period between December and February (Lillebø et al., 2015). Tides are characterized by semi-diurnal regime, ranging, at the ocean boundary, from 0.6 m at neap tide to 3.5 m at spring tide, with an average amplitude of 2 m (IH, 2019).

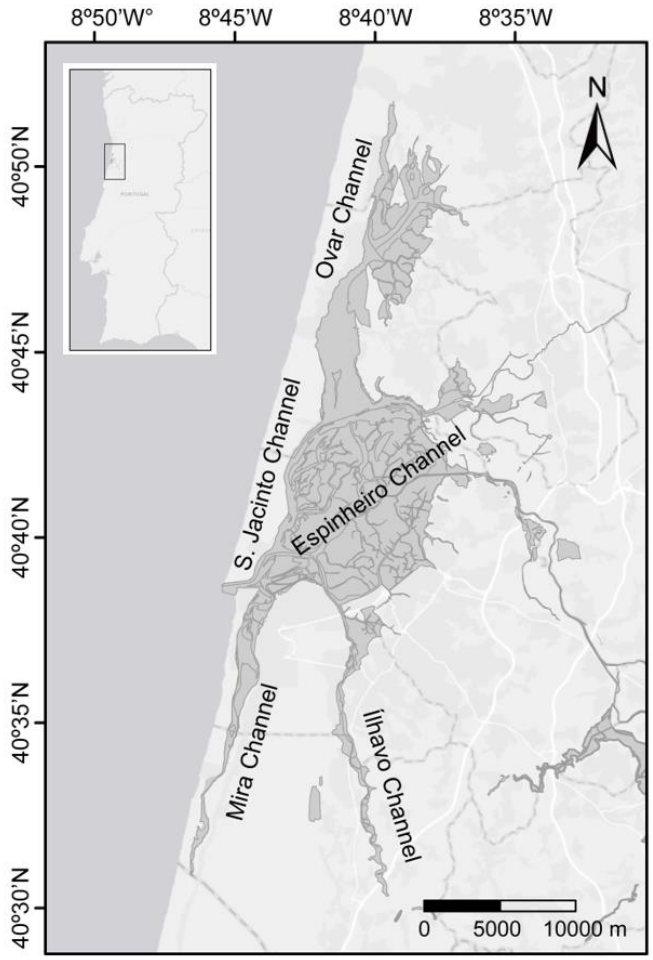

Figure 2.1 - Geographic map of Ria de Aveiro lagoon. The map was created using the software ArcMap v10.2.

Ecologically, it provides a wide variety of habitats with high biodiversity and it is possible to find several species with economic value such as clams, shrimps, crabs and migrant and resident fish well adapted to the lagoon (Lillebø et al., 2015). Polychaetes also present some economic value being used as bait in recreational and commercial fishing. Moreover, polychaetes play a major role in the ecosystem, especially in the food-webs being a major food source for animals in higher trophic-levels, e.g. fish and birds (Figure 2.2) (Abrantes et al., 1999; Breton et al., 2003). They are also bioturbators, contributing to ecosystem engineering by reworking the water-sediment interface (Gillet et al., 2012). The lagoon's natural capital, including the variety of ecosystem services and biodiversity, is essential for the development of the region and for the well-being of the local population.

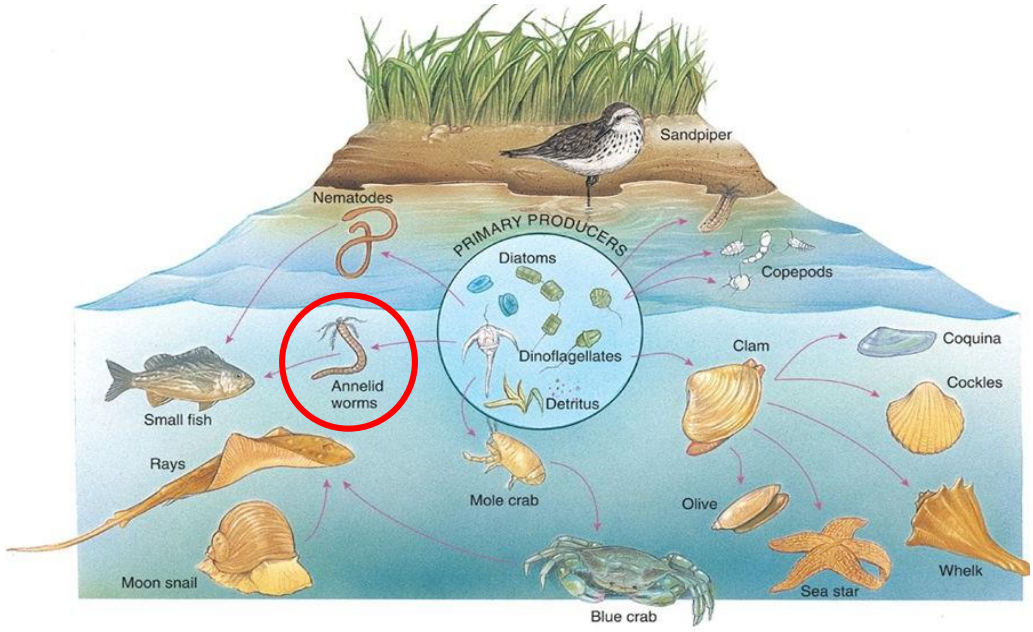

Figure 2.2 - Example of an estuarine food-web © Cengage (2010).

2.3 Environmental data collection

Field environmental data were measured in the Espinheiro channel at Ria de Aveiro (40°38'2"N, 8°39'42"W) in order to determine within season and tide cycle variations. All the measurements (water temperature, salinity and pH) were taken between July 2019 and September 2019. Water temperature was measured every half an hour using a HOBO® data logger (HOBO® Water Temp Pro v2 U22 – 001, Onset, USA) (Figure 2.3 - 1). Salinity and water pH were measured once a week during low tide, and during one tide cycle (10h) per month between 7 am (sunrise) and 21 pm (sunset) with a conductivity and pH portable meter 3110 SET 1 WTW® (Xylem Analytics, Germany) (Figure 2.3 - 2).

Figure 2.3 – Probes for environmental data: 1) HOBO® data logger (HOBO® Water Temp Pro v2 U22 – 001, Onset, USA) 2) conductivity and pH portable meter 3110 SET 1 WTW® (Xylem Analytics, Germany).

2.4 Study species – *Hediste diversicolor*

The common ragworm *Hediste diversicolor* (Figure 2.4 -1) is a benthic polychaete species belonging to the Nereididae family (Müller, 1776). It inhabits shallow marine and brackish waters particularly in estuaries and coastal lagoons, under tidal influence along the temperate coast of North Atlantic Ocean (Figure 2.5) (Scaps, 2002). *H. diversicolor* is euryoecious and euryhaline, adapting to a wide range of environmental conditions, e.g. hypoxia and low salinities. This species builds burrows in sandy mud, gravel and clay with U- or Y-shaped galleries that can reach 30 cm deep and it can be found in densities from 35 to 3700 individuals per m^2 (Durou et al., 2008). Individuals grow up to 20 cm in length, comprising a maximum of 120 segments. *H. diversicolor* is a gonochoristic species, with maturation occurring between the ages of 1 and 3 years old. However, warmer temperatures can accelerate this development. When mature, individuals change their colour from reddish brown (Figure 2.4 2a) to dark green - females (Figure 2.4 2b) and bright green - males (Figure 2.4 2c) (Scaps, 2002). *H. diversicolor* is a monotelic species with individuals dying after reproduction (Scaps, 2002).

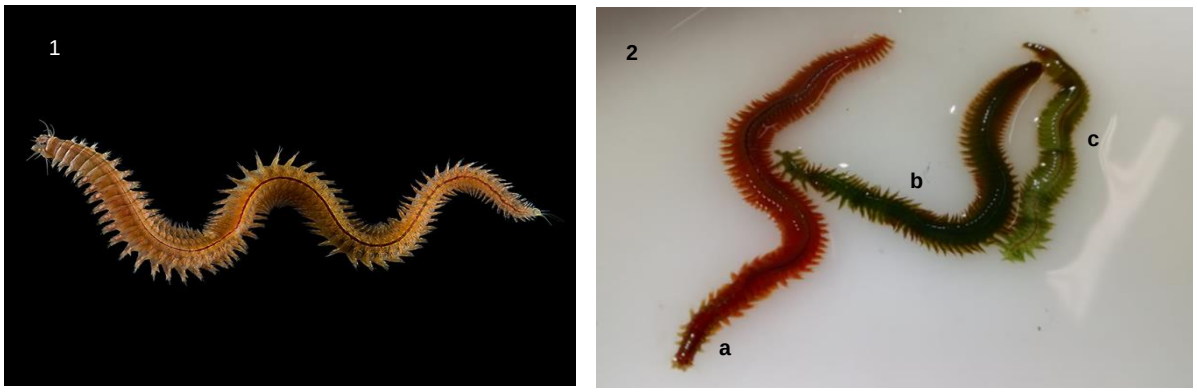

Figure 2.4 - 1) Ragworm *Hediste diversicolor*; 2) Colour changes in maturation of *Hediste diversicolor* a) adult with undetermined sex, b) female and c) male.

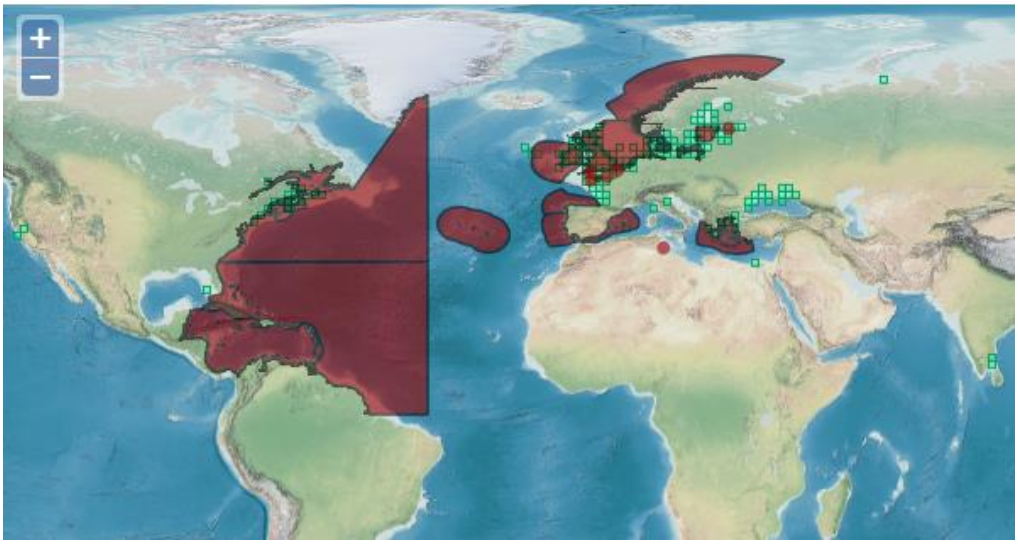

Figure 2.5 - Total range of *Hediste diversicolor* species distribution © WoRMS (2019).

Hediste diversicolor present three different feeding modes, i.e. filter feeding, deposit feeding and active feeding that change depending on the environmental conditions, seasonality and primary production (Sturdivant et al., 2015). For example, filter feeding is triggered by high concentrations of phytoplankton (Riisgård, 1994) whilst deposit feeding may be facilitated by the absence of predators (Fidalgo e Costa et al., 2006) and active feeding, i.e. this species predates different animals inhabiting the surrounding sediment. *H. diversicolor* excretes a mucus net and creates an irrigation current in its burrows with undulated body movements; suspended particles are retained in the net and the net is subsequently

ingested (Riisgård, 1994). As prey, this species constitutes a key element in the trophic webs being predated by numerous species of fish, crustaceans and birds (Scaps, 2002; Gillet and Torresani, 2003; Nesto et al., 2012).

2.5 Field work and animal husbandry

Hediste diversicolor individuals (n=1470, weight 0.22 g ± 0.08 g, measured in a sub-sample of 60 individuals) used in this study were collected in the Espinheiro channel at Ria de Aveiro (40°38'2"N, 8°39'42"W) during the Autumn of 2018 (Figure 2.6). Sampling was performed during low tide, with water at 15 ºC and 25 of salinity (recorded using Conductivity portable meter 3110 SET 1 WTW® probe). Individuals were accommodated in plastic containers filled with macroalgae to keep a moist environment and transported to the laboratory in the end of the sampling period.

At the laboratory, specimens were housed during 2 weeks in plastic containers (600 mm x 400 mm x 250 mm, 47L) with artificial seawater and sand from WhiteMinerals® to simulate the natural environment and continuous aeration. Seawater was made using reverse osmosis water and Red Sea® salt (25 salinity). The water temperature was maintained at 15 ± 0.5 ºC with the individuals being fed every 2 days with commercial fish food Goldfish Tetra® (42% protein and 11% fat). The photoperiod was 12 h L: 12 h D cycle.

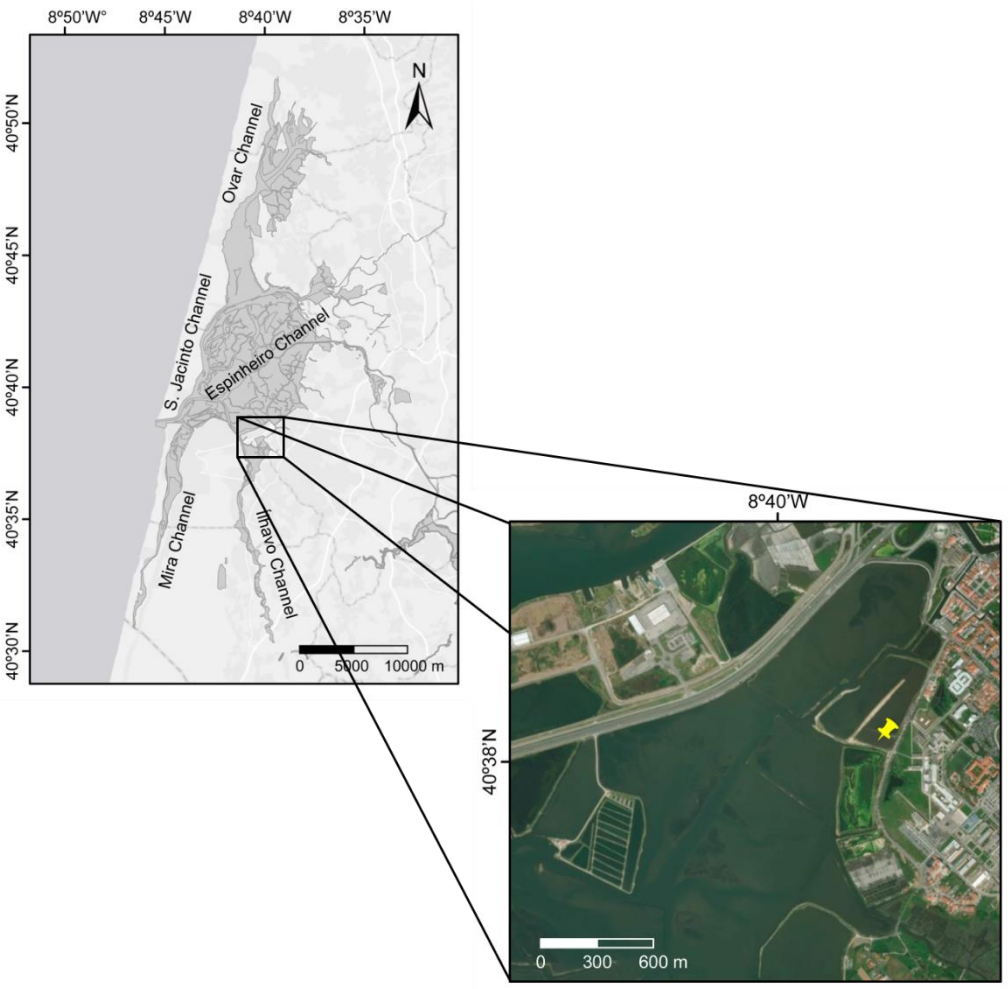

Figure 2.6 - Specimen collection area in Espinheiro channel, Ria de Aveiro, Portugal (40°38'2"N, 8°39'42"W) marked with yellow pin. The maps were created using the software ArcMap v10.2.

2.6 Experimental design and acclimation assay

After housing (see section 2.5), specimens were subjected to a thermal ramp in order to reach the experimental temperatures of 24, 27 and 30 ºC, predicted by IPCC for 2100 (IPCC, 2014) (salinity was kept stable at 25). In each thermal ramp, water temperature was steadily increased (2 °C h^{-1}) from the housing to the treatment temperature in six plastic containers (25 L, n=81-82 individuals *per* container, until a total n= 490 polychaetes *per* temperature) placed inside a thermostatized bath with a digital controlled heater device (JUMO Quantrol PID LC300 with a temperature probe PT1000 coupled to an aluminum box IP66 and 3kW resistance) designed by AQUALGAE SOC. LDA. Successive thermal ramps were carried out until all individuals had reached the correct experimental temperature. Specimens were randomly distributed into small plastic boxes with different combinations of water temperature (24 ºC, 27 ºC and 30 ºC) and salinity (20 and 30) in full factorial design (total of six treatments, n=7 boxes $treatment^{-1}$, n=35 individuals box^{-1}, box size 300 mm x 200 mm x 170 mm, 6.5 L (Figure 2.7 and 2.8). The total number of individuals per treatment was 245. Each box contained artificial seawater (see above), 80 mm of sand (see above) and was wrapped in black plastic to mimic environmental conditions, as no light enters the sediment column in the natural environment. Individuals were exposed to the six different treatments during 29 days. To activate de nitrogen cycle, one month before individual's distribution in boxes, 13 mL of a solution of ammonium chloride (5 mg L^{-1}) was added in each box once a week, and the parameters (ammonia, nitrite and nitrates' concentrations) were measured to evaluate if the cycle was working.

To maintain the water temperature in the boxes, heating thermostats were used (Eheim® thermocontrol 3612 Aquarium Heater 50W). Boxes were covered by a lid to prevent evaporation and water temperature and salinity variations. Oxygen levels were maintained using an aeration device (Hailea® vortex blower) with a diffuser stone. Boxes had been previously assigned to treatments in a random way and randomly placed in the experimental bench. There was one depuration tank per treatment (600 mm x 400 mm x 250 mm, 47 L) maintained at the correct temperature with a thermostat (Eheim® thermocontrol 3612 Aquarium Heater 100 W). In each

depuration tank, there were seven glass flasks (100 mm x 100 mm x 180 mm, 1 L) filled with 40 mm of sand and aerated sea water, corresponding to the seven replicate boxes per treatment.

After 14 and 28 days of acclimation (D14 and D28 respectively), 9 individuals per plastic box were sampled (*per* time point). Specimens were pooled for each analysis type (n=3 pooled specimens for biomarker analyses, n=3 for glycogen quantification and n=3 for fatty acid profiles). Specimens were sampled frozen in liquid nitrogen and kept at -80 ºC for posterior biochemical analyses. Sampled organisms were previously depurated for 24 h in the depuration tanks, as described above.

During the acclimation assay, individuals were fed in a 2-day period with commercial fish food (see section 2.5). To assure that no additional stress, other than temperature and salinity, were induced to the organisms, salinity, temperature, pH and the dissolved oxygen were measured using specific probes (Conductivity portable meter 3110 SET 1 WTW®, Oxygen portable meter 3210 SET 2 WTC®, pH portable meter 3110 SET WTC®) and ammonia and nitrites' concentrations using water tests (Salifert Profi Test NH_4 Ammonia, Salifert Profi Test NO_2 Nitrite). The water of each plastic box was partially (50 %) renewed every week, using previously prepared seawater (the day before) with the different treatment characteristics and stored in 6 drums (π350 mm x 60 mm, 65 L). Water temperature in each drum was maintained using a thermostat (Eheim® thermocontrol 3612 Aquarium Heater 300W).

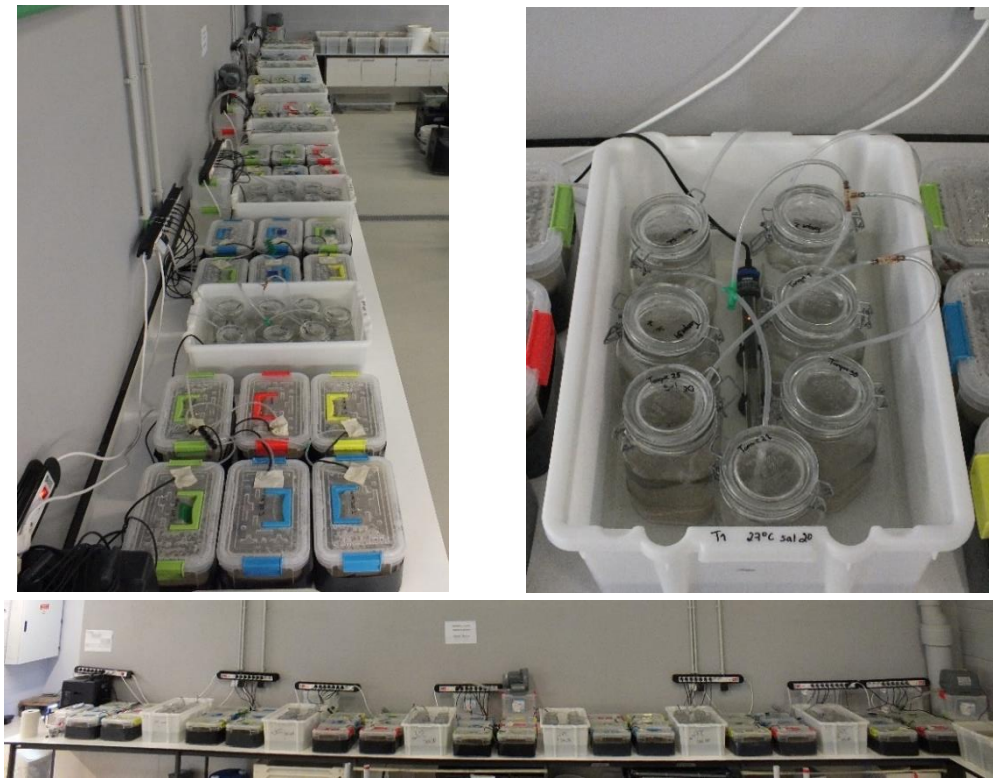

Figure 2.7 - Experimental setup a) view from the top b) bigger containers with flasks for depuration purposes (depuration tank) c) panoramic view.

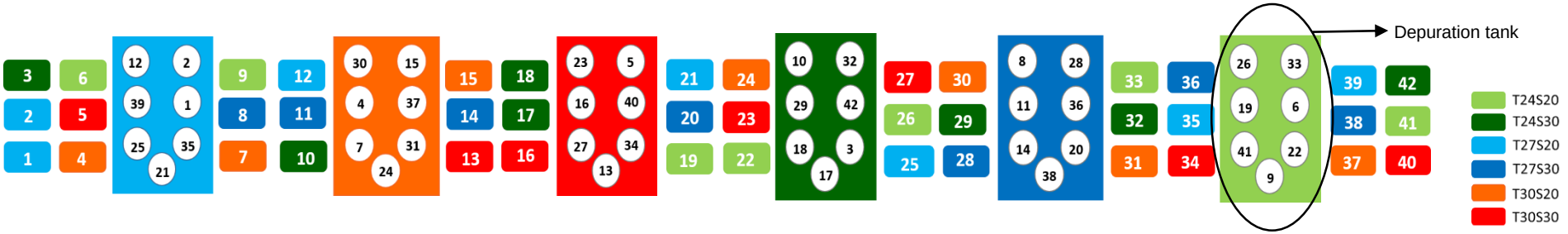

Figure 2.8 - Scheme of the experimental design. Polychaetes (*Hediste diversicolor*) were subjected to six treatments in a full factorial randomized design combining three levels of temperature (indicated by T24, T27 and T30 ºC) and two levels of salinity (S20 and S30). There were 7 replicate boxes per treatment (highlighted with different colours), each one containing 35 individuals. Tank and depuration flask numbers are specified.

2.7 Thermal tolerance limits

Thermal tolerance of *H. diversicolor* was determined following a dynamic method widely used for the determination of upper thermal limits in ectothermic animals (Madeira et al., 2012a; Kaspari et al., 2015; Vinagre et al., 2016).

To determine the Critical Thermal Maximum (CT_{Max}), after the acclimation assay and 24 hours of depuration (same procedure as in 2.1), three different individuals *per* replicate box (n=21 per treatment) were moved to other glass flasks (100 mm x 100 mm x 180 mm, 1 L) with 0.5 cm high of sand, seawater (1L) and aeration. These glass flasks were grouped by temperature treatment and assigned to the correct salinity value and randomly placed in a thermostatized bath (Figure 2.9) with a digitally controlled heater device (see above) (Figure 2.10). In summary, three CT_{Max} assays were run, each starting at the tested acclimation temperatures (24, 27 and 30 °C).

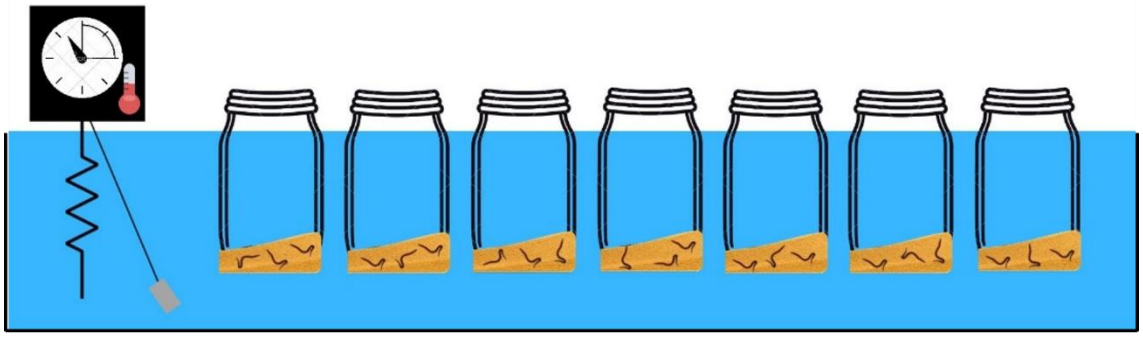

Figure 2.9 - Scheme of a CT_{Max} assay per treatment, n=7. Total n= 7 replicates x 6 treatments.

Figure 2.10 – Digitally controlled heater used in CT_{Max} assays.

During the trial, individuals were exposed to a constant rate of water temperature increase (2 ºC h^{-1}), until they reached the end-point, defined as loss of equilibrium, i.e. individuals were not in their gravitational position, showing loss of righting response (previously tested in a pilot trial). The CT_{Max} trial was run under continuous observation and the individuals' behaviour such as digging activity (i.e. individual attempted to burrow or emerged from the substrate, possibly in an attempt to find more favourable temperatures) and the response to prodding (i.e. individual was motionless and moved only after being gently prodded with a tip) was also registered, as defined by Massamba-N'Siala et al. (2012).

After reaching the end-point, the individuals were weighted using the scale Kern EMB 200-3.

2.8 Biomarkers' analyses

2.8.1 Protein extraction

The 84 samples previously collected, weighed and frozen, at D14 and D28 (ca. 0.65-0.70 g), from the acclimation assay (see section 2.6) were stored in 5 mL microtubes. After thawing, samples were homogenized in a phosphate buffered saline solution (5 mL PBS – 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH = 7.4) using a Dremel® Model 3000 homogenizer. Crude homogenates (1 mL) were centrifuged at 4 °C for 15 min at 10 000 ×*g* and the supernatant was collected and transferred to new microtubes and frozen at -80 ºC for biomarkers analyses. Biomarker assays were undertaken using colorimetric methods and adapted for 96-well microplates (Nunc-Roskilde, Denmark), specified in each analysis.

2.8.2 Total protein quantification

Total protein was quantified following the Bradford methodology (Bradford, 1976) for 96-well microplates. Briefly, 10 µL of each sample, diluted to 1:20, was added to each well (in duplicates). Subsequently, 190 µL of Bradford reagent

(Comassie Blue G250, methanol, phosphoric acid, distilled water) were added to each well and absorbance was read at 595 nm in Synergy™ HTX Multi-Mode Microplate BioTek® reader. An eight-point calibration curve was built using BSA (Bovine Serum Albumin, Sigma-Aldrich, USA) as standards (0 to 1 mg mL^{-1}). Total protein measurements were used to normalize all biomarker levels (Heat shock protein 70 kDa – Hsp70, total ubiquitin - Ub, antioxidant enzymes: catalase - CAT, glutathione-S-transferase - GST, superoxide dismutase - SOD, non-enzymatic antioxidant agents: total antioxidant capacity – TAC and oxidative damage products: lipid peroxidation – LPO).

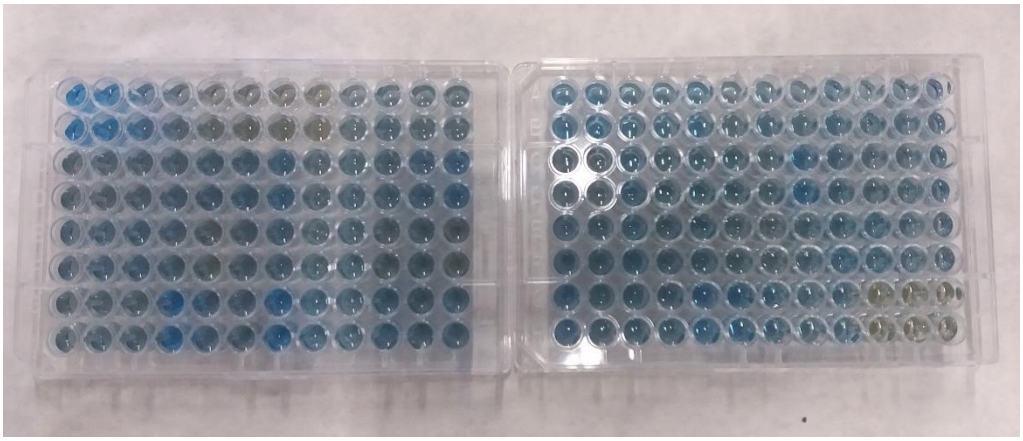

Figure 2.11 - Bradford assay (Bradford, 1976) for 96-well microplates.

2.8.3 Hsp70 and total ubiquitin quantification

For heat shock protein 70kDa (Hsp70) and total ubiquitin (Ub) quantification, an enzyme linked immunosorbent assay (ELISA) (Njemini et al., 2005; Madeira et al., 2014b) was carried out. Hsp70 was quantified using a 1:100 dilution of the original sample, whereas Ub was quantified using a non-diluted sample. One hundred microliters of each sample (diluted for Hsp70 and non-diluted for Ub) were placed in microplates' wells (in duplicate) and incubated overnight at 4 °C. After incubation, both microplates were washed 3× in PBS (see above) 0.05 % Tween-20 and then blocked by adding 200 µL of 1 % BSA in PBS. Both microplates were then incubated at 37 °C for 90 min. After washing, the primary antibodies (mouse monoclonal Hsp70/Hsc70, # TA326357, OriGene, USA - for Hsp70 and mouse monoclonal Ubi-1, #ab7254, Abcam, UK - for Ub),

were diluted to 2 µg mL^{-1} (Hsp70) and 1.5 µg mL^{-1} (Ub) in 1 % BSA in PBS, and added to the microplates' wells (100 µL of each). Subsequently, both microplates were incubated overnight at 4 ºC. After incubation and another washing stage in both microplates (see above), 100 µL of a secondary antibody, the same for Hsp70 and Ub, anti-mouse IgG (Fab specific) conjugated to alkaline phosphatase (#A1293, Sigma-Aldrich, USA) previously diluted to 2.5 µg ml^{-1} (Hsp70) and 2 µg mL^{-1} (Ub) in 1 % BSA in PBS was added to each well and incubated at 37 °C for 90 min. After incubation and another washing stage, 100 µL of substrate (157 mg - Tris-HCl, 58 mg - NaCl, - 50 uL $MgCl_2$, 10 mg - PnPP, 10 mL pH = 9) was added to each well in both microplates and incubated for 30 min at 37 ºC. After incubation, the absorbance was read in 96-well microplate reader (see above) at 405 nm. For quantification purposes, within the 0 to 2 µg mL^{-1} range, calibration curves were constructed using serial dilutions of purified HSP70 active protein (#AR03018PU-N, OriGene, USA) and purified Ubiquitin (UbpBio, E-1100, USA).

2.8.4 Catalase activity

Catalase assay was adapted from Madeira et al. (2019b). Duplicates of 20 µL were taken from each original sample and transferred to the microplates' wells. Then, 100 µL of assay buffer (100 mM potassium phosphate), 30 µL of methanol and 20 µL of 0.035 M hydrogen peroxide were added to each well. Following, the microplates were incubated for 20 min in the shaker (80 rpm). After incubation, 30 µL of potassium hydroxide 10 M and 30 µL of Purpald 34.2 mM in 0.5 M HCl were added to the wells and microplates were incubated for 10 more min in the shaker (80 rpm). Posteriorly, 10 µL of potassium periodate 65.2 mM in 0.5 M HCl was added to the microplates and absorbance was read at 540 nm in the same reader (see above). As a positive control, the activity from a standard bovine catalase solution of 1523.6 U mL^{-1} was used. Formaldehyde standards were used to create the calibration curve (ranging from 0 to 75 µM). The catalase activity was calculated considering that one unit of catalase is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C.

2.8.5 Glutathione-S-transferase activity

GST activity was calculated following a protocol by Madeira et al. (2019b). Duplicates of 20 µL were taken from each original sample and transferred to the microplates' wells. In each well, 180 µL of a reagent mix containing 200 mM of reduced L-glutathione, 100 mM CNDB and buffer Dulbecco (Sigma Aldrich, USA®) were added. The absorbance was read at 340 nm each minute for a total of 6 min, in the same reader (see above). GST activity was calculated using a molar extinction coefficient of 0.00503 µM (adapted for microplates) following the equations:

1) GST Abs_{340}/min = (Abs_{340} final read – Abs_{340} initial read) / reaction time (min)

2) GST specific activity = (GST Abs_{340}/min x 0.2 mL) / (0.00503 µM × 0.02mL)

2.8.6 Superoxide dismutase inhibition

SOD activity was calculated following a protocol by Madeira et al. (2019b). In each sample, duplicates of 10 µL were taken and transferred to the microplates' wells. In each well, 230 µL of a reagent mix were added containing: EDTA (ethylenediaminetetraacetic acid) 3 mM, xanthine 3 mM and NBT (nitro blue tetrazolium chloride) 0.75 mM. In the end, 10 µL of xanthine oxidase were added to the wells to start the reaction. Negative controls (mix without sample) were included. After reading the absorbance at 560 nm every 5 min for a total period of 20 min, superoxide dismutase activity (SOD) was calculated using the equations for the % inhibition:

1) SOD Abs_{560}/min = (Abs_{560} final read – Abs_{560} initial read) / reaction time (min)

2) SOD % inhibition = ((Abs_{560}/min negative control – Abs_{560}/min sample) / (Abs_{560}/min negative control)) × 100

2.8.7 Total antioxidant capacity

To determine total antioxidant capacity (TAC) duplicates of 10 µL were taken from the samples to the microplates' wells. Additionally, in each well were added 10 µL of myoglobin 90 µM and 150 µL of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) 600 µM. To start the reaction, 40 µL of hydrogen peroxide 500 µM (0.0017 %) were added. Trolox standards (0 to 0.330 mM) were used to create the calibration curve (Kambayashi et al., 2009).

2.8.8 Lipid peroxidation

Lipid peroxidation (LPO) was quantified following the thiobarbituric acid reactive substances method (TBARS, (Uchiyama and Mihara, 1978). First, 50 µL of each original sample were placed in a microtube and 12.5 µL of sodium dodecyl sulfate (SDS) 8.1 %, 93.5 µL of trichloroacetic acid (TCA) 20 % and 93.5 µL of thiobarbituric acid (TBA) 1 % were added. To this mixture, 50.5 µL of Milli-Q grade ultrapure water was added to each microtube and vortexed for 30 s. Microtubes' lids were punctured with a needle and were incubated in boiling water for 10 min. Subsequently, they were placed on ice for a few minutes to cool and then, 62.5 µL of Milli-Q grade ultrapure water was added. Microtubes were then vortexed and centrifuged at 10.000 ×*g* for 5 min. Two portions of supernatant each with 150 µL were put into a 96-well microplate and absorbance was read at 530 nm. To quantify lipid peroxides, an eight-point calibration curve (0 to 0.3 µM) was calculated using malondialdehyde bis(dimethylacetal) standards (Merk, Germany) (Madeira et al., 2015).

2.9 Sample treatment for the quantification of glucose and glycogen

The 84 samples previously collected, weighed and frozen, at D14 and D28, from the acclimation assay (see section 2.6) were stored in 5 mL microtubes. After thawing, samples were homogenized in 4 mL of distilled water using a Dremel® Model 3000 homogenizer. To inactivate enzymatic activity, the

samples were boiled for 10 min (Huijing, 1970). Then, the supernatant was collected into another microtube and centrifuged at 10 000 x *g* for 15 min. After that the supernatant was transferred to new 5 mL tubes and frozen at -80ºC for posterior analyses. Sample wet weight was used to normalize glucose and glycogen quantifications.

2.9.1 Glucose quantification

Glucose was quantified according to Huijing (1970). First, 200 µL of each sample and 200 µL of 0.1 M of sodium acetate buffer pH 4.8 were mixed in a microtube and vortexed. Additionally, 400 µL of glucose oxidase mix (0.3 mg mL^{-1} glucose oxidase (Sigma–Aldrich #G6125-10kv, 1500 U mg^{-1}), 0.03 mg mL^{-1} peroxidase (Sigma–Aldrich #P8375-2kv, 250 U mg^{-1}), 0.1 mg mL^{-1} O-dianisidine dihydrochloride (Sigma – Aldrich #F5803-50 mg) in Tris-phosphate-glycerol buffer (300 mM trizma, 362 mM sodium phosphate monobasic monohydrate, 400 mL Glycerol from Sigma® and 600 mL ultrapure water, pH = 7) was added and incubated for one hour at 30 ºC. To stop the reaction, 800 µL of 5 M HCl was added, and two portions each with 200 µL of each sample were put into a 96-well microplate and absorbance was read in a microplate reader at 530 nm (BIO-RAD, Benchmark, USA). To quantify glucose units, an eight-point calibration curve (0 to 80 ug mL^{-1}) was calculated using glucose standards (Merck® Millipore).

2.9.2 Glycogen quantification

Glycogen was quantified through its degradation by enzymatic activity in glucose units (Huijing, 1970). Briefly, 200 µL of each sample, diluted to 1:40 previously, was added to each microtube. Subsequently, 200 µL of 50 µg mL^{-1} α-glucosidase (Sigma–Aldrich #10115-1G-F, 70 U mg^{-1}) and 80 µg mL^{-1} α-amylase (Sigma–Aldrich #A6380 – 25 mg, 1500 U mg^{-1}) in 0.1 M of sodium acetate buffer pH 4.8 were added, vortexed and incubated for one hour at 30 ºC in order to degrade glycogen into glucose. Then, to detect glucose units, 400 µL of glucose oxidase mix was added (see above section 2.9.1) and incubated for one more hour at 30 ºC. The reaction was stopped by adding 800 µL of 5 M HCl and two

duplicates each with 200 µL of each sample were put into a 96-well microplate and absorbance was read in a microplate reader at 530 nm (BIO-RAD, Benchmark, USA). To quantify glucose units, an eight-point calibration curve (0 to 80 ug mL^{-1}) was calculated using glycogen standards (Type II G8751–5g Lot#SLBX2021 from Sigma®). In addition, the final glycogen content (in glucose units) was calculated by subtracting the glucose background measured previously.

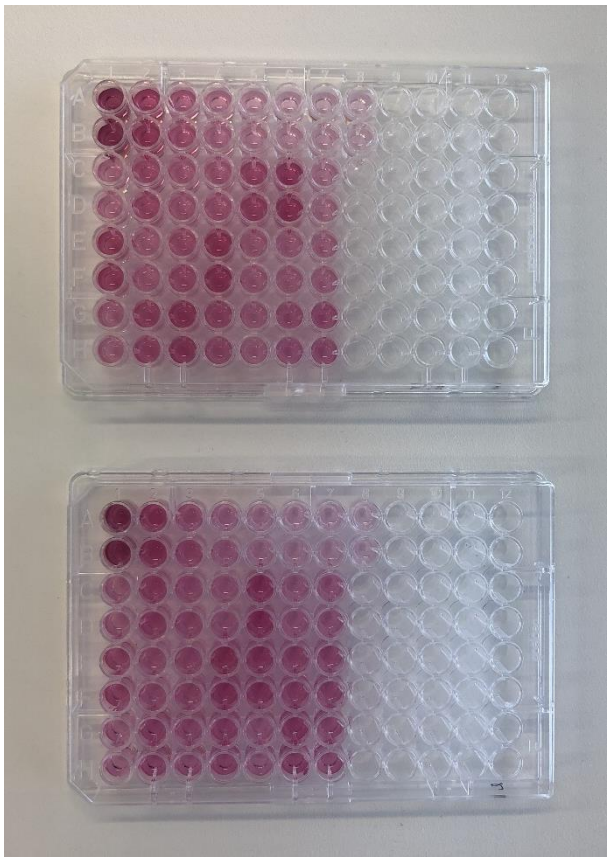

Figure 2.12 – Glycogen and glucose assays (Huijing, 1970) in 96-well microplates.

2.10 Sample treatment for the quantification of total lipids and fatty acid profiling

The remaining 84 samples collected, weighed and frozen, at D14 and D28, from the acclimation assay (see section 2.5) and 7 fish feed aliquots were stored in 5 mL microtubes and were freeze-dried in freeze dryer (Unicryo MC4L – 60ºC) connected to a Balston® vacuum pump during 3 days. Afterwards, the samples

were ground in a mortar with pestle to make the sample homogeneous and kept at -80ºC for posterior analyses.

2.10.1 Lipid extraction

Lipids in polychaetes and fish feed were extracted according to Bligh and Dyer (1959). Approximately 30 to 35 mg of freeze-dried and ground polychaete and fish feed aliquots were weighed in 10 mL glass test tubes with tight screw caps. To avoid degradation of lipids, the test tubes were kept in ice throughout the whole extraction. Following, 3.75 mL of CH_2Cl_2:MeOH (1:2 v/v) solution was added to the tubes, vortexed very well (2x) and left on ice during 15 min. After that, the tubes were vortexed again during 30 s and kept on ice for more 15 min. This step was repeated one more time and then 1.25 mL of CH_2Cl_2 was added and vortexed during 1 min. Thereafter, 1.25 mL of miliQ water was added, 1 min more of vortexing to homogenize and the tubes were centrifuged during 5 min at 98 ×*g* in order to separate organic and aqueous phases. After that, due to the lipids' higher density, the dichloromethane phase (lower phase) was collected with a glass pipet to another tube. To make sure that no lipids are discarded, 1.88 mL of CH_2Cl_2 was added again to the aqueous phase and was vortexed and centrifuged again. The organic phase was collected again to the other tube used previously. Tubes were then placed in a nitrogen stream to evaporate the CH_2Cl_2. Afterwards, 0.4 mL of CH_2Cl_2 were added and vortexed to dissolve the lipids and then transferred to vials previously dried in the lab oven at 105 ºC for 2h, in order to remove all existing humidity, and weighed. Then, 0.5 mL of CH_2Cl_2, were added and the last step was repeated. After all, the vials were placed to dry in nitrogen stream and subsequently weighed. The amount of lipid extract was determined following the equation:

Lipid extract = Initial vial weight – Final vial weight

In the end, the vials were kept on the freezer (-20°C) for posterior fatty-acid analysis.

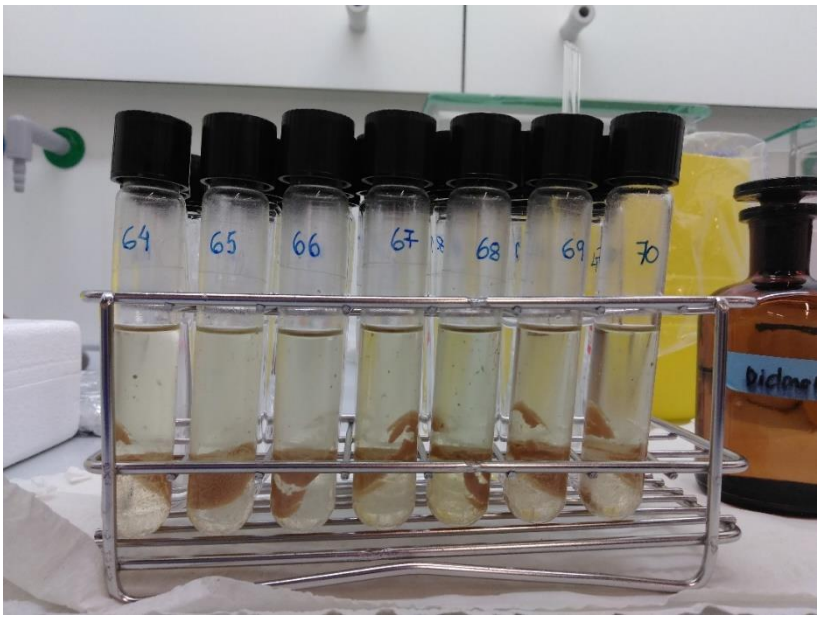

Figure 2.13 – Lipid extraction in the step with the organic phase (lower phase) ready to collect.

2.10.2 Fatty-acid analysis

Fatty acid methyl esters (FAMEs) of the total lipid extracts were obtained by transmethylation according to the method described by Aued-Pimentel et al. (2004). The lipid extracts frozen previously, were resuspended in 1 mL of dicloromethane and vortexed, and the volume that corresponded to 30 µg in each sample was transferred to dry test tubes (previously washed with *n*-hexane) and placed in nitrogen stream to evaporate the CH_2Cl_2. Then, 1 mL of the internal standard solution of a methyl ester fatty acid C19:0 in *n*-hexane (1 µg mL^{-1}) was added. In the same tube, 0.2 mL of methanolic solution KOH (2 M) and 2 mL saturated NaCl solution was added, followed by intense vortexing during 2 min. After centrifugation at 392 ×*g* for 5 min, the organic phase (higher phase) was collected to a microtube (previously washed with *n*-hexane) and dried under a nitrogen stream.

The resulting FAMEs were dissolved in hexane (60 µL) prior to injection and analysed by gas chromatography with a flame ionization detector (GC-FID) on an Agilent Technologies 7890 B. The detector and injector were kept at 250°C,

with hydrogen as carrier gas. Fatty acids (FAs) were separated in a fused-silica capillary column, DB-FFAP (30 m length, 0.32 mm internal diameter, 0.25 µm film thickness, J & W Scientifics) with the following temperature program: initial temperature of 75 ºC, 20 °C min^{-1} to 155 °C (for 4 min), 2 °C min^{-1} to 180 °C (for 16.5min), 4 °C min^{-1} to 250 °C (for 17.5 min) and a hold time in 250 ºC during 10min. The identification of FAMEs was done by matching retention times with a previously injected standards mixture (Supelco® 37 component FAMEs mix, Sigma-Aldrich) and by analysing one sample under identical chromatographic conditions by gas chromatography–mass spectrometry (GC–MS) with a mass spectrometer Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range m/z 50–550 in a 1 s cycle in a full scan mode acquisition and comparing the resulting mass spectra to reference spectra in the The LipidWeb© library (Christie, 2019).

Individual FAMEs peaks were integrated and normalized using the equipment's software, and identified considering the retention time of each FA relative to the standards mixture. In order to calculate absolute concentration of each FA, the area of each FA was divided by the area of the internal FA standard (C19:0) following Marques et al. (2018). Then, was multiplied by the internal FA standard concentration added in the beginning, and normalized by their dry weight (final concentrations of FA expressed as µg mg^{-1} of dry weight). Values of each FA in each treatment were reported as mean values ± standard deviation (SD) of final concentrations of FA expressed as µg mg^{-1} of dry weight. PUFA are defined as all FAMEs with ≥2 double bonds, but highly unsaturated fatty acids (HUFA, FAMEs with ≥4 double bonds) are considered separately.

2.11 Data analyses

2.11.1 Environmental data

Temperature data was transformed in digital support from the probes previously placed in the field. After that, descriptive statistics (means and SD, minimum and maximum values) were computed and data were plotted using the software GraphPad Prism v6.01 and edited in Adobe® Illustrator CC 2015.

2.11.2 Survival

After 28 days of exposure, a Two-way ANOVA, using temperature and salinity as independent variables, was performed to test significant differences between survival percentages data of the six different treatments. A bar graph was constructed to visualize de results and the tests were performed using a significance level (α) of 0.05, using the software GraphPad Prism v6.01.

2.11.3 Thermal tolerance limits

CT_{Max} was calculated as the arithmetic mean of all end points reached by the individuals (Mora and Ospína, 2001; Madeira et al., 2012a; Massamba-N'Siala et al., 2012), following the equation:

$$CT_{Max}\,(\text{treatment}) = \sum(T_{end\text{-}point}\,n) / n,$$

where $T_{end\text{-}point}$ is the temperature at which the end-point was observed for any given individual and *n* the number of individuals that were in the treatment.

Coefficients of variation (% CV) were calculated to estimate intraspecific variability in CT_{Max} variation as follows:

$$\%\ CV = (SD/Mean) \times 100,$$

where SD is the standard deviation of all CT_{Max} individual values and Mean is the respective average of CT_{Max}.

Acclimation capacity was also calculated as:

$$\text{Acclimation capacity } (^{o}C) = CT_{Max}\ \text{treatment} - CT_{Max}\ \text{control},$$

where CT_{Max} treatment means the CT_{Max} of each treatment group subtracted by CT_{Max} of control group.

The thermal safety margin (TSM, i.e. warming tolerance) was calculated (usually used for the intertidal and subtidal environments) as the difference

between the mean CT_{Max} previously determined and the Maximum Habitat Temperature (MHT) determined through field measurements at the study area (Madeira et al., 2019a). TSM indicates how near this species is from its upper thermal limit.

Acclimation response ratio (ARR) is a measure of heat tolerance plasticity and calculated as the change in heat tolerance (ΔCT_{Max}) for a given change in acclimation temperature (ΔT_{acc}) (Claussen, 1977):

$$ARR = \frac{\Delta CT_{Max}}{\Delta T_{acc}},$$

An ARR = 0 means no plasticity whereas ARR = 1 means complete physiological compensation for environmental warming.

Exploratory analyses were performed on the dataset to test normality (D'Agostino-Pearson normality test) and homoscedasticity (Bartlett's test). A Two-way ANOVA, using temperature and salinity as independent variables, was performed to test significant differences between the thermal limits of the six different treatments and a box & whiskers graphs were plotted to visualize the results. All tests were performed using a significance level (α) of 0.05 and using the software GraphPad Prism v6.01.

2.11.4 Biomarkers' analyses

Biomarker's data were analyzed in order to detect differences in biomarkers' levels between treatments in each time point.

A Principal Component Analysis (PCA) was carried out for each time point to explore data structure and detect biomarkers that contribute to explain the variance in the dataset. This analysis allows the visualization of inter-individual and inter-group differences in biomarker's response, representing differences between all treatments from each biomarker along the first two axes.

The resemblance matrix among samples was obtained with the Euclidean distance, following a log (x+1) transformation in order to make data distributions less skewed and reduce heterocedasticity. The variables were also normalized using autoscaling in PRIMER-E (normalization corresponds to the ratio of the

difference between each data entry (x_i) and the respective mean ($\bar{x}$), and the standard deviation (sd) of a given variable ($\frac{xi - \bar{x}}{sd}$) thus rescaling different types of variables into a common measurement scale). The Euclidean similarity matrix was analysed using the PERMANOVA + add-on in PRIMER-E v.6 (Anderson et al., 2008) following unrestricted permutation of the raw data (9999 permutations) and the calculation of type III sums of squares. The null hypotheses tested were: 1) there are no significant differences in terms of biomarkers' response among treatments in each time point; 2) there are no significant differences in terms of biomarkers' response among time points for each treatment.

Similarity percentages (SIMPER) in PRIMER-E v.6, were determined to describe the differences in individual biomarkers among the treatments. SIMPER identifies the biomarkers that contribute the most to the variations in the assemblage patterns recorded based on an Euclidean distance matrix. Only the biomarkers that cumulatively contributed up to 80% of the dissimilarities recorded were selected to characterize the differences between the treatments.

Factorial ANOVAs followed by Tukey's post-hocs were then carried out for each biomarker to assess how temperature, salinity, day and their interactions influence each specific biomarker response.

Additionally, in MetaboAnalyst (v5.0), a clustered heatmap was constructed to provide an intuitive visualization of the concentration pattern of each biomarker between treatments in each timepoint. The data matrix of their absolute contents was pre-processed by generalized log transformation and auto scaling and the distance measure was Euclidean distance, while the clustering algorithm between the biomarkers was the Ward's linkage. A significance level of 0.05 was considered in all analyses. Graphs were edited using Adobe® Illustrator CC 2015.

2.11.5 Classes and fatty acids' profiles

The concentration of each fatty acid was represented by the absolute abundance per replicate, per treatment in each time point.

Fatty acids' (FAs) profiles data were analyzed in order to detect differences in each FA and classes (saturated fatty acids ∑SFA, monounsaturated fatty acids

∑MUFA, polyunsaturated fatty acids ∑PUFA and highly unsaturated fatty acids ∑HUFA), between treatments in each time point. The resemblance matrix among samples was obtained with the Bray-Curtis similarity coefficient, following a log (x+1) transformation (see above). A preliminary one-way analysis of similarity (ANOSIM) was performed in PRIMER-E v.6 with PERMANOVA + to detect significant differences in FA profiles and classes of *H. diversicolor* between treatment groups in both time points. Moreover, in MetaboAnalyst (v5.0), a clustered heatmap was generated to provide an intuitive visualization of the concentration pattern in FA profiles and between classes in each time point. The data matrix of their absolute contents was pre-processed by generalized log transformation and auto scaling and the distance measure was Pearson correlation, while the clustering algorithm between the FA was the Ward's linkage. Additionally, assumption tests were performed on the ∑*n*-3 PUFA, ∑*n*-6 PUFA, ratio ∑*n*-3/*n*-6 all FA, ARA (arachidonic acid, 20:4*n*-6), EPA (eicosapentaenoic acid, 20:5*n*-3) and DHA (docosahexaenoic acid, 22:6*n*-3) datasets to test normality (Shapiro-Wilk normality test) and homoscedasticity (Bartlett's test). Then, factorial ANOVAs followed by Tukey's post-hocs were carried out in Statistica (version 12.0, Statsoft Inc.USA), to assess how temperature, salinity, day and their interactions influence each specific variable. A significance level of 0.05 was considered in all analyses. Graphs were edited using Adobe® Ilustrator CC 2015.

2.11.6 Energy reserves

Data of energy reserves (total protein, total lipid and glycogen content) was tested for normality (D'Agostino-Pearson normality test) and homoscedasticity (Bartlett's test). Since, at least, one of the assumptions of a specific test (e.g. normality or homoscedasticity) were not met, a nonparametric test was used (Kruskal-Wallis). The objective was to evaluate if the quantify of energy reserves differs between the treatments, in both time points and additionally, if there are differences in the same treatment between two time points. All tests were performed using a significance level of 0.05 and using the software GraphPad Prism v6.01 and graphs were edited using Adobe® Ilustrator CC 2015.

3. Results

3.1 Environmental data

3.1.1 Temperature

Water temperature in the intertidal zone was highly variable during summer months (July, August and September 2019), with steep daily fluctuations (Fig. 3.1). The maximum – MHT (Maximum Habitat Temperature) (35.7 ºC) was registered on 2nd of August and the minimum (12.4 ºC) on 11th of September (Fig. 10). Mean daily water temperatures derived from half-hourly readings ranged from 17.5 °C to 27.7 °C during the three sampling months (Fig. 3.2).

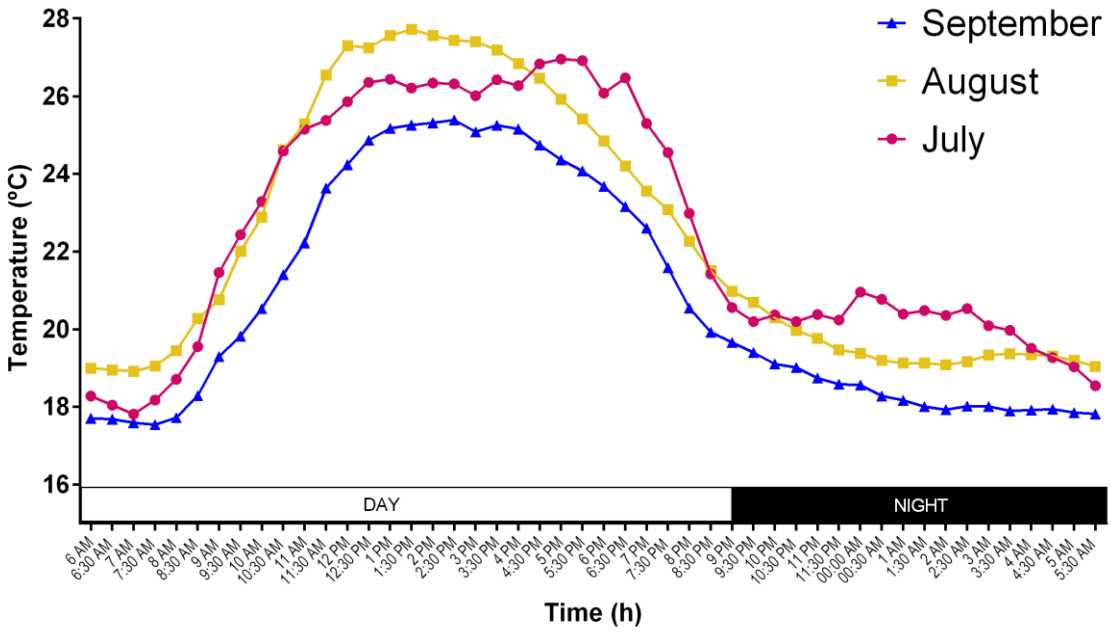

Figure 3.1 - Mean daily water temperatures derived from half-hourly readings (HOBO® data logger Water Temp Pro v2 U22 – 001) in the three sampling months (July, August and September 2019).

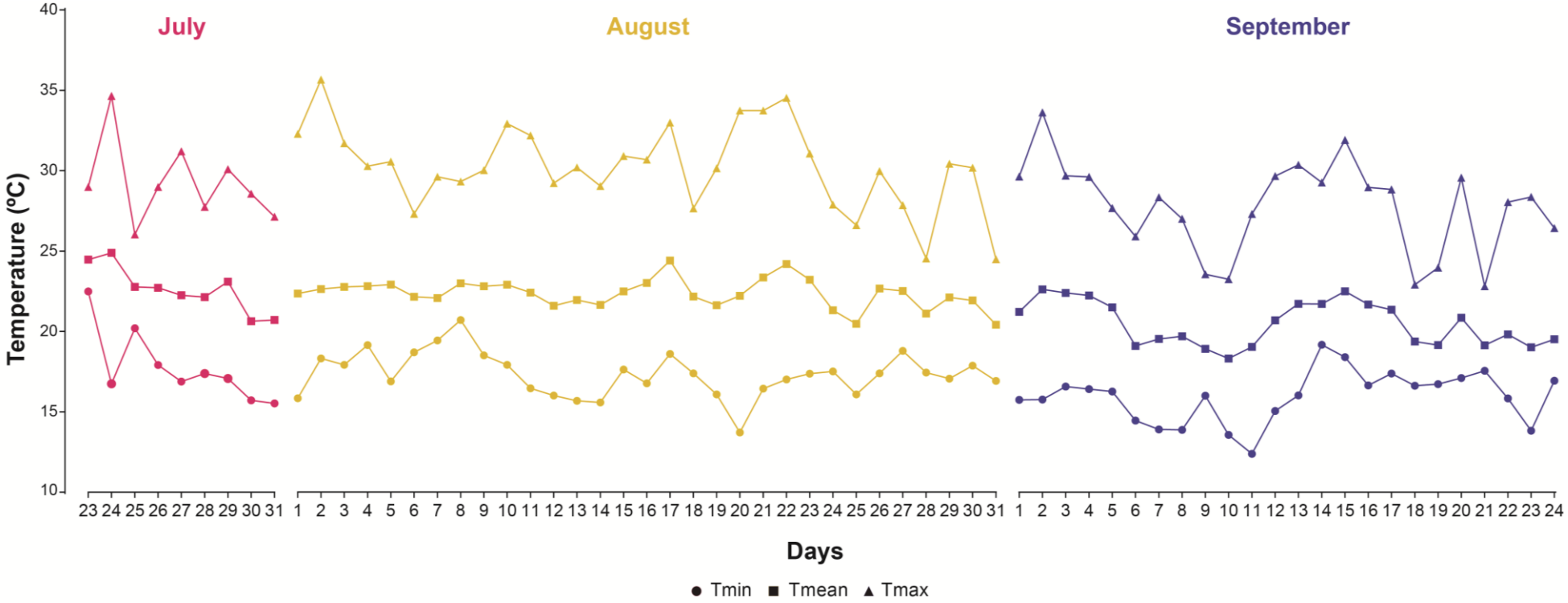

Figure 3.2 – Temperature variations (minimum (Tmin), mean (Tmean), maximum (Tmax) temperatures) from each sampling day during July, August and September 2019.

3.1.2 Salinity and pH

Salinity data collected in the sampling location showed a mean of 34.96 ± 0.82, a minimum of 32.9 and a maximum of 36.3 during the sampling months. For pH data, the mean value was 8.11 ± 0.37, a minimum of 7.41 and a maximum of 8.694. Generally, these two factors did not vary so much, as opposed to temperature, which is a fairly variable parameter.

3.2 Survival

The two-way ANOVA output, showed significant differences in survival percentages between salinity values (F = 5.28, *df* = 1, *p* value = 0.03). However, Tukey's post-hocs were non-significant for all pairwise comparisons. No significant differences between acclimation temperatures (F = 3.03, *df* = 2, *p* value = 0.06) and no significant interaction between both factors (F = 0.40, *df* = 2, *p* value = 0.68) was detected (Figure 3.3).

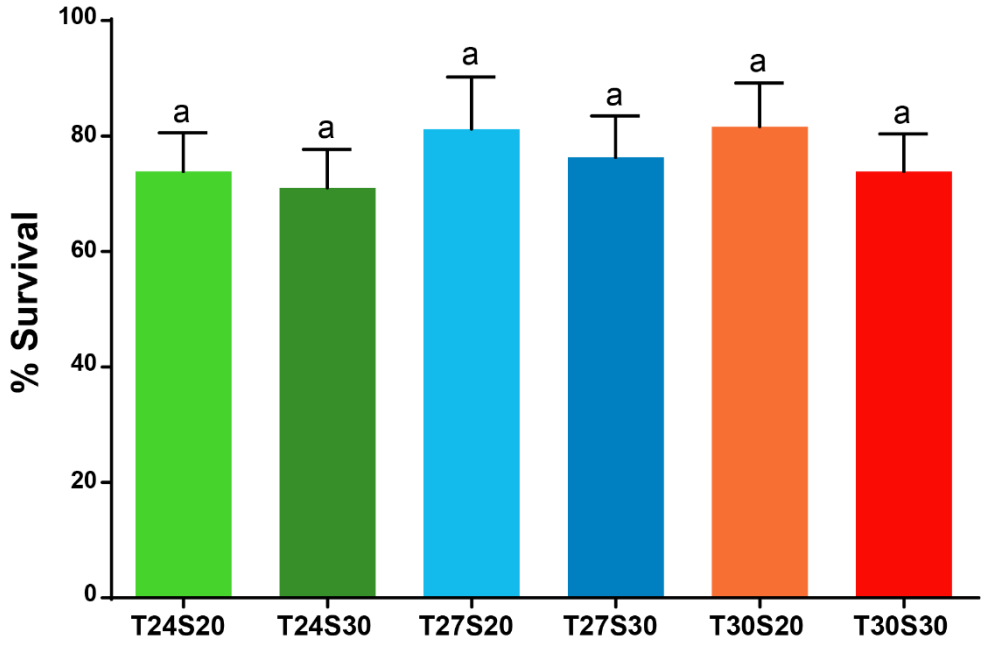

Figure 3.3 – Graph representation (mean ± SD, n=7 biological replicates per treatment) of survival percentages of *Hediste diversicolor* of the six treatments after 28 days exposed to different combinations of temperature (T 24, 27 and 30 ºC) and salinity (S 20 and 30) No significant differences were detected between treatment groups (Tukey's post hocs *p* value > 0.05 in all pairwise comparisons).

3.3 Thermal tolerance limits

Hediste diversicolor specimens showed CT_{Max} values ranging from 37.3 to 39.1 ºC between all treatments (Table 3.2). Species intraspecific variability, given by % CV, was generally low (1.1 %) including within each treatment (≤ 0.85 %) (Fig. 3.4).

Significant differences in CT_{Max} were observed between acclimation temperatures (*p* value < 0.0001) and between salinity values (*p* value = 0.02), however no significant interaction was detected between both factors (*p* value = 0.09) (Table 3.1). CT_{Max} values were higher in treatments with salinity of 30, when compared to treatments with salinity of 20, within the same temperature group. Moreover, TSM (Thermal Safety Margin) also increased as the acclimation temperature of the treatment was higher. Thus the highest TSM value was detected in the group exposed to 30 ºC and salinity 30 (TSM=2.94 ºC). In opposite, Acclimation Response Ratio (ARR) values decreased as the acclimation temperature was higher (Table 3.2).

Table 3.1 – Main results of Two-way ANOVA of Critical Thermal Maximum (CT_{Max}) values of *Hediste diversicolor* after 28 days of exposure to different combinations of temperature (T 24, 27 and 30 ºC) and salinity (S 20 and 30). Significant results are marked in bold.

	df	*F*	*p*
Temperature	**2**	**56.00**	**< 0.0001**
Salinity	**1**	**5.267**	**0.0235**
Temperature x Salinity	2	2.465	0.0893

Table 3.2 - CT_{Max} (Critical Thermal Maximum) values (minimum, maximum and mean and SD). Acclimation capacity (CT_{Max} treatment - CT_{Max} control) and ARR (Acclimation Response Ratio) for temperatures tested for different scenarios (+3 and +6 ºC) combined with salinity (20 and 30). TSM (Thermal Safety Margin) for each treatment, where TSM is the difference between CT_{Max} mean from each treatment and MHT (Maximum Habitat Temperature).

		Treatments (n = 21)					
		T24S20	**T24S30**	**T27S20**	**T27S30**	**T30S20**	**T30S30**
CTMax (ºC)	Minimum	37.40	37.30	37.60	37.80	38.10	38.0
	Maximum	38.20	38.30	38.60	38.80	38.90	39.10
	Mean ± SD	37.83 ±0.29	37.79 ±0.31	38.08 ±0.31	38.31 ±0.33	38.41 ±0.23	38.60 ±0.32
	Acclimation capacity (ºC)	------	------	0.25	0.52	0.58	0.81
	TSM (ºC)	2.17	2.13	2.42	2.65	2.75	2.94
	ARR	------	------	0.33	0.33	0.13	0.18

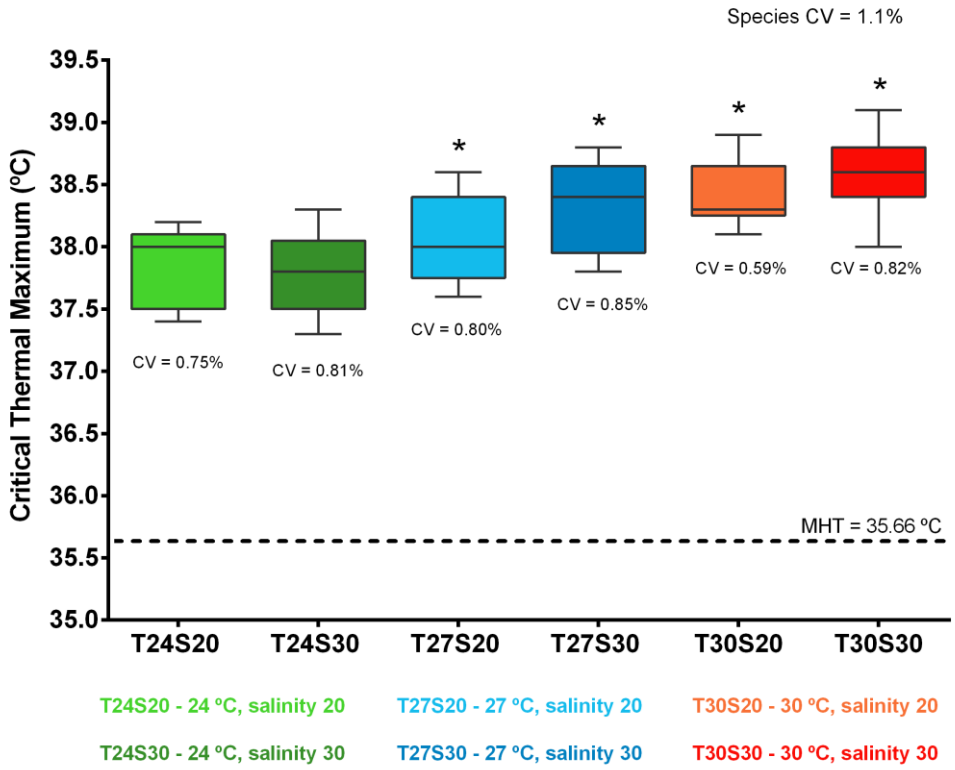

Figure 3.4 – Box and whiskers plots representing Critical Thermal Maximum (CT_{Max}) values (minimum, maximum, median and quartiles) from each treatment (n = 21). Species % CV (Coefficient of Variation) of all specimens of *Hediste diversicolor* on top (1.1 %) and for each treatment under each box. MHT (Maximum Habitat Temperature) value collected from sampling location (dashed line). Significant differences (T27S30, T30S20, T30S30 p-value < 0.0001 and T27S20 p-value = 0.0239) when compared to control group (T24S30) are marked with an asterisk (*).

3.4 Biomarkers' analyses

Overall, *Hediste diversicolor* showed physiological changes in response to increased temperature combined with salinity along the 28 days of acclimation.

Based on the Principal Component Analysis (PCA) done for both time points (Figure 3.5) the variance explained by PC1 and PC2 at day 14 (53.6% and 19.30%, for PC1 and PC2, respectively) was higher than in at day 28 (43.7% and 19.3% for PC1 and PC2, respectively). Overall, in both PCAs, the same biomarkers had a positive or negative correlation with the PCA axes. At day 14, CAT, SOD, TAC, Hsp70 and Ub had a highly positive correlation (Pearson r > |0.7|) to PC1 while at day 28, those biomarkers had a highly negative correlation (Pearson r > | -0.7|) (Fig. 3.5). Additionally, at day 14, LPO was negatively correlated to PC2, whereas at day 28 it was positively correlated to this axis (PC2) (Figure 3.5). A slight differentiation of temperature groups, especially 30 ºC, is visible at D14.

Main results from PERMANOVA, showed a significant effect of the factor temperature (p = 0.001), factor salinity (p = 0.0467), factor day (time point) (p = 0.0001) and the interaction of temperature with salinity (p = 0.0441), temperature with day (p = 0.0065) and the interaction of the three factors (temperature, salinity and day, - p = 0.0172), rejecting the null hypothesis of additive effects (Table 3.3). Since the interaction of the three factors was significant, the pair-wise tests showed which treatments are different from each other for that interaction. At day 14 (D14), when comparing the same salinity but different temperatures, the treatment groups T24S20 *vs* T30S20, T24S30 *vs* T27S30 and T27S30 *vs* T30S30 showed significant differences between them (Table 3.4). However, at day 28 (D28), no significant differences were observed between temperature treatments within salinity of 30, but all the temperature treatments within salinity of 20 were significantly different (Table 3.4). On the other hand, at day 14, significant differences between salinity groups (20 *vs* 30) were only observed within temperature of 24 ºC (p = 0.0031, Table 3.4). An opposite response was detected, at day 28, in which significant differences between salinity groups (20 *vs* 30) were observed for the temperatures of 27 (p = 0.0123) and 30 ºC (p = 0.0096) (Table 3.4). Comparisons between the different time points showed

significant differences in almost all treatments, except in the control group T24S30 and the high temperature and low salinity group T30S20 (Table 3.4).

Table 3.3 – Main test results from PERMANOVA based on biomarkers' data with factors: temperature (24, 27 and 30 ºC), salinity (20 and 30) and day (time points 14 and 28) and their interaction in species *Hediste diversicolor*. Significant results are shown in bold ($p < 0.05$).

	Pseudo – F	*p*
Temperature	**3.4992**	**0.0010**
Salinity	**2.5774**	**0.0467**
Day	**18.39**	**0.0001**
Temperature x Salinity	**2.1912**	**0.0441**
Temperature x Day	**3.0598**	**0.0065**
Salinity x Day	0.6626	0.5926
Temperature x Salinity x Day	**2.6718**	**0.0172**

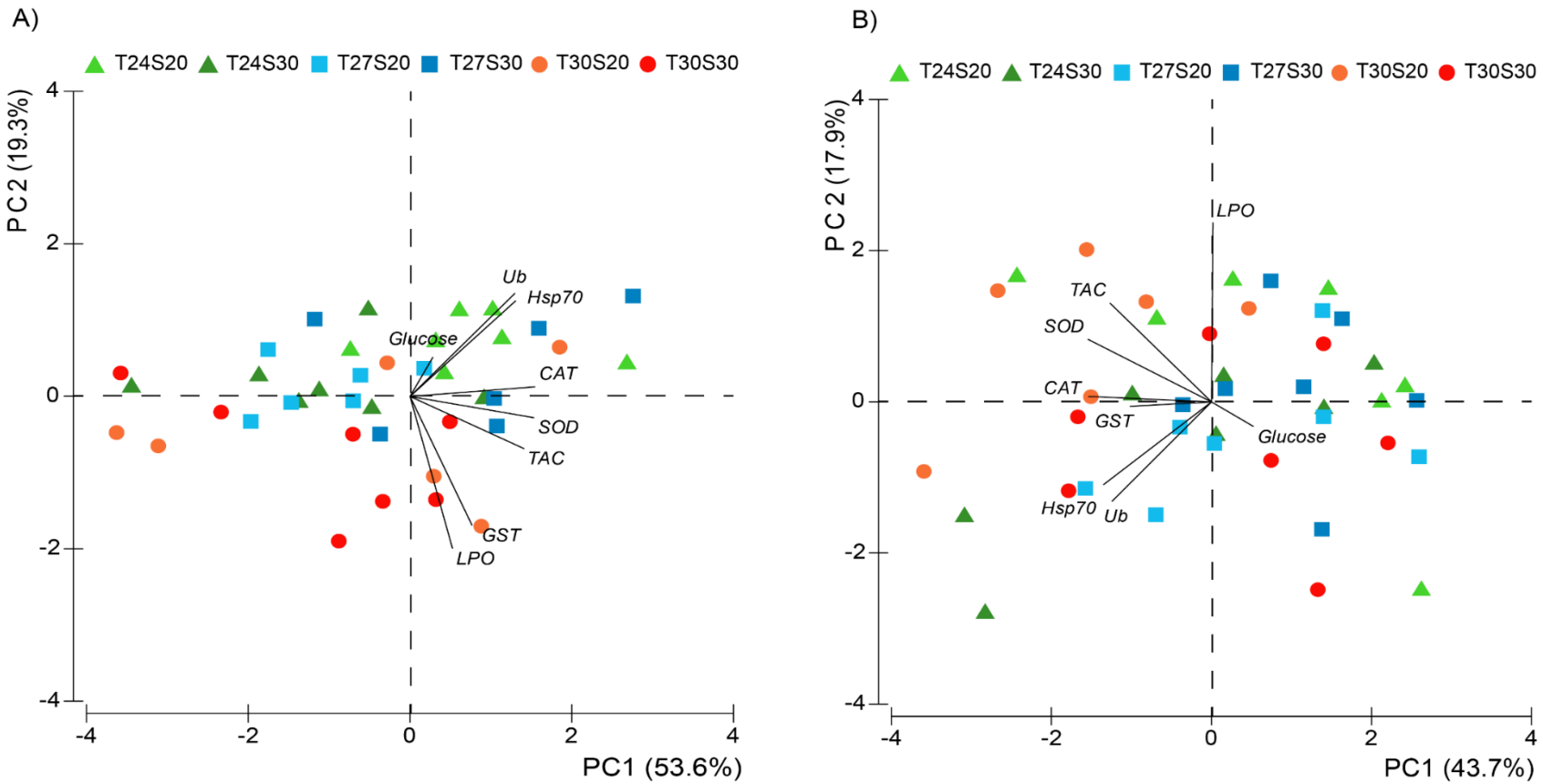

Figure 3.5 – Principal Component Analysis (PCA) of the biomarkers' response in *Hediste diversicolor* carried out for each sampled time point of the experiment: A) day 14 (D14) B) day 28 (D28). Levels of factors: Temperature (T24, T27, T30) Salinity (S20, S30). Biomarkers: Hsp70 – heat shock protein 70 kDa, Ub – total ubiquitin, GST – glutathione-S-transferase, CAT – catalase, SOD – superoxide dismutase, TAC – total antioxidant capacity, LPO – lipid peroxidation.

Table 3.4 - Pairwise tests from PERMANOVA for the significant interaction (Temperature x Salinity x Day) shown above in main test results from PERMANOVA (Table 3.3). Significant results are shown in bold ($p < 0.05$).

Pairwise tests			
Levels of factor	**Within levels of factors**	Groups	*p*
Temperature (24, 27 and 30 ºC)	Salinity 20 Day 14	T24S20D14 vs T27S20D14	0.073
		T24S20D14 vs T30S20D14	**0.033**
		T27S20D14 vs T30S20D14	0.218
	Salinity 20 Day 28	T24S20D28 vs T27S20D28	**0.0038**
		T24S20D28 vs T30S20D28	**0.0016**
		T27S20D28 vs T30S20D28	**0.017**
	Salinity 30 Day 14	T24S30D14 vs T27S30D14	**0.0102**
		T24S30D14 vs T30S30D14	0.0873
		T27S30D14 vs T30S30D14	**0.0079**
	Salinity 30 Day 28	T24S30D28 vs T27S30D28	0.2117
		T24S30D28 vs T30S30D28	0.319
		T27S30D28 vs T30S30D28	0.1475
Salinity (20 and 30)	Temperature 24 ºC Day 14	T24S20D14 vs T24S30D14	**0.0031**
	Temperature 24 ºC Day 28	T24S20D28 vs T24S30D28	0.1642
	Temperature 27 ºC Day 14	T27S20D14 vs T27S30D14	0.1692
	Temperature 27 ºC Day 28	T27S20D28 vs T27S30D28	**0.0123**
	Temperature 30 ºC Day 14	T30S20D14 vs T30S30D14	0.4727
	Temperature 30 ºC Day 28	T30S20D28 vs T30S30D28	**0.0096**
Day (14 and 28)	Temperature 24 ºC Salinity 20	T24S20D14 vs T24S20D28	**0.0007**
	Temperature 24 ºC Salinity 30	T24S30D14 vs T24S30D28	0.0799
	Temperature 27 ºC Salinity 20	T27S20D14 vs T27S20D28	**0.0159**
	Temperature 27 ºC Salinity 30	T27S30D14 vs T27S30D28	**0.0002**
	Temperature 30 ºC Salinity 20	T30S20D14 vs T30S20D28	0.3687
	Temperature 30 ºC Salinity 30	T30S30D14 vs T30S30D28	**0.0145**

After pairwise tests from the PERMANOVA, the SIMPER was done for the groups that were significantly different, in order to detect which biomarkers contribute the most for those differences. In general, the biomarkers that contribute the most for the groups' average squared distance were Glucose, GST, Ub and CAT (Table 3.6 and 3.7). The first three highest percentages that had contributed the most were all from biomarker Glucose, between treatments at day 28 (T24S20 *vs* T27S20 – 42.2%, T27S20 *vs* T27S30 – 31.3% and T24S20 *vs* T30S20 – 28.28% see Table 3.6 and 3.7). When different temperatures were compared, the biomarkers that contributed more were Glucose, GST, Ub and Hsp70 (Table 3.6), whereas when salinities were compared only Glucose and Ub were the most contributing biomarkers (Table 3.7). Between treatments in different time points the most contributing biomarkers were Glucose, CAT and LPO.

Overall, significant effects were observed for all biomarkers in specific factors or interaction of factors in factorial ANOVAs (Table 3.5). Main effects of temperature were significant for GST and LPO, while main effects of salinity were significant for GST and Glucose. Main effects of day were significant for all biomarkers except Ub. Two-way interactions between temperature and day were detected for Hsp70, CAT and GST. Two-way interactions between temperature and salinity were detected for SOD and LPO. Particularly, significant temperature-salinity-time interactions were recorded for Hsp70, Ub and CAT (Table 3.5). Focused on the significant results of factorial ANOVAs and SIMPER, Tukey's post- hocs were used to confirm major changes in relevant biomarkers. With regards to temperature, there was a significant increase in GST with rising temperature at both salinity levels, especially evident in day 14. In the same day, both Hsp70 and Ub maintain stable levels across temperatures (within salinity 20). However, at control salinity (30), Hsp70 shows a trend of increase at moderate temperature (27 ºC) followed by a significant decrease at extreme temperature (30 ºC). Focusing on day 28, there are no changes in Hsp70 or Ub across temperatures for both salinity levels. Still, over exposure time, Hsp70 decreased at control and moderate temperature (24 and 27 ºC), but the same does not happen at 30 ºC, as levels remain stable throughout time. Moreover,

glucose was depleted after 28 days at higher temperature, especially under low salinity (20) (Annex 7.1).

Regarding salinity, Tukey's post-hocs showed that glucose levels were lower in salinity 20 when compared to salinity 30, with a concomitant increase in GST activity at salinity 20. However, no significant differences were detected for Ub, despite its relevant contribution to discriminate salinity groups in the SIMPER. Finally, with regards to exposure time, there was an overall decrease in biomarker levels throughout time, except in glucose, which was significantly higher at day 28 when compared to day 14.

To further understand the overall metabolic changes in *H. diversicolor*, under these treatments over the course of time, clustered heatmaps were constructed for each time point. The biomarkers analysed clearly distinguish the metabolic profile of ragworms exposed to different temperatures and salinities and were visualized by changing colours, ranging from red (relative increase) or to blue (relative decrease) in the treatments (Figure 3.6).

Table 3.5 - Statistical analyses factorial ANOVAs showing main effects of temperature (24ºC, 27ºC, 30ºC) salinity (20,30) and time (14 and 28 days) and their interactive effects on selected biomarkers from the species *Hediste diversicolor*. Levels of significance: *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001, ns: non-significant (p > 0.05). Significant results (p value ≤ 0.05) are indicated (asterisk) and bold. Hsp70 heat shock protein 70, Ub total ubiquitin, CAT catalase, GST glutathione-S-transferase, SOD superoxide dismutase, LPO lipid peroxidation, TAC total antioxidant capacity, Temp Temperature, T*S*D temperature*salinity*day.

	Hsp70		Ub		CAT		GST		SOD		TAC		LPO		Glucose	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Temperature	1.34	ns	1.77	ns	0.35	ns	**26.89**	***	0.06	ns	0.00	ns	**3.95**	*	1.20	ns
Salinity	0.26	ns	0.31	ns	0.45	ns	**6.22**	*	1.54	ns	2.07	ns	0.14	ns	**10.64**	**
Day	**10.56**	**	3.91	ns	**47.73**	***	**16.44**	***	**25.92**	***	**19.75**	***	**18.52**	***	**11.33**	**
Temp*Salinity	2.68	ns	0.56	ns	0.94	ns	3.06	ns	**3.68**	*	1.48	ns	**3.69**	*	1.93	ns
Temp*Day	**8.42**	***	1.86	ns	**3.55**	*	**4.69**	*	1.59	ns	0.88	ns	2.27	ns	2.55	ns
Salinity*Day	1.55	ns	1.11	ns	0.45	ns	1.44	ns	0.45	ns	0.03	ns	0.47	ns	0.06	ns
T*S*D	**4.49**	**	**5.10**	**	**3.76**	*	0.72	ns	2.84	ns	0.92	ns	0.22	ns	2.80	ns

Table 3.6 – Similarity percentage analysis (SIMPER) identifying which biomarkers contribute to the differences recorded between treatments with different temperatures in species *Hediste diversicolor*. Groups of treatments are the significantly different ones showed in pairwise tests in PERMANOVA (Table 3.3). Levels of factors: Temperature (T24, T27, T30) Salinity (S20, S30) Day (D14, D28). Hsp70 heat shock protein 70, Ub total ubiquitin, CAT catalase, GST glutathione-S-transferase, SOD superoxide dismutase, LPO lipid peroxidation, TAC total antioxidant capacity,

Different temperature																		
T24S20D14 ***vs*** **T30**S20D14			**T24**S20D28 ***vs*** **T27**S20D28			**T24**S20D28 ***vs*** **T30**S20D28			**T27**S20D28 ***vs*** **T30**S20D28			**T24**S30D14 ***vs*** **T27**S30D14			**T27**S30D14 ***vs*** **T30**S30D14		
Biomarker	**Ind (%)**	**Cum (%)**	**Biomarker**	**Ind (%)**	**Cum (%)**	**Biomarker**	**Ind (%)**	**Cum (%)**	**Biomarker**	**Ind (%)**	**Cum (%)**	**Biomarker**	**Ind (%)**	**Cum (%)**	**Biomarker**	**Ind (%)**	**Cum (%)**
GST	26.54	26.54	Glucose	42.2	42.2	Glucose	28.28	28.28	GST	21.23	21.23	Ub	18.11	18.11	Hsp70	26.02	26.02
TAC	16.71	43.25	LPO	12.09	54.29	GST	18.23	46.51	SOD	16.75	37.98	TAC	16.94	35.06	Ub	15.15	41.17
LPO	15.73	58.99	Ub	11.1	65.39	CAT	13.34	59.85	Ub	14.7	52.68	SOD	15.76	50.82	TAC	13.2	54.36
SOD	10.53	69.52	SOD	8.96	74.35	SOD	12.81	72.65	Hsp70	13.4	66.08	GST	13.74	64.55	Glucose	11.61	65.98
Ub	9.67	79.18	GST	7.81	82.16	Hsp70	9.07	81.73	CAT	11.87	77.95	CAT	13.26	77.81	SOD	11.47	77.45
CAT	8.04	87.22							Glucose	9.48	87.43	Hsp70	11.77	89.58	CAT	11.11	88.56

Table 3.7 - Similarity percentage analysis (SIMPER) identifying which biomarkers contribute to the differences recorded between treatments with different salinities (left) and in different time points (days) (right) in species *Hediste diversicolor*. Groups of treatments are the significantly different ones showed in pairwise tests in PERMANOVA (Table 3.3). Levels of factors: Temperature (T24, T27, T30) Salinity (S20, S30) Day (D14, D28). Hsp70 heat shock protein 70, Ub total ubiquitin, CAT catalase, GST glutathione-S-transferase, SOD superoxide dismutase, LPO lipid peroxidation, TAC total antioxidant capacity,

Different salinity									**Different time points (days)**											
T24**S20**D14 ***vs*** T24**S30**D14			T27**S20**D28 ***vs*** T27**S30**D28			T30**S20**D28 ***vs*** T30**S30**D28			T24S20**D14** ***vs*** T24S20**D28**			T27S20**D14** ***vs*** T27S20**D28**			T27S30**D14** ***vs*** T27S30**D28**			T30S30**D14** ***vs*** T30S30**D28**		
Biomarker	**Ind (%)**	**Cum (%)**	**Biomarker**	**Ind (%)**	**Cum (%)**	**Biomarker**	**Ind (%)**	**Cum (%)**	**Biomarker**	**Ind (%)**	**Cum (%)**	**Biomarker**	**Ind (%)**	**Cum (%)**	**Biomarker**	**Ind (%)**	**Cum (%)**	**Biomarker**	**Ind (%)**	**Cum (%)**
Ub	22.33	22.33	Glucose	31.83	31.83	Glucose	22.33	22.33	Glucose	21.85	21.85	CAT	20.64	20.64	CAT	20.82	20.82	LPO	20.85	20.85
SOD	16.22	38.55	LPO	14.86	46.7	GST	16.95	39.29	CAT	18.03	39.88	Hsp70	18.37	39.02	Hsp70	17.7	38.52	Glucose	18.03	38.88
TAC	14.83	53.38	Ub	14.36	61.06	SOD	15.62	54.91	Hsp70	14.01	53.89	Ub	17.85	56.87	TAC	16.15	54.67	Hsp70	13.7	52.58
Glucose	13.21	66.59	GST	13.45	74.5	Hsp70	14.19	69.1	SOD	13.63	67.52	SOD	15.4	72.27	SOD	13.56	68.23	SOD	13.3	65.88
Hsp70	11.93	78.52	Hsp70	12.65	87.15	Ub	11.33	80.43	Ub	12.97	80.49	Glucose	10.36	82.63	Ub	12.37	80.6	GST	12.09	77.97
CAT	10.34	88.87																CAT	11.3	89.27

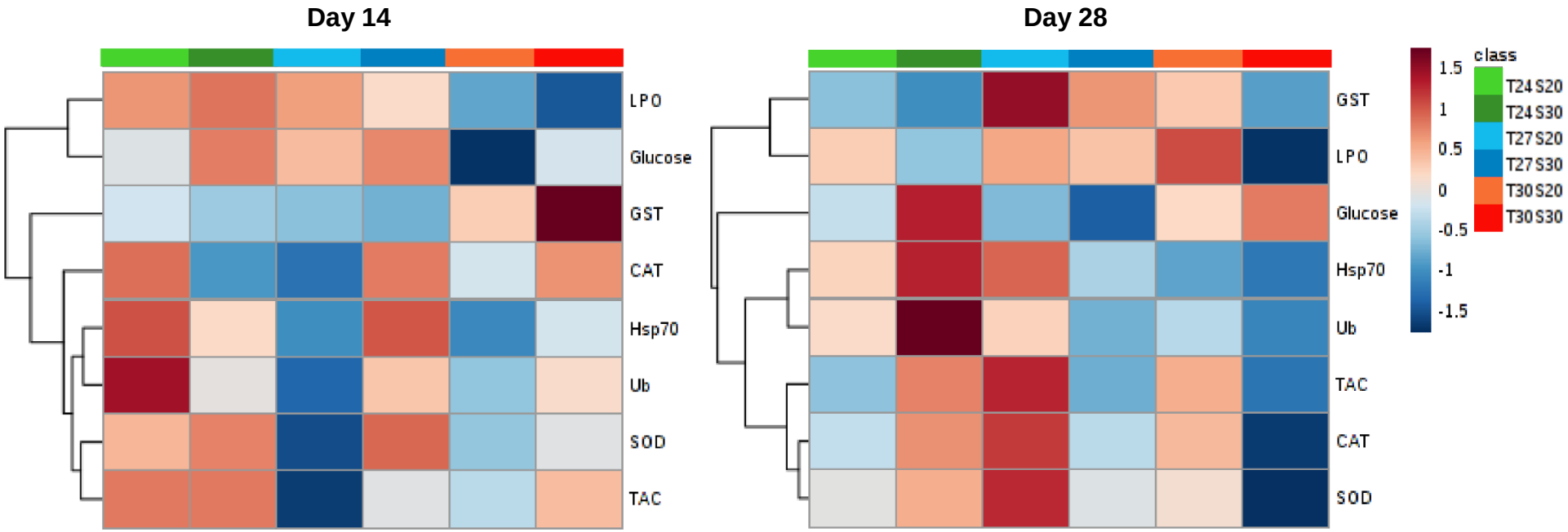

Figure 3.6 - Clustered heatmaps (distance measure: Euclidean; clustering algorithm: Ward's linkage) of biomarkers concentrations in *Hediste diversicolor* exposed to temperature (24, 27 and 30 ºC) and salinity combinations (20 and 30) in each sampling time point (14 and 28 days). The data matrix represents the glog and auto-scaled biomarkers concentrations' in which red cells represent a relative increase (up-accumulation) and blue cells represent a relative decrease in biomarkers concentrations (down-accumulation). Hsp70 heat shock protein 70, Ub total ubiquitin, CAT catalase, GST glutathione-S-transferase, SOD superoxide dismutase, LPO lipid peroxidation, TAC total antioxidant capacity,

3.5 Classes and fatty acids' profiles

Saturated FA (SFA) ranged from 0.07 to 0.20 µg mg $^{-1}$, monounsaturated FAs (MUFA) from 0.07 to 0.21 µg mg $^{-1}$, polyunsaturated FA (PUFA) from 0.06 to 0.17 µg mg $^{-1}$ and highlyunsaturated FA (HUFA) from 0.07 to 0.22 µg mg $^{-1}$ of all fatty acids identified in *Hediste diversicolor* under different treatments along the sampled time points (Table 3.8). The major SFA was palmitic acid (16:0; PA) ranging from 0.05 to 0.13 µg mg $^{-1}$ and the dominant MUFA was vaccenic acid (18:1*n*-7) ranging from 0.02 to 0.08 µg mg $^{-1}$. In PUFA the most abundant FA was linoleic acid (18:2*n*-6) with a minimum of 0.02 µg mg $^{-1}$ and a maximum of 0.07 µg mg $^{-1}$. Eicosapentaenoic acid (EPA) (20:5*n*-3) was the FA with the higher values between all the HUFA (from 0.01 to 0.10 µg mg $^{-1}$) (Table 3.8).

The ANOSIM performed using all FA and their classes (i.e. SFA, MUFA, PUFA and HUFA) showed significant differences among some treatments. Between different time points, the treatments that were significantly different in almost all classes were those of salinity 30 (Table 3.9). Except in SFA class, the T30S20 treatment showed that it is significantly different between the two time points (p = 0.022). In the same time point, first at day 14, temperature groups within salinity 20 showed significant differences in FA quantities. For instance, the groups of treatments T24S20 *vs* T27S20 and T27S20 *vs* T30S20 were both significantly different in all FAs and classes. The groups T24S20 and T30S20 were different in overall FA, especially MUFA, PUFA and HUFA. Differences were also detected between temperature groups within salinity 30, namely T24S30 *vs* T27S30 (differences in all FA, and HUFA class) and T24S30 *vs* T30S30 (which differed in HUFA). When comparing salinity treatments within each temperature, the group of treatments T24S20 *vs* T24S30 showed only a significant difference in HUFA class. However, at higher temperature levels, differences between salinities are stronger. For instance, all FA and all classes differ between groups T27S20 *vs* T27S30. Moreover, all FA and MUFA and PUFA classes are significantly different between T30S20 *vs* T30S30 (Table 3.9). At day 28, the ANOSIM done for MUFA class had a main result not significant (R = 0.075, p = 0.085) (grey columns in Table 3.9), however, the treatments T24S20 *vs* T24S30 showed a significant difference in this class and all other classes (Table 3.9).

Moreover, the group of treatments T30S20 *vs* T30S30 showed significant differences in all classes, except in MUFA and PUFA (Table 3.9). At day 28, differences between temperatures within each salinity were only detected for HUFA, between groups T24S20 *vs* T27S20.

Table 3.8 - Fatty acid profile (data presented as absolute abundances of total fatty acids expressed as µg mg^{-1} of dry weight) of the species *Hediste diversicolor* (values are means of 7 replicates ± SD) from the six treatments (T24S20 – 24 ºC salinity 20, T24S30 – 24 ºC salinity 30, T27S20 – 27 ºC salinity 20, T27S30 – 27 ºC salinity 30, T30S20 – 30 ºC salinity 20 and T30S30 – 30 ºC salinity 30) sampled in two time points (D14 – day 14 and D28 – day 28). SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; and HUFA - highlyunsaturated fatty acids.

	T24S20		T24S30		T27S20		T27S30		T30S20		T30S30	
Fatty Acid	**Day 14**	**Day 28**	**Day 14**	**Day 28**	**Day 14**	**Day 28**	**Day 14**	**Day 28**	**Day 14**	**Day 28**	**Day 14**	**Day 28**
14:0	0.0032 ± 0.0010	0.0031 ± 0.0006	0.0039 ± 0.0018	0.0069 ± 0.0036	0.0067 ± 0.0023	0.0052 ± 0.0037	0.0024 ± 0.0012	0.0064 ± 0.0019	0.0055 ± 0.0009	0.0041 ± 0.0021	0.0032 ± 0.0015	0.0063 ± 0.0030
15:0	0.0016 ± 0.0005	0.0012 ± 0.0003	0.0019 ± 0.0006	0.0045 ± 0.0025	0.0043 ± 0.0016	0.0038 ± 0.0024	0.0021 ± 0.0010	0.0038 ± 0.0015	0.0028 ± 0.0008	0.0031 ± 0.0015	0.0022 ± 0.0009	0.0043 ± 0.0016
16:0	0.0630 ± 0.0154	0.0505 ± 0.0136	0.0606 ± 0.0086	0.1015 ± 0.0448	0.1302 ± 0.0446	0.0957 ± 0.0574	0.0521 ± 0.0235	0.1251 ± 0.0358	0.0770 ± 0.0214	0.0585 ± 0.0151	0.0597 ± 0.0239	0.1043 ± 0.0434
17:0	0.0026 ± 0.0008	0.0028 ± 0.0006	0.0035 ± 0.0005	0.0064 ± 0.0026	0.0077 ± 0.0023	0.0053 ± 0.0026	0.0039 ± 0.0018	0.0057 ± 0.0020	0.0043 ± 0.0015	0.0045 ± 0.0011	0.0037 ± 0.0010	0.0058 ± 0.0017
18:0	0.0347 ± 0.0117	0.0234 ± 0.0067	0.0293 ± 0.0073	0.0415 ± 0.0105	0.0518 ± 0.0180	0.0421 ± 0.0237	0.0249 ± 0.0085	0.0550 ± 0.0125	0.0353 ± 0.0114	0.0287 ± 0.0041	0.0291 ± 0.0110	0.0432 ± 0.0168
∑SFA	**0.1051 ± 0.0270**	**0.0809 ± 0.0210**	**0.0993 ± 0.0184**	**0.1608 ± 0.0613**	**0.2007 ± 0.0685**	**0.1521 ± 0.0882**	**0.0732 ± 0.0443**	**0.1960 ± 0.0524**	**0.1249 ± 0.0345**	**0.0990 ± 0.0178**	**0.0978 ± 0.0377**	**0.1639 ± 0.0661**
16:1*n*-9	0.0050 ± 0.0032	0.0044 ± 0.0012	0.0068 ± 0.0040	0.0123 ± 0.0060	0.0112 ± 0.0036	0.0082 ± 0.0059	0.0043 ± 0.0019	0.0099 ± 0.0033	0.0089 ± 0.0031	0.0084 ± 0.0038	0.0055 ± 0.0021	0.0107 ± 0.0053
18:1*n*-9	0.0114 ± 0.0033	0.0146 ± 0.0033	0.0142 ± 0.0037	0.0288 ± 0.0164	0.0355 ± 0.0168	0.02676 ± 0.0166	0.0160 ± 0.0079	0.0300 ± 0.0106	0.0192 ± 0.0064	0.0153 ± 0.0040	0.0132 ± 0.0050	0.0224 ± 0.0064
18:1*n*-7	0.0250 ± 0.0086	0.0384 ± 0.0116	0.0275 ± 0.0107	0.0684 ± 0.0311	0.0822 ± 0.0308	0.0600 ± 0.0306	0.0308 ± 0.0150	0.0743 ± 0.0295	0.0467 ± 0.0152	0.0524 ± 0.0198	0.0322 ± 0.0083	0.0751 ± 0.0324
18:1*n*-5	0.0080 ± 0.0025	0.0097 ± 0.0021	0.0099 ± 0.0020	0.0219 ± 0.0112	0.0257 ± 0.0075	0.0162 ± 0.0092	0.0099 ± 0.0047	0.0201 ± 0.0076	0.0131 ± 0.0042	0.0128 ± 0.0044	0.0100 ± 0.0033	0.0172 ± 0.0067
20:1*n*-7	0.0085 ± 0.0028	0.0071 ± 0.0015	0.0107 ± 0.0016	0.0208 ± 0.0105	0.0244 ± 0.0105	0.0192 ± 0.0108	0.0111 ± 0.0053	0.0207 ± 0.0075	0.0138 ± 0.0058	0.0115 ± 0.0028	0.0109 ± 0.0046	0.0185 ± 0.0052
20:1*n*-11	0.0085 ± 0.0031	0.0092 ± 0.0021	0.0089 ± 0.0027	0.0223 ± 0.0092	0.0283 ± 0.0099	0.0225 ± 0.0128	0.0104 ± 0.0050	0.0253 ± 0.0098	0.0152 ± 0.0049	0.0165 ± 0.0060	0.0106 ± 0.0023	0.0223 ± 0.0097
$22:1^{(7)}$	0.0069 ± 0.0011	0.0395 ± 0.0078	0.0051 ± 0.0019	0.0091 ± 0.0042	0.0114 ± 0.0030	0.0077 ± 0.0036	0.0047 ± 0.0019	0.0095 ± 0.0042	0.0090 ± 0.0027	0.0099 ± 0.0033	0.0064 ± 0.0025	0.0116 ± 0.0047
∑MUFA	**0.0733 ± 0.0224**	**0.0956 ± 0.0209**	**0.0832 ± 0.0246**	**0.1836 ± 0.0815**	**0.2187 ± 0.0796**	**0.1606 ± 0.0859**	**0.0747 ± 0.0481**	**0.1899 ± 0.0670**	**0.1259 ± 0.0370**	**0.1267 ± 0.0404**	**0.0888 ± 0.0184**	**0.1777 ± 0.0681**
18:2*n*-6	0.0250 ± 0.0070	0.0362 ± 0.0126	0.0280 ± 0.0100	0.0712 ± 0.0338	0.0776 ± 0.0296	0.0601 ± 0.0338	0.0323 ± 0.0150	0.0730 ± 0.0299	0.0443 ± 0.0133	0.0475 ± 0.0166	0.0292 ± 0.0059	0.0706 ± 0.0288
18:3*n*-3	0.0055 ± 0.0017	0.0071 ± 0.0015	0.0059 ± 0.0020	0.0135 ± 0.0063	0.0144 ± 0.0048	0.0107 ± 0.0065	0.0055 ± 0.0025	0.0131 ± 0.0045	0.0089 ± 0.0027	0.0094 ± 0.0034	0.0062 ± 0.0014	0.0129 ± 0.0053
$20:2^{(5,11)}$	0.0043 ± 0.0013	0.0126 ± 0.0026	0.0044 ± 0.0012	0.0113 ± 0.0059	0.0139 ± 0.0066	0.0128 ± 0.0082	0.0055 ± 0.0022	0.0124 ± 0.0049	0.0083 ± 0.0026	0.0078 ± 0.0024	0.0053 ± 0.0011	0.0105 ± 0.0034
20:2*n*-6	0.0203 ± 0.0066	0.0058 ± 0.0009	0.0124 ± 0.0039	0.0428 ± 0.0213	0.0419 ± 0.0158	0.0394 ± 0.0272	0.0157 ± 0.0077	0.0398 ± 0.0149	0.0198 ± 0.0065	0.0217 ± 0.0077	0.0167 ± 0.0066	0.0327 ± 0.0142
$22:2^{(7,13)}$	0.0057 ± 0.0017	0.0068 ± 0.0027	0.0061 ± 0.0015	0.0145 ± 0.0078	0.0161 ± 0.0083	0.0148 ± 0.0096	0.0073 ± 0.0031	0.0141 ± 0.0049	0.0100 ± 0.0034	0.0086 ± 0.0026	0.0078 ± 0.0025	0.0127 ± 0.0034
$22:3^{(7,13,16)}$	0.0020 ± 0.0005	0.0064 ± 0.0013	0.0021 ± 0.0009	0.0069 ± 0.0034	0.0067 ± 0.0041	0.0067 ± 0.0054	0.0033 ± 0.0012	0.0061 ± 0.0021	0.0051 ± 0.0017	0.0047 ± 0.0012	0.0045 ± 0.0013	0.0067 ± 0.0017
∑PUFA	**0.0626 ± 0.0105**	**0.0767 ± 0.0176**	**0.0590 ± 0.0169**	**0.1602 ± 0.0755**	**0.1706 ± 0.0681**	**0.1445 ± 0.0890**	**0.0597 ± 0.0374**	**0.1586 ± 0.0590**	**0.0965 ± 0.0278**	**0.0998 ± 0.0317**	**0.0696 ± 0.0130**	**0.1461 ± 0.0549**
20:4*n*-6 (AA)	0.0102 ± 0.0023	0.0189 ± 0.0044	0.0157 ± 0.0033	0.0300 ± 0.0162	0.0376 ± 0.0152	0.0281 ± 0.0139	0.0185 ± 0.0103	0.0306 ± 0.0126	0.0189 ± 0.0055	0.0155 ± 0.0024	0.0133 ± 0.0049	0.0248 ± 0.0047
20:5*n*-3 (EPA)	0.0332 ± 0.0067	0.0140 ± 0.0033	0.0404 ± 0.0043	0.0914 ± 0.0536	0.1079 ± 0.0414	0.0775 ± 0.0484	0.0465 ± 0.0229	0.0854 ± 0.0322	0.0531 ± 0.0156	0.0423 ± 0.0071	0.0363 ± 0.0164	0.0606 ± 0.0163
22:4*n*-6	0.0091 ± 0.0026	0.0024 ± 0.0003	0.0149 ± 0.0031	0.0242 ± 0.0134	0.0334 ± 0.0127	0.0209 ± 0.0105	0.0162 ± 0.0079	0.0229 ± 0.0096	0.0162 ± 0.0054	0.0133 ± 0.0021	0.0137 ± 0.0056	0.0196 ± 0.0043
22:5*n*-3	0.0076 ± 0.0015	0.0104 ± 0.0029	0.0089 ± 0.0016	0.0167 ± 0.0086	0.0205 ± 0.0071	0.0150 ± 0.0097	0.0102 ± 0.0042	0.0164 ± 0.0056	0.0136 ± 0.0045	0.0124 ± 0.0037	0.0104 ± 0.0040	0.0152 ± 0.0050
22:6*n*-3 (DHA)	0.0120 ± 0.0048	0.0069 ± 0.0012	0.0109 ± 0.0033	0.0257 ± 0.0108	0.0258 ± 0.0092	0.0215 ± 0.0142	0.0125 ± 0.0046	0.0236 ± 0.0084	0.0159 ± 0.0052	0.0162 ± 0.0045	0.0132 ± 0.0020	0.0208 ± 0.0063
∑HUFA	**0.0721 ± 0.0161**	**0.0826 ± 0.0171**	**0.0907 ± 0.0094**	**0.1881 ± 0.1000**	**0.2252 ± 0.0844**	**0.1630 ± 0.0957**	**0.0890 ± 0.0578**	**0.1788 ± 0.0676**	**0.1177 ± 0.0337**	**0.0998 ± 0.0162**	**0.0869 ± 0.0314**	**0.1410 ± 0.0345**

Table 3.9 - Similarity values (ANOSIM) between all fatty acids (FA), saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) and highlyunsaturated (HUFA) fatty acids of the species *Hediste diversicolor* subjected to different treatments of: temperature (24, 27, 30 ºC) combined with salinity (20 and 30) over the time of the experiment (day 14 - D14 and day 28 - D28. The grey columns mean a non-significant main result for MUFA (R = 0.075, p = 0.085).

		All FA		SFA		MUFA		PUFA		HUFA	
		R	***p***	**R**	***p***	**R**	***p***	**R**	***p***	**R**	***p***
	T24S20D14 *vs* T24S20D28	0.089	0.166	-0.013	0.436	0.083	0.178	0.079	0.197	0.025	0.317
	T24S30D14 *vs* T24S30D28	**0.350**	**0.009**	0.140	0.083	**0.375**	**0.014**	**0.463**	**0.005**	**0.257**	**0.011**
	T27S20D14 *vs* T27S20D28	0.172	0.086	0.186	0.071	0.132	0.109	0.085	0.176	0.173	0.084
	T27S30D14 *vs* T27S30D28	**0.314**	**0.024**	**0.417**	**0.004**	**0.274**	**0.04**	**0.292**	**0.03**	0.123	0.112
	T30S20D14 *vs* T30S20D28	0.082	0.165	**0.279**	**0.022**	-0.046	0.605	0.01	0.342	0.031	0.270
	T30S30D14 *vs* T30S30D28	**0.395**	**0.017**	0.135	0.116	**0.487**	**0.006**	**0.566**	**0.004**	**0.358**	**0.027**
Day 14	T24S20 *vs* T24S30	0.059	0.21	-0.097	0.927	-0.086	0.929	0.091	0.191	**0.391**	**0.022**
	T24S20 *vs* T27S20	**0.812**	**0.0006**	**0.480**	**0.004**	**0.818**	**0.0006**	**0.821**	**0.0006**	**0.894**	**0.0006**
	T24S20 *vs* T30S20	**0.457**	**0.005**	0.117	0.097	**0.410**	**0.017**	**0.593**	**0.002**	**0.362**	**0.015**
	T24S30 *vs* T27S30	**0.156**	**0.05**	0.190	0.051	0.03	0.248	-0.019	0.478	**0.192**	**0.013**
	T24S30 *vs* T30S30	0.128	0.121	0.05	0.250	-0.027	0.473	0.094	0.146	**0.329**	**0.022**
	T27S20 *vs* T27S30	**0.384**	**0.01**	**0.407**	**0.01**	**0.412**	**0.01**	**0.377**	**0.013**	**0.313**	**0.023**
	T27S20 *vs* T30S20	**0.283**	**0.01**	**0.235**	**0.014**	**0.202**	**0.029**	**0.246**	**0.013**	**0.353**	**0.012**
	T27S30 *vs* T30S30	0.012	0.322	-0.054	0.67	0.123	0.101	0.137	0.08	0.001	0.348
	T30S20 *vs* T30S30	**0.206**	**0.05**	0.017	0.33	**0.319**	**0.011**	**0.421**	**0.005**	0.075	0.173
Day 28	T24S20 *vs* T24S30	**0.275**	**0.017**	**0.296**	**0.005**	**0.293**	**0.028**	**0.279**	**0.024**	**0.271**	**0.009**
	T24S20 *vs* T27S20	0.08	0.146	0.086	0.164	0.038	0.261	0.086	0.144	**0.135**	**0.048**
	T24S20 *vs* T30S20	0.078	0.184	0.035	0.296	0.092	0.179	0.082	0.195	0.029	0.261
	T24S30 *vs* T27S30	-0.043	0.555	0.042	0.256	-0.047	0.598	-0.073	0.716	-0.081	0.686
	T24S30 *vs* T30S30	-0.038	0.57	-0.038	0.55	-0.097	0.896	-0.057	0.653	0.038	0.307
	T27S20 *vs* T27S30	0.079	0.195	0.217	0.061	-0.017	0.43	-0.021	47.1	0.004	0.352
	T27S20 *vs* T30S20	0.019	0.323	0.018	0.367	-0.042	0.602	-0.026	0.53	0.003	0.42
	T27S30 *vs* T30S30	-0.027	0.528	-0.048	0.746	-0.077	0.83	-0.09	0.948	0.02	0.288
	T30S20 *vs* T30S30	**0.238**	**0.041**	**0.394**	**0.016**	0.089	0.176	0.141	0.98	**0.334**	**0.027**

To further understand the overall metabolic changes in *H. diversicolor*, under these treatments over the course of time, clustered heatmaps (using correlation analysis) were constructed for each time point, using fatty acid absolute concentrations (µg mg $^{-1}$), illustrating the relative increase (red) or decrease (blue) values in the treatments (Figure 3.7, 3.8 and 3.9). The identified FA and classes clearly distinguish the metabolic profile between treatments. The first main result observed from the heatmap at day 14, is that the treatment T27S20 had the highest amount of all fatty acids between all the treatments (Figure 3.7 and 3.8). Looking at each treatment, the treatments with salinity of 20

(T24S20, T27S20 and T30S20) showed a decrease in FA class quantity over the course of time (from day 14 to day 28) (Figure 3.9). In the other hand, the treatments with salinity 30 (T24S30, T27S30, T30S30) increase their quantity in the 2nd time point (D28) (Fig. 3.7 and 3.9). When comparing salinity treatments, the heatmap showed that FA levels are similar between S20 and S30 within the 24 ºC treatment at day 14, corroborating ANOSIM results. However, differences were detected between salinities within the 27 and 30 ºC treatments (Fig. 3.7 and 3.8). Overall, at day 28, there were differences between salinity treatments within each tested temperature (especially at 24 ºC and 30 ºC), suggesting that salinity is a determining factor shaping FA quantities in *Hediste diversicolor* (Figure 3.7, 3.8 and 3.9).

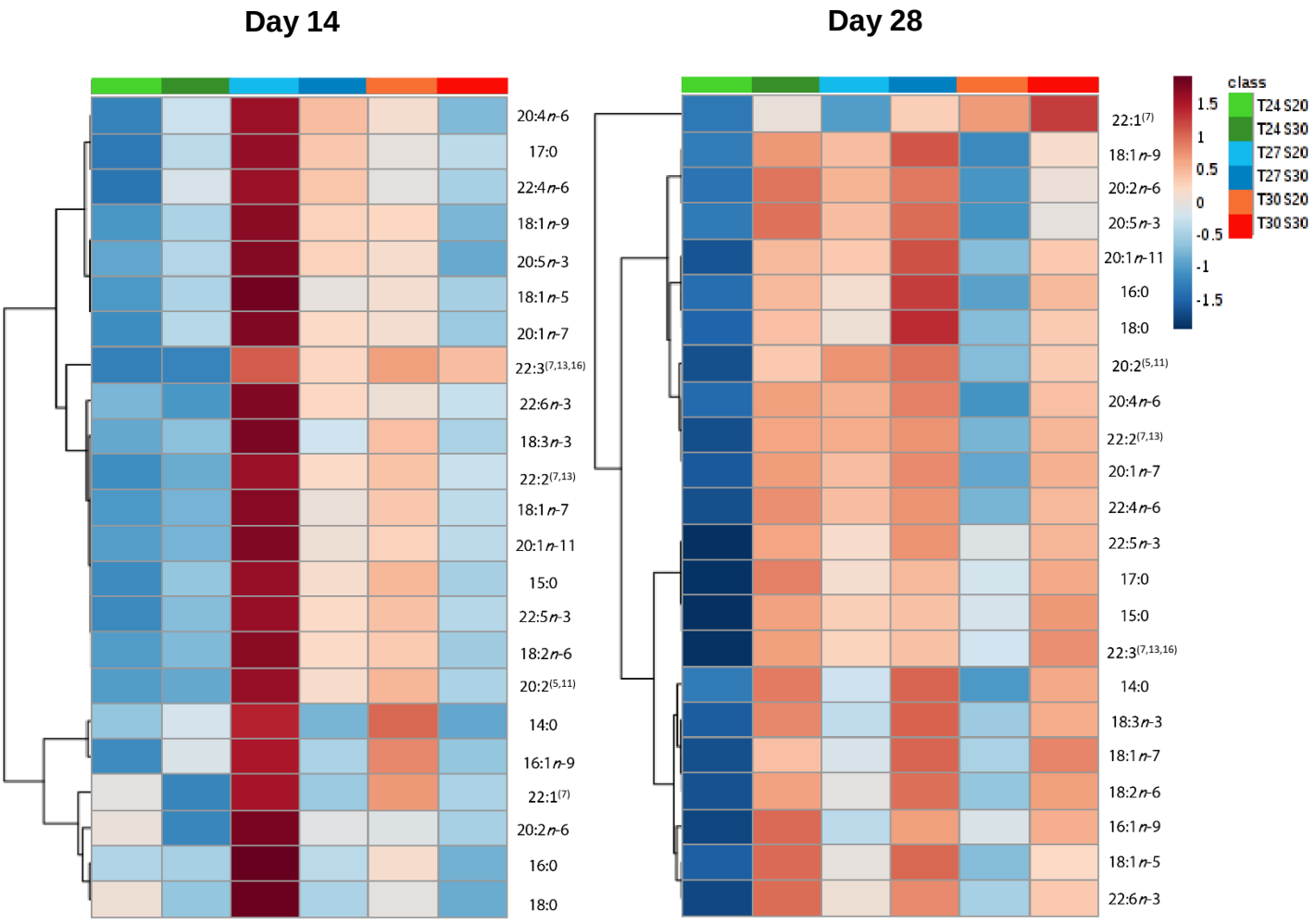

Figure 3.7 – Clustered heatmaps (distance measure: Pearson correlation; clustering algorithm: Ward's linkage) of fatty acid concentrations in *Hediste diversicolor* exposed to temperature (24, 27 and 30 ºC) and salinity combinations (20 and 30) in each sampling time point (14 and 28 days). All FAs identified are represented. The data matrix represents the glog and auto-scaled FA concentrations in which red cells represent a relative increase in FA concentrations (up-accumulation) and blue cells represent a relative decrease in FA concentrations (down-accumulation).

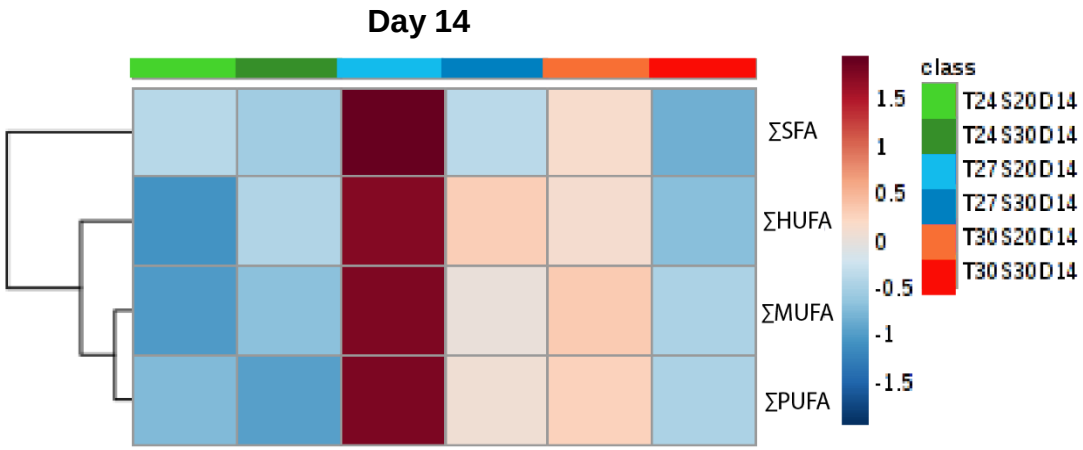

Figure 3.8 - Clustered heatmap (distance measure: Pearson correlation; clustering algorithm: Ward's linkage) of concentrations sum of FA classes in *Hediste diversicolor* exposed to temperature (24, 27 and 30 ºC) and salinity combinations (20 and 30) after 14 days of exposure. The data matrix represents the glog and auto-scaled FA class concentrations in which red cells represent a relative increase in FA class concentrations (up-accumulation) and blue cells represent a relative decrease in FA class concentrations (down-accumulation). SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; and HUFA - highlyunsaturated fatty acids.

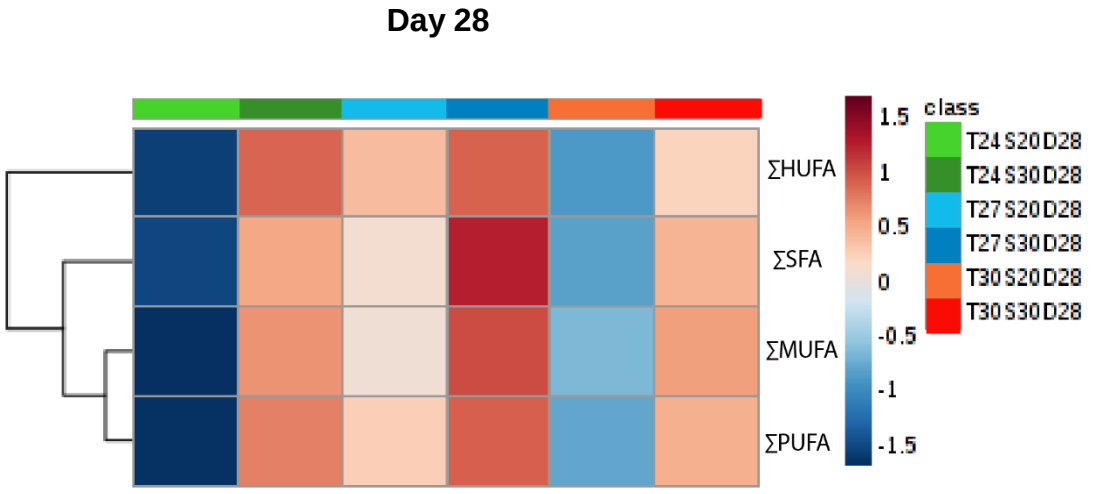

Figure 3.9 - Clustered heatmap (distance measure: Pearson correlation; clustering algorithm: Ward's linkage) of concentrations sum of FA classes in *Hediste diversicolor* exposed to temperature (24, 27 and 30 ºC) and salinity combinations (20 and 30) after 28 days of exposure. The data matrix represents the glog and auto-scaled FA class concentrations in which red cells represent a relative increase in FA class concentrations (up-accumulation) and blue cells represent a relative decrease in FA class concentrations (down-accumulation). SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; and HUFA - highlyunsaturated fatty acids.

Overall, significant effects were observed for ∑*n*-3 PUFA, ∑*n*-6 PUFA, ratio of total ∑*n*-3/*n*-6, ARA, EPA and DHA FAs in specific factors or interaction of factors in factorial ANOVAs (Table 3.10). Main effects of temperature were significant for all, except for ratio of total ∑*n*-3/*n*-6, while main effects of salinity were non-significant for all the tested FA or classes. Main effects of day were significant for ∑*n*-3 PUFA, ratio of total ∑*n*-3/*n*-6, ARA and DHA. Two-way interactions between temperature and salinity were detected for all, except for ratio of total ∑*n*-3/*n*-6. Particularly, non-significant temperature-salinity-time interactions were recorded for all the variables (Table 3.10).

Regarding the significant results of factorial ANOVAs, Tukey's post- hocs were used to confirm major changes in relevant variables (Annex 7.2). With regards to temperature, all variables show a significant increase from control temperature (24 ºC) to moderate temperature (27 ºC) followed by a significant decrease at extreme temperature (30 ºC). This pattern is especially evident at salinity 20, given the significant interaction found between temperature and salinity for all variables except ratio ∑*n*-3/*n*-6. Overall, at salinity of 30, all remained constant until moderate temperature (27 ºC) and then a slight decrease to extreme temperature (30 ºC). With regards to the day, ∑*n*-3 PUFA, ARA and DHA fatty acids increase from day 14 to day 28, as opposed to the ratio ∑*n*-3/*n*-6, which tends to decrease from day 14 to day 28. For the two way interaction between salinity and day, all the significant variables (∑*n*-3 PUFA, ∑*n*-6 PUFA, ARA, EPA and DHA) at salinity of 20, showed a constant pattern, instead of salinity 30 in which the variables increase from day 14 to day 28 (Annex 7.2). Given the interaction of temperature, salinity and day, ∑*n*-3 PUFA and ∑*n*-6 PUFA showed significant differences between treatments at different time points. The treatment of 27 ºC and salinity 20 at day 14 showed be the mostly different from the rest of the treatments in both types of omega (∑n-3 PUFA and ∑n-6 PUFA) (Figure 3.10 and 3.11).

Table 3.10 - Statistical analyses factorial ANOVAs showing main effects of temperature (24 ºC, 27 ºC, 30 ºC) salinity (20,30) and time (14 and 28 days) and their interactive effects on ∑*n*-3 PUFA, ∑*n*-6 PUFA, ratio of total ∑*n*-3/*n*-6, ARA (arachidonic acid), EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) fatty acids from the species *Hediste diversicolor*. Levels of significance: *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001, ns: non-significant (p > 0.05). Significant results (p value ≤ 0.05) are indicated

	∑*n*-3 PUFA		∑*n*-6 PUFA		Total ∑*n*-3/*n*-6		20:4 *n*-6 (ARA)		20:5 *n*-3 (EPA)		22:6 *n*-3 (DHA)	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Temperature	**7.503**	***	**9.883**	***	0.522	ns	**10.448**	***	**8.756**	***	**3.958**	*
Salinity	0.049	ns	0.477	ns	0.001	ns	0.399	ns	0.031	ns	0.122	ns
Day	**4.566**	*	2.288	ns	**11.983**	***	**4.620**	*	3.929	ns	**7.952**	**
Temp*Salinity	**5.774**	**	**6.780**	**	1.549	ns	**6.016**	**	**5.897**	**	**4.000**	*
Temp*Day	1.109	ns	1.301	ns	0.351	ns	1.031	ns	1.362	ns	0.494	ns
Salinity*Day	**14.321**	***	**11.818**	***	0.067	ns	**12.365**	***	**13.823**	***	**13.341**	***
T*S*D	0.543	ns	0.691	ns	0.063	ns	0.519	ns	0.574	ns	0.589	ns

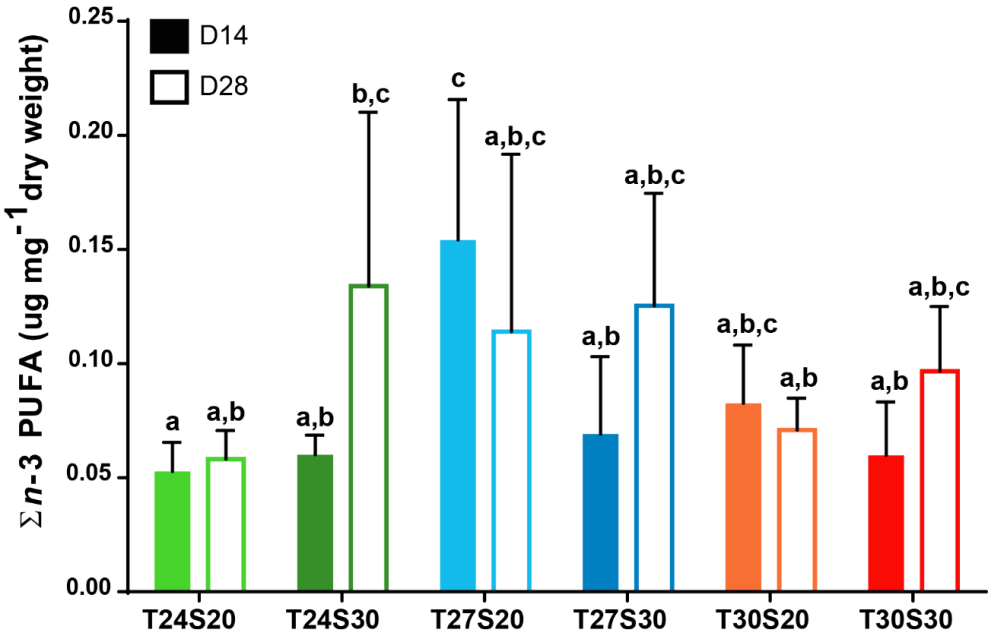

Figure 3.10 - ∑*n*-3 polyunsaturated fatty acids (PUFA) (µg mg^{-1} dry weight) of *Hediste diversicolor* exposed to temperature (24, 27 and 30 ºC) and salinity combinations (20 and 30). Filled graphs correspond to day 14 of exposure (D14) and unfilled graphs to day 28 of exposure (D28). Significant differences are shown with letters between treatments in each time point.

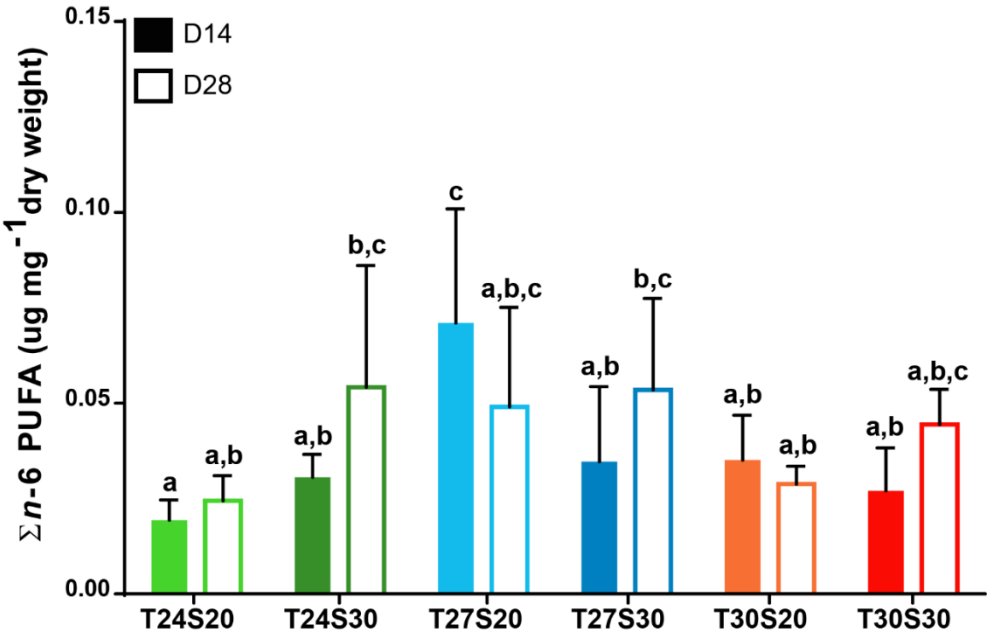

Figure 3.11 - ∑*n*-6 polyunsaturated fatty acids (PUFA) (µg mg^{-1} dry weight) of *Hediste diversicolor* exposed to temperature (24, 27 and 30 ºC) and salinity combinations (20 and 30). Filled graphs correspond to day 14 of exposure (D14) and unfilled graphs to day 28 of exposure (D28). Significant differences are shown with letters between treatments in each time point.

3.6 Energy reserves

Overall, energy reserves did not change throughout the time-course of the experiment, with the exception of T27S20 and T27S30, in which there was an increase in total protein (from day 14 to day 28), indicating protein accretion throughout time (Figure 3.12). No significant differences were found for glycogen and total lipid in each treatment between different time points (Figure 3.13 and 3.14).

At D28, significant differences were found in total protein concentration between T24S20 and T30S30 (p = 0.0152) (Figure 3.12). However, looking in more detail to each day, at D14 significant differences were found in total lipid concentration between the groups of treatments T24S20 *vs* T27S20 (p = 0.0009) and T27S20 *vs* T30S30 (p = 0.0030) (Fig. 3.14). Moreover, significant differences were found between groups T24S20 *vs* T27S30 (p = 0.0102) and T27S30 *vs* T30S30 (p = 0.0399) (Figure 3.14).

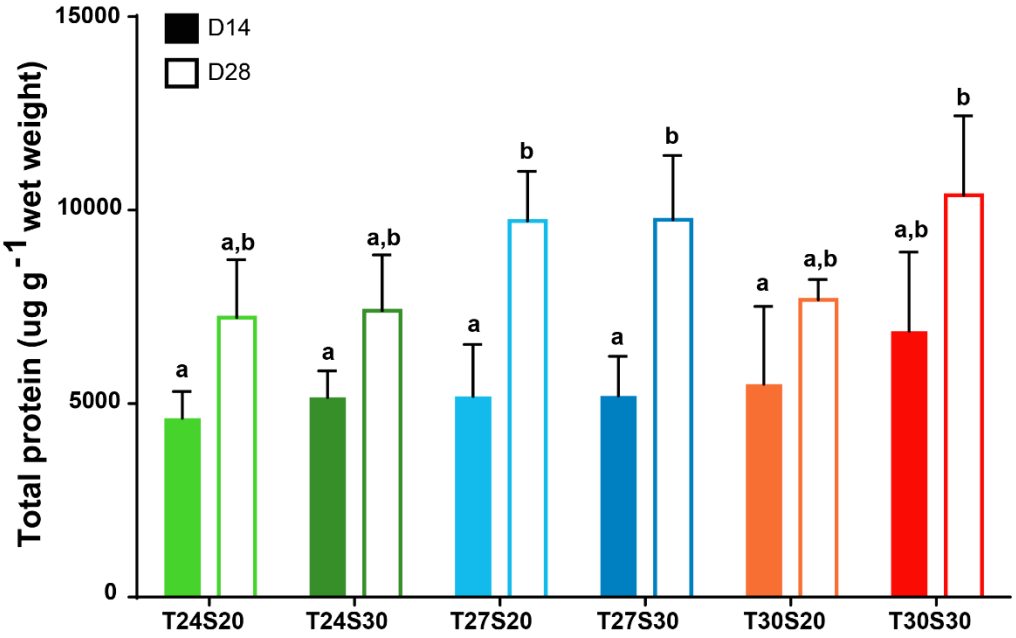

Figure 3.12 - Graph representation (mean ± SD, n=7 biological replicates per treatment) of the concentration of total protein in *Hediste diversicolor* exposed to different combinations of temperature (T 24, 27 and 30 ºC) and salinity (S 20 and 30) throughout time points (D 14 and 28 days). Significant differences are shown with letters between treatments in each time point.

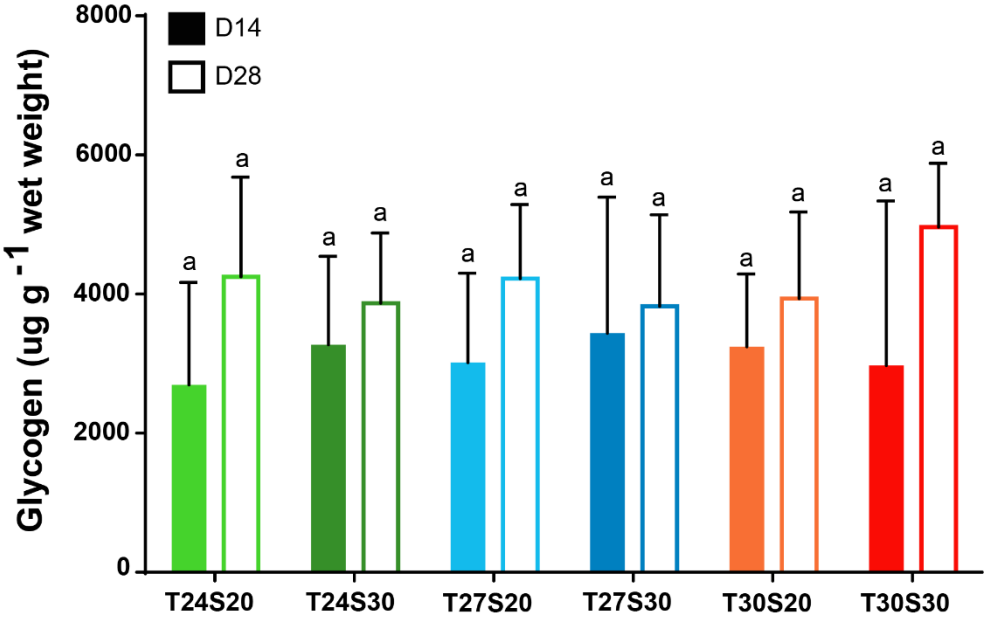

Figure 3.13 - Graph representation (mean ± SD, n=7 biological replicates per treatment) of the concentration of glycogen in *Hediste diversicolor* exposed to different combinations of temperature (T 24, 27 and 30 ºC) and salinity (S 20 and 30) throughout time points (D 14 and 28 days). No significant differences were found between treatments in each time point.

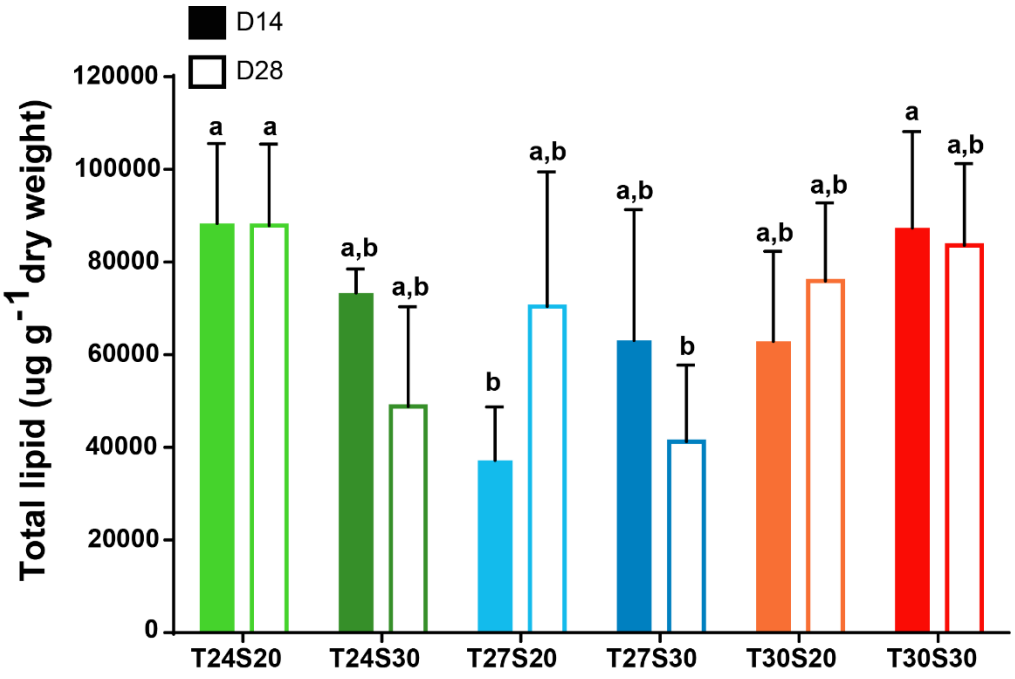

Figure 3.14 – Graph representation (mean ± SD, n=7 biological replicates per treatment) of the concentration of total lipid in *Hediste diversicolor* exposed to different combinations of temperature (T 24, 27 and 30 ºC) and salinity (S 20 and 30) throughout time points (D 14 and 28 days). Significant differences are shown with letters between treatments in each time point.

4. Discussion

Predicted increases in mean sea surface temperature and salinity fluctuations, as well as the predicted increase in frequency, duration, intensity and spatial extent of heat waves, will impose both long and short-term multiple stress on marine ectotherms. Therefore, in the present study, the mechanisms involved in sub lethal, as well as whole body responses to increased temperature and salinity variations over exposure time, were evaluated in the intertidal worm species *H. diversicolor*. The main highlights elucidated from this study are discussed in the following subsections.

4.1 Environmental data

As expected, the most variable parameter studied in the sampling location was temperature. Summer mean water temperatures in Ria de Aveiro lagoon, are around 23 – 31 ºC (Almeida et al., 2005; Sousa et al., 2017) during high and low tide, which is in good agreement with our results. Intertidal areas and estuaries, have large temperature variations due to tide cycles (O'Dea and Okamura, 1999; Harrison and Whitfield, 2006; Lillebø et al., 2015). In *situ* data collection in this study, showed that *H. diversicolor* experiences wide temperature variations. Considering that this ragworm inhabits intertidal mudflats (Scaps, 2002), which are naturally prone to abiotic variation, this species could be likely equipped with plastic and adaptive mechanisms that allow them to withstand extremely variable environments. Moreover, the highest and lowest temperature values recorded *per* day, suggest that these organisms live in an environment with large daily thermal ranges, i.e. they must acclimatize quickly in a short time frame. In fact, tolerance to temperature fluctuations has already been proposed as a relevant physiological trait that might underlie successful species' survival (Kim et al., 2016).

Salinity showed no considerable variations in the sampling location, despite being a coastal lagoon environment, where some variation was expected (Vaz and Dias, 2008). However, major salinity variations are expected to occur between seasons, due to variations in temperature and pluviosity (Lillebø et al., 2015). Within season data are actually in agreement with previous studies in Ria de Aveiro lagoon (Lillebø et al., 2015). During summer, low freshwater inflow is

expected, leading to saltwater propagation further upstream. Thus oceanic saline water is found at the extreme ends of the lagoon, and the main lagoon channels are mostly filled with high saline water (Vaz and Dias, 2008). Some of these channels can reach salinity values over 40, in summer, at low tide (Sousa et al., 2017). Since this factor does not vary so much in this channel in the summer, presumably, in the winter with rainfall, salinity may have larger variations, and species from this specific location, can have mechanisms to acclimatize to salinity fluctuations.

4.2 Species upper thermal limits, intraspecific variation and acclimation capacity

In this study we have determined the Critical Thermal Maximum (CT_{Max}) of *H. diversicolor* after 30 days of acclimation in the tested global change scenarios. In addition we have calculated the intraspecific variability given by coefficient of variation in percent (% CV), Acclimation Capacity (º C), Thermal Safety Margin (TSM) and Acclimation Response Ratio (ARR), for each treatment, providing an overview of the capacity of ragworms to deal with environmental changes.

Organisms that were acclimated to higher temperatures had a higher CT_{Max} value than organisms acclimated to lower temperatures. Higher tolerance for organisms that were acclimated to higher temperatures has also been found in other ectotherms such as fish and shrimps (Stillman, 2003; Chown et al., 2009; Peck et al., 2014; Rodríguez-Fuentes et al., 2017; Madeira et al., 2019a). *Hediste diversicolor* was capable of shifting CT_{Max} by a maximum of 0.81 ºC, following a change of +6 ºC in environmental temperature for 1 month. Such shift is comparable to values reported in other invertebrate species, for instance in the fruit fly *Drosophila* (shifts in CT_{Max} are ≤ 1 ºC, van Heerwaarden et al. (2016) and intertidal crab *Pachygrapsus marmoratus* (~1 ºC shift in CTMax, Vinagre et al. 2016) but lower than reported in the intertidal shrimp *Palaemon elegans* (2.8 ºC shift in CT_{Max},Vinagre et al. (2016), intertidal fish *Coryphoblennius galerita* (4.0 ºC shift in CT_{Max}, Vinagre et al. (2016) and intertidal snail *Echinolittorina malaccana* (~2 ºC shift in lethal thermal limit LT50, Brahim et al. 2019). Acute heat shock has also been shown to increase CT_{Max} in the order of 0.83 to 2.42 ºC

in several marine crustaceans, depending on species and season (Hopkin et al. 2006).

These results confirm that acclimation temperature (i.e. recent thermal history) affects the thermal tolerance plasticity helping organisms to be more tolerant to a sudden change in water temperature (Galvez, 2018).

However, this may not be a general pattern as decreases in CT_{Max} after heat stress have been reported as well (Hopkin et al. 2006,(Vinagre et al., 2018). Nevertheless, overall positive shifts in CT_{Max} should increase population survival, at least in the short-term (Kellermann and Sgrò, 2018). Accordingly, phenotypic plasticity in physiological and life-history traits, occurring within- and trans-generationally, is known to buffer the negative effects of climate change (Gibbin et al., 2017). If adaptive, it allows species to persist in a specific habitat/environment by having a positive effect on fitness (Reed et al., 2011; Grenier et al., 2016; Bonamour et al., 2019) Thus, as suggested by Massamba-N'Siala et al. (2014) and Chakravarti et al. (2016), this improved capacity response may facilitate the ability of populations and species to face and surmount the challenges raised by climate change within and across generations. However, phenotypic plasticity is a trait that is subject to evolutionary change itself (Gibbin et al 2017). For instance, phenotypic plasticity should be selected for when the environment shows sustained space and/or time heterogeneity, leading to different phenotypes in different environments (see Reed et al. 2011, Chevin et al. 2017, Brahim et al. 2019). However, global change may influence the evolutionary trajectories of phenotypic plasticity by altering the reliability, perception and interpretation of environmental cues, associated with climate stochasticity (Bonamour et al 2019). Moreover, (i) the production cost of plasticity and (ii) genetic and physiological constraints leading to a saturation state of plastic phenotypic responses, may also limit population persistence under extreme conditions (see Chevin et al 2010). In fact, several studies report that intertidal/eurythermal organisms, despite having high thermal tolerances, have low acclimation capacities as they are already living on the edge of their physiological limits (Hopkin et al. 2006, Stillman 2003). At higher temperatures, plasticity of CT_{Max} might be constrained (as shown by the decrease in ARR with a rise in temperature), suggesting a trade-off exists between high thermal

tolerance and phenotypic plasticity, as shown in studies with invertebrates including *Drosophila* (van Heerwaardeen et al. 2016, Kellerman et al. 2017) and marine organisms (Vinagre et al. 2018). Thus, some authors suggest that plastic adjustments in CT_{Max} are somewhat beneficial but may be too small to decrease the risk of overheating under global change scenarios (Heerwaarden et al. 2016, Gunderson and Stillman 2015, Barria and Bacigalupe 2017, Gunderson et al. 2017).

Curiously, the CT_{Max} increased with rising salinity, suggesting that the species may prefer a higher salinity (30) than lower salinity (20). Such result matches well with the salinity measurements carried out in the sampling location (section 3.1.2), that were always above 30. Thus, a small variation in salinity can be critical in extreme events (e.g. heatwaves), allowing or not, the species' survival by shifting their thermal tolerance (i.e. CT_{Max}). Additionally, this correlates fairly well with Freitas et al. (2015) and further supports the idea of optimum salinity around 30 for this ragworm species, similarly to other polychaetes such as *Diopatra neapolitana* (optimum salinity range of 28 to 35). If living within their optimum salinity, polychaetes may have an increased capacity to cope with other stressors, as shown in *D. neapolitana* which was able to regenerate tissue after injury if held at optimum salinity.

However, there was no significant interaction between temperature and salinity on CT_{Max} values, suggesting that the pattern of response across the levels of one factor is the same at all levels of the other factor (e.g. CT_{Max} increases as temperature rises, in both salinities, moreover, CT_{Max} increases as salinity rises, at all temperatures. Considering these facts, *H. diversicolor* may have some capacity to cope with predicted extreme events, but further studies are necessary to reach any congruent conclusions.

Overall, in our study, species intraspecific variability in thermal tolerance was low, including within each treatment, suggesting that individuals have a very similar reaction and behavior, even under different conditions. Similar results were obtained for thermal tolerance measurements in crustaceans and fish, in which the CV varied between 0 and 5 % (Madeira et al. 2012, Vinagre et al. 2016). Interestingly, intraspecific variation may be greater if populations from different geographic regions are compared, suggesting that heat tolerance should be

assessed over the full geographical range of the species in order to accurately estimate climate change impacts on biodiversity (e.g. see Herrando-Pérez et al. 2019, Bennett et al., 2019, Barria and Bacigalupe 2017, Shaw et al. 2016). Accordingly, some authors report that organisms undergo local adaptation or acclimation to the temperature range on a regional scale i.e. conspecifics in different geographic locations can have different responses under environment stressors (Freitas et al., 2010), suggesting that populations may differ in climate susceptibility.Other thermal performance related traits also show intraspecific variation, for example aerobic performance in elasmobranchs (Di Santo 2016) and net calcification rates in corals (Shaw et al. 2016), suggesting that intraspecific genetic variability within a population may play an important role in determining adaptive capacity to future conditions.

The increase of acclimation capacity and Thermal Safety Margin (TSM) as the temperature of the treatment was higher, showed the ability of this species to acclimate to new conditions and increase their TSM, by increasing their CT_{Max}, allowing them to have a larger thermal window to survive extreme events as seen in previous studies (Vinagre et al., 2016). Moreover, since the MHT registered is lower than CT_{Max} values, all the specimens could survive even in the worst-case scenario predicted for 2100, i.e. they can withstand higher values than those found in the environment. These results suggest that *H. diversicolor* may have an advantage over other organisms, since in other species the TSM can be negative, showing that some of them are already under pressure at present-day temperatures (Madeira et al., 2017a). This is especially true for tropical and polar species, which are at a greater risk of overheating than species from intermediate latitudes (e.g. Peck et al. 2014, Tewksbury et al. 2008). In fact, tide pools have been characterized as potential ecological traps in tropical habitats (Vinagre et al. 2018).

Acclimation response ratio decreased as the acclimation temperature was higher, proving that the organism has some plasticity, but it is not further increased when it is acclimated to higher temperatures. This is because the degree of plasticity often decreases at relatively high acclimation temperatures, reaching a plateau (i.e. the line explaining changes in CT_{Max} with changing

acclimation temperature reaches an asymptote); as shown in other ectotherms (Otto, 1973; Fangue et al., 2006; Gunderson and Stillman, 2015).

In summary, “physiological plasticity may manifest as chronic acclimation ability to altered environmental baselines and ability to compensate for acute exposures to new environmental extremes” (Galvez, 2018). This ability may be crucial for organisms inhabiting variable and extreme environments such as estuarine and intertidal areas, as these organisms must endure harsh conditions associated with multiple stressors acting concomitantly (periodic and stochastic changes in pH, dissolved oxygen, temperature and salinity) (Galvez 2018).

Each single variable may impose significant stress on inhabiting organisms, but the combined exposure to several interacting variables may produce additive and synergistic effects (Wolanski 2007). Even when animals are adapted to tolerate these extreme environments and populations can persist, the environmental variability characteristic of estuaries is potentially stressful to animals inhabiting these conditions (Schulte 2014). More species need to be tested to confirm these findings, as acclimation capacity can vary among and within taxa, and to allow more complex future studies that also consider species’ interactions, community structure and ecosystem function.

4.3 Stress biomarkers: combined influence of temperature and salinity over time of exposure

Molecular mechanisms underpinning *H. diversicolor’s* acclimation capacity to global and local change drivers were evaluated by quantifying specific biomarkers involved in the heat shock response, antioxidant defense, oxidative damage and energy utilization. Stress biomarkers levels varied with thermal load, salinity gradients and over time of exposure, as observed in other ectotherms (Ivanina et al., 2009; Dimitriadis et al., 2012; Madeira et al., 2015; Rodríguez-Fuentes et al., 2017; Madeira et al., 2019a). In fact, a three-way interaction between temperature, salinity and time was detected in the PERMANOVA, indicating that the interactive effects of temperature and salinity on biomarker

levels change over time. Thus, exposure time is a determinant factor in order to detect molecular changes induced by environmental stressors in organisms as also suggested by Deschaseaux et al. (2011) and Ellison et al. (2017). Anestis et al. (2007) and Madeira et al. (2019b) showed that some biomarkers tend to increase expression over time, especially under stressful conditions. In this study, most biomarkers showed a decrease throughout the experimental period, except for glucose, which increased over time. This pattern is possibly associated with successful acclimation to the treatment conditions. Interestingly, two groups did not show significant differences over time, namely the control (T24S30) and the high temperature and low salinity group (T30S20), indicating that overall biomarker response patterns are steadily maintained throughout time in these groups. Such results could be expected, especially in the control group in which organisms are not under stressful conditions and thus do not need to change biomarker levels over time. With regards to the group exposed to high temperature and low salinity, the results show that there is a change in biomarker levels in relation to control conditions and thus this change should be maintained at both 14 and 28 days.

Overall, Glucose was the biomarker that was most affected by the tested treatments over the time of exposure. This may be related to the fact that it is a simple sugar, abundant in organisms, and easier to convert to energy, that in stressful situations, it can start the process of glycolysis (process that converts glucose into pyruvate), to get more adenosine triphosphate (ATP) i.e. energy to combat stress caused by environmental stressors (Fothergill-Gilmore and Michels, 1993) or to meet the higher energetic demands associated with higher metabolic rates at elevated temperature. In living organisms, glucose is converted to other several chemical compounds that are the starting material for various metabolic pathways. In addition, glucose metabolites can produce some nonessential amino acids, sugar alcohols such as mannitol and sorbitol, fatty acids, cholesterol and nucleic acids (Heinrich et al., 2014).

Besides glucose, the other biomarkers that contributed to discriminate between temperature groups were GST, Ub and Hsp70, suggesting that the antioxidant enzyme, ubiquitin and the chaperone played an important role during thermal stress protecting cells from the toxic effects of ROS (Lesser, 2006), and

managing the integrity of the protein pool (Chen et al., 2011), thereby allowing the proper management of cellular functioning. Differences between temperature groups were more evident and sustained in time at lower salinity. At control salinity (30), the differences detected between temperature groups at day 14 are no longer sustained at day 28, suggesting that ragworms can easily acclimate to temperature if kept at optimum salinity. Accordingly, glucose was depleted after 28 days at higher temperature, under salinity 20, indicating that the combination of elevated temperature and low salinity increases energy demand, probably associated with the maintenance of osmotic balance and thermal stress protection, as reported for other invertebrates (e.g. amphipods, Vereshchagina et al. (2016). Such results are corroborated by an increase in the antioxidant activity of GST with rising temperature and lower salinity, indicating that antioxidants are important for intertidal organisms to deal with thermal load and salinity variations. Such pattern has been extensively reported in other studies in marine organisms such as fish (Madeira et al., 2013; Madeira et al., 2016), crustaceans (Vinagre et al., 2014) and corals (Dias et al., 2019). Other authors also state that (Matozzo et al., 2013), suggesting that the response pattern is species dependent. Interestingly, other authors report that high temperature, low salinity or their combination increase the deleterious effects of other stressors such as metal pollution (Ozoh, 1992) and carbon nanotubes (De Marchi et al., 2018) on worms. Altogether, such results potentially suggest that the occurrence of heatwaves in low-salinity and contaminated areas of estuaries could have an impact on *H. diversicolor*. Still, long-term field studies suggest that these ragworms are somewhat tolerant to heatwaves (Dolbeth et al., 2011), but this may depend on geographic location and the potential for local adaptation.

According to the literature, Hsp70 and Ub are usually induced in ectotherms exposed to thermal stress (including cold and heat stress) until an extreme point is reached and protein levels start to decrease, potentially due to denaturation (Madeira et al., 2012b; Madeira et al., 2014a; Madeira et al., 2014b; Meng et al., 2014). However, the response pattern is also dependent on tissue-specific metabolism and thus variation between tissue types is expected (Dietz and Somero, 1993; Madeira et al., 2014b). Although main effects of temperature were non-significant for Hsp70 and Ub in this study, a three-way interaction of

temperature, salinity and day was detected for both biomarkers. Hsp70 and Ub show similar response patterns, which makes sense considering their interaction when managing the integrity of the protein pool (Shiber and Ravid, 2014). For instance, at day 14 both Hsp70 and Ub maintain stable levels across temperatures (within salinity 20). At control salinity (30), Hsp70 shows a trend of increase at moderate temperature (27 ºC) followed by a significant decrease at extreme temperature (30 ºC), following the typical pattern expected from literature. Such results may suggest that there may be moderate protein damage associated with temperature at day 14. However, at day 28, there are no changes in Hsp70 or Ub across temperatures for both salinity levels, corroborating the hypothesis of successful acclimation of *H. diversicolor* to elevated temperature. These results are in accordance to other studies which state that molecular adjustments to thermal stress are usually greatest within 7 to 14 days of exposure, being restored to control levels after approximately one month, if the animal shows acclimation (Madeira et al., 2016; Madeira, 2016). The high acclimation capacity of *H. diversicolor* to environmental change has been highlighted in other studies; for instance, these ragworms are known to reproduce successfully within a broad range of temperatures (tested range: 16 to 25 ºC, Bagarrão (2013) and juvenile specific growth rate is also supported under different salinities (from 15 to 25, Bagarrão (2013).

Another highlight from the results was that ubiquitin, in addition to glucose, contributed to discriminate between salinity groups. This can be explained by the fact that ubiquitin may be involved in osmotic stress signaling, which is in a good agreement with a previous study which shows that ubiquitin promotes environmental salinity tolerance by supporting cell function during hyperosmotic stress (Fiol et al., 2011). Overall, *H. diversicolor* is an euryhaline species that can maintain osmotic balance by osmoregulation (at salinities below 14) or acting as osmoconformer (at higher salinities) (Hohendorf 1963 as cited in Schiedek (1998). At low salinities, osmotic balance can be achieved by several mechanisms, including the active absorption of salt from the surrounding medium, shifting the permeability of the body wall to both water and salts, the production of hypotonic urine to excrete the excess water and regulation of osmolyte concentrations such as free amino acids to adjust volume (Generlich

and Giere, 1996). Interestingly, acclimation of this ragworm to hypoosmotic shock is known to depend on temperature, with faster acclimation occurring at moderate when compared to cold temperatures (Generlich and Giere, 1996).

Overall, biomarker responses are often suitable indicators of the species condition and health status and are thus used to support management policies by prioritizing areas and species that need local or regional intervention. However, physiological biomarkers should ideally be used in combination with physiological (e.g. thermal limits) and molecular approaches (e.g. molecular markers, metabolomics and proteomics) to integrate lines of evidence of global change effects.

4.4 Fatty acid profiles: combined influence of temperature and salinity over time of exposure

Our results indicate that in a future ocean scenario, temperature and salinity will affect *H. diversicolor's* fatty acid profiles' and their classes. The most abundant FA in each registered class (palmitic acid (16:0) in SFA, vaccenic acid (18:1*n*-7) in MUFA, linoleic acid (18:2*n*-6) in PUFA and EPA in HUFA (20:5*n*-3) were coincident to the ones found in other marine organisms under environmental variations (Luis and Passos, 1995; Anacleto et al., 2014; Lopes et al., 2016).

Exposure time had a significant impact on FA class concentrations, especially within salinity 30. Most classes showed an increase throughout time (independently of the tested temperature), suggesting that worms can increase FA classes, especially MUFA and PUFA, throughout time if held at optimum salinity conditions. If held at low salinity, such an accumulation is not observed. Moreover, a significant interaction between salinity and day was detected for omega fatty acids and essential fatty acids, with an overall increase in ∑*n*-3 PUFA, ∑*n*-6 PUFA, ARA, EPA and DHA throughout time, at salinity 30. Some authors suggest that fatty acid accumulation is typical of optimum culture conditions, as minimal stress levels are imposed (Rasdi et al., 2019). Thus, ragworms retained these FA, instead of consuming them as energy sources. This suggests that at optimum salinity conditions, worms can keep in good physiological condition, since they are still able to increase FA concentrations.

These results may also be related to variations in feeding rate, food conversion efficiency and fatty acid bioconversion efficiency under different environmental conditions. Even though these performance parameters were not measured in this study, several other authors have reported that temperature and salinity have a direct effect on food conversion efficiency and growth of marine worms. For instance, Neuhoff (1979) showed that an increase in salinity (from 15 to 30) leads to higher food conversion efficiency in *H. diversicolor*, resulting in a higher energy content at higher salinity. In another study using the rockworm *Marphysa sanguinea* as model, weight gain and crude fat content were higher at salinities of 30 and 35 when compared to salinities of 15, 20 and 25 (Thu et al., 2019). Lower growth rates at lower salinity were also reported for *Perinereis rullieri* (Prevedelli and Vandini, 1997) and *Capitella* sp. (Pechenik et al., 2000), possibly due to reduced feeding rates. Thus, changes in feeding behavior and feed assimilation could explain the accumulation/non-accumulation patterns of FA classes under different salinities; however, further studies should confirm this hypothesis.

A detailed analysis of each time-point further reinforces the idea that salinity is a determinant factor shaping FA concentrations in *H. diversicolor*, despite contrasting results reported in Bagarrão (2013). This contrast may be due to the range of salinities tested, as the author exposed *H. diversicolor* to salinities between 15 to 25 (versus salinities of 20 and 30 tested in this study).

Fatty acid content was significantly affected by salinity at both sampling days (14 and 28) but in opposite patterns. Differences were detected for all classes at day 14, namely an overall decrease in SFA, MUFA, PUFA and HUFA with rising salinity. However, after 28 days of experiment, there was an overall increase of all FA classes at salinity 30, when compared to 20, suggesting that, in the long-term, their physiological status was better under salinity 30. A study by Frolov et al. (1991) in rotifers also found an increased proportion of SFA with rising salinity, but the authors also report a decreased proportion of PUFA, which was not detected in this study using *H. diversicolor* as model. The authors state that such alterations may be important to shift membrane permeability and energy utilization. Long et al. (2019) also stated that SFA and MUFA are preferably accumulated as energy sources by crabs under long-term salinity adaptation (6-

18 compared to 0), as these conditions are optimal due to matching osmolality of the environment *vs* body. However, long chain PUFA may be mobilized to the membrane to adjust fluidity and maintain osmotic balance (Long et al., 2019). Indeed, the properties of membranes are intimately related to the fluidity of the constituent lipids (Thompson, 1992). High levels of these PUFAs in cellular membranes preserve membrane fluidity of the organisms and in particular, salinity changes can induce elongation and desaturation of FAs chains (Lodish et al., 2000; Hernando et al., 2018). This capacity may be species-specific, but there is evidence of *de novo* biosynthesis of ARA and EPA in nereid polychaetes (Pairohakul, 2013; Monroig and Kabeya, 2018) suggesting that they could be able to adjust PUFA as needed. However, salinity alone did not affect essential fatty acid concentrations (ARA, EPA and DHA) neither ∑*n*-3 PUFA, ∑*n*-6 PUFA nor the ratio of total ∑*n*-3/*n*-6, suggesting that all omegas (*n*-3 and *n*-6) and their ratio remain balanced at both salinities. This is not the case of previous studies in invertebrates. For instance, on the Pacific oyster, *Crassostrea gigas*, the ∑*n*-3/∑*n*-6 ratio decreased markedly with increasing salinities in whole tissue (Fuhrmann et al., 2018). Moreover, ∑*n*-6 PUFA have also been shown to decrease in *C. gigas* (Fuhrmann et al., 2018) and the Chinese mitten crab (Long et al., 2017) under hypoosmotic conditions.

Temperature effects on fatty acid content were detected at both time-points. A greater variation in fatty acid content was noteworthy on day 14, while on day 28, almost no differences were found. Moreover, temperature-induced differences were greater under low salinity, when compared to optimum salinity (30). After 14 days of exposure, under salinity 30, HUFA increased from 24 ºC to 27 ºC and then decreased below control levels at 30 ºC. However, no differences were found between temperature groups after 28 days, suggesting that worms were able to acclimate to warm water, if held at optimum salinity. Previous studies on the dynamics of shifts in the lipid composition of cell membranes, suggest that temperature increase affects the balance between PUFAs and SFA (Imbs and Yakovleva, 2012). Thermal stress may induce a reduction in multiple PUFAs over time, leading to structural modifications in bio-membranes and causing leakiness (Hillyer et al., 2017). However, other authors state that a stable unsaturated to

saturated FA ratio may be important to mitigate lipid damage under high temperature and low salinity conditions (Hernando et al. 2018).

In this study, if a temperature increase occurs under low salinity conditions, there is an increase in SFA, MUFA, PUFA and HUFA at both 27 ºC and 30 ºC, when compared to 24 ºC, after 14 days of exposure. After 28 days of exposure, differences were only found in HUFA, which were still elevated at 27 ºC. Overall, these results suggest that organisms may have a need for saturated and shorter-chain fatty acids for (i) energy supply in thermal protection and osmoregulation at low salinity as proposed recently by Chen et al. (2019) and/or (ii) cellular membrane adjustments to keep homeostasis, taking into account that SFAs are usually incorporated into the membrane under high temperature but PUFAs may be needed to keep membrane permeability under low salinity. According to the literature, the energy yield that an organism can obtain from a fatty acid beta oxidation is much higher (129 ATP) (Schulz, 1991) than, for example, the glycolysis of glucose (section 4.3). This may suggest that saturated fatty acids are an important energy source during acclimation to fluctuating environmental conditions. At a later stage, organisms may be able to balance which energy source will be the best to achieve metabolic homeostasis.

Temperature also had a significant main effect on omega fatty acids (∑*n*-3 PUFA, ∑*n*-6 PUFA, ARA, EPA, DHA) and interacted with salinity to modulate their concentrations on *H. diversicolor*. At optimum salinity, levels of omega fatty acids remained stable at 24 ºC and 27 ºC and then a slight decrease was detected at 30 ºC. Under low salinity, omega fatty acids increase from 24 ºC to 27 ºC and then decrease at extreme temperature (30 ºC). The overall decrease detected at the extreme temperature (30 ºC) follows the expected pattern described in the literature, namely a decrease in PUFA at elevated temperatures (e.g. Valles-Regino et. al 2015) potentially associated with membrane fluidity adjustments. Cellular membranes are thermally sensitive structures, and membrane lipid reorganization can act as a thermal sensor mechanism, regulating the activation of cellular stress responses and cellular homeostasis (see Balogh et. al 2013 for a review). Interestingly, temperature increases have been shown to directly reduce the expression or enzymatic activity of desaturases in fish, affecting fatty acid bioconversion processes (Mellery et al., 2016). The increase in omega fatty

acids observed at moderate temperature (27 ºC) only under low salinity conditions may be more related to osmotic regulation needs (as no change was detected at 27 ºC under optimum salinity). This pattern may be explained by the fact that some fatty acids, for example DHA, may increase tolerance to multiple stressors (Kanazawa, 1997).

4.5 Energy reserves: combined influence of temperature and salinity over time of exposure

Matching metabolic fuel supply with energy utilization rates is crucial to support animal activities (see Weber 2011). The maintenance of energy balance under fluctuating environments may rely on different strategies including i) adjustment of feeding rate and energy expenditure, ii) adjustment of feeding rate but not energy expenditure, iii) no adjustment of feeding rate but compensation of energy expenditure (Newell and Branch, 1980). Ultimately, if animals have to adjust energy expenditure, fuel selection (carbohydrates *vs* lipids *vs* proteins) for a specific pathway or activity will depend on stored quantities, energy density, conversion speed, solubility in water, and activity duration and intensity (Weber, 2011).

Acclimation to multiple stressors is often thought to impose considerable metabolic costs to organisms (Angilletta et al., 2002), as it may induce an increased use of energetic reserves (Sandblom et al., 2014), otherwise used for different functions such as growth and reproduction. However, such costs are difficult to quantify (Somero, 2011). Our study, in overall, showed no changes in energy reserves throughout the time-course of the experiment, with the exception of treatments of 27 ºC in both salinities, in which there was an increase in total protein, indicating protein accretion throughout time. Protein synthesis and accretion is the major biochemical process underpinning organismal growth (Schröer et al., 2006). Thus, our results indicate that adult *H. diversicolor* worms were able to maintain homeostasis and grow at 27 ºC. In order to do so, they must be able to keep energy balance and oxygen consumption within optimum ranges as observed for other invertebrate species (Pörtner, 2010; González et al., 2015). Verberk et al. (2016) explained this balance based on the fact that

organisms depend on a constant and sufficient flux of oxygen from their environment to their metabolizing tissues, in order to ensure an adequate ATP supply to cover all physiological demands and still maintain the energy status (Rodríguez-Fuentes et al., 2017). Interestingly, many intertidal organisms like crabs, fish, mollusks and polychaetes (including *H. diversicolor*) have a bimodal breathing strategy, being able to exchange gases in water or air along the body surface or gill-like structures, (Newell, 1976; Fusi et al., 2016). Such organisms may be able to extend thermal tolerance by moving within the habitat and switching from water-breathing to air-breathing strategies during low tide/warm conditions, contributing to the maintenance of oxygen partial pressure in tissues and keeping aerobic metabolism stable during warm events (see Fusi et al. 2016). The maintenance of aerobic metabolism under stress is highly relevant, as aerobic metabolism yields much more energy than anaerobic metabolism (the conversion efficiency of sugar molecules into high energy phosphates is 40 % under aerobic versus 3-7 % under anaerobic conditions) (Newell 1976). This bimodal breathing strategy could be an important mechanism enabling intertidal organisms like *H. diversicolor* to maintain an efficient energy use upon environmental variation, without significantly depleting all energy sources.

Lipids and carbohydrates are considered cellular fuel, besides their important function as structural constituents of membranes. Hence, their decrease can negatively affect growth and metabolism of cells (Araújo and Garcia, 2005). Curiously, glycogen showed no differences in any treatment, not even over time of exposure, as found in previous study in bivalves under ocean warming (Anacleto et al., 2014). This supports the idea that polychaetes can maintain energy efficiency and could be related to the fact that, as organisms had glucose available for energy production, they did not need to degrade the carbohydrate, keeping their reserves stable. However total lipid content, had differences in each time point taking into consideration different temperatures (significant differences were only found between 24 and 27 ºC). The depletion of lipid content at 27 ºC may be associated with two potential processes i) as the worms were accumulating protein (proxy for growth) under those conditions, lipids could be used as main energy fuels for protein synthesis (Gonzalez-Manchon et al., 1990). This is corroborated by the fact that fatty acids, especially

PUFAs, are essential to maintain growth in marine organisms (Müller-Navarra et al., 2000). Moreover, ii) since metabolic rates generally increase at higher temperatures (as shown for *H. diversicolor*, (Galasso et al., 2018) and under stressful conditions, a higher demand is placed on metabolic reserves, such as the stored lipid, potentially leading to its depletion. Under these circumstances, reduced lipid reserves could have significant implications for the long-term viability of *H. diversicolor* populations (Valles-Regino et al., 2015). Still, no differences were detected at 30 ºC, suggesting that elevated temperature is not imposing major energetic costs on worms.

5. Conclusions and Future Perspectives

The main objective of this thesis was to study the performance, physiological and molecular responses of *H. diversicolor* exposed to global and local change scenarios drivers (water temperature increase and salinity variations). To investigate the survival, upper thermal limits (CT_{Max}), acclimation capacity, and thermal safety margins of this species, the organisms were exposed to different treatments of water temperature (24 ºC, 27 ºC and 30 ºC) and salinity (20 and 30), showing that the survival of this species is not affected by these changes. Also, organisms that were acclimated to higher temperatures and salinity had a higher CT_{Max} than those in lower temperatures and salinity. Furthermore, higher acclimation temperatures led to higher thermal tolerance limits, confirming that *H. diversicolor* has some physiological plasticity, acclimation capacity and a positive thermal safety margin.

At a molecular level, selected biomarkers and fatty acid profiles were analysed to test *H. diversicolor's* stress responses to the different combined temperature and salinity scenarios throughout the exposure time (14 and 28 days). Here, a significant interaction of the three factors (temperature, salinity and day) suggested a non-additive effect of the factors on the biomarkers' concentrations. Thus, the effect of one factor on cellular stress responses depends on the levels of the other two factors. Regarding the fatty acids, a significant interaction of temperature and salinity was also detected, as well as between salinity and day. Overall, a better physiological condition was detected for polychaetes exposed to salinity 30, when compared to 20. When elevated temperature was combined with low salinity, stress responses were activated concomitantly with a decrease in glucose, suggesting an increased energy demand related to the maintenance of homeostasis. Moreover, fatty acid accumulation was observed throughout time in treatments with salinity 30 but not salinity 20, corroborating the hypothesis that salinity 30 imposes minimal stress levels on *H. diversicolor*. Additionally, fatty acid profile changes induced by warming were especially evident at low salinity conditions. For instance, if *H. diversicolor* is exposed to an extreme temperature (30 ºC) under the preferred salinity condition (30), a decrease in HUFA is observed (only at day 14), probably to adjust membrane fluidity. However, if an extreme warm event is combined with low salinity, all FA classes increase at day 14 and HUFA remain elevated after

28 days, suggesting that HUFA may be needed to keep osmotic balance. The analysis of energy reserves of the ragworm between the different tested scenarios over time of acclimation confirmed that the amount of total proteins and total lipids were affected by treatment, namely an increase in total protein and a decrease in total lipids at 27 ºC were detected, suggesting increased protein synthesis potentially fuelled by lipids. On the opposite, glycogen content in the organisms were similar between the treatments.

This study improves the knowledge about climate change impacts on marine invertebrates that play major ecological roles in their habitats, concluding that *H. diversicolor* can easily acclimate to increased water temperature and salinity fluctuations. This is especially relevant as this estuarine and coastal species plays an important role on ecosystem processes and biological and environment interactions, being an important prey item in trophic webs and a major bioturbator in coastal environments. Additionally, knowledge on the ecophysiology of *H. diversicolor* may be useful to the blue bioeconomy sector and associated stakeholders due to by its commercial value as bait for fishing and its role in effluent bioremediation and added value in integrated multi-trophic aquaculture. The need to, understand how climate change influences the physiological condition, survival and performance of species provides valuable data to identify vulnerable and resistant populations and ultimately define what are the best breeds and conditions to maintain this species for a future use in the aquaculture industry. Moreover, this study can contribute for a better climate-smart conservation, helping the scientific community and relevant organizations to develop management plans that take into account the impacts of climate change on marine biodiversity, contributing to the sustainability of marine resource use. To this end, knowledge transfer to relevant stakeholders and political organizations is crucial, in order to take action to face global changes.

Nevertheless, some gaps were identified and should be considered in future studies to improve the management and conservation of the ecosystems, but also to increase the knowledge of their functioning. Future studies on the current topic are therefore needed in order to verify the plasticity of organisms to new global change conditions. Moreover, longer experimental trials, including all the life cycle stages and trans-generational experiments, are needed to identify

vulnerable life stages and to detect changes on an evolutionary scale. These will certainly be at play in the survival and adaptation of species to future global changes. The integration of physiology into ecological modelling will allow scientists to detect global change impacts at higher levels of biological complexity, improving global change risk assessment in marine environments.

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7. Annexes

7.1 Tukey's post- hocs tests (Biomarkers)

7.1.1 Temperature

Table 7.1 - Tukey's post-hocs tests for the biomarkers that were significant in factorial ANOVAs for the factor Temperature. Significant results are marked in bold (≤ 0.05).

	Biomarkers	
Groups	GST	LPO
Temperature	*p*	
24 ºC *vs* 27 ºC	**0.01**	0.08
24 ºC *vs* 30 ºC	**0.00**	0.91
27 ºC *vs* 30 ºC	**0.00**	**0.03**

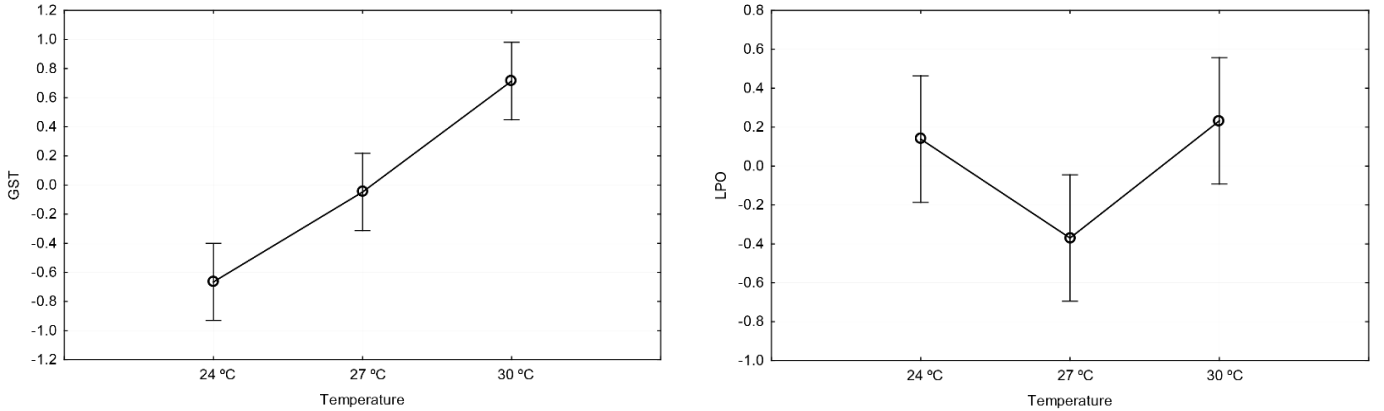


Figure 7.1 - Graphical representation of patterns of biomarkers that were significant in factorial ANOVAs for the factor Temperature.

7.1.2 Salinity

Table 7.2 - Tukey's post-hocs tests for the biomarkers that were significant in factorial ANOVAs for the factor Salinity. Significant results are marked in bold (≤ 0.05).

	Biomarkers	
Groups	GST	Glucose
Salinity	*p*	
20 *vs* 30	**0.02**	**0.00**

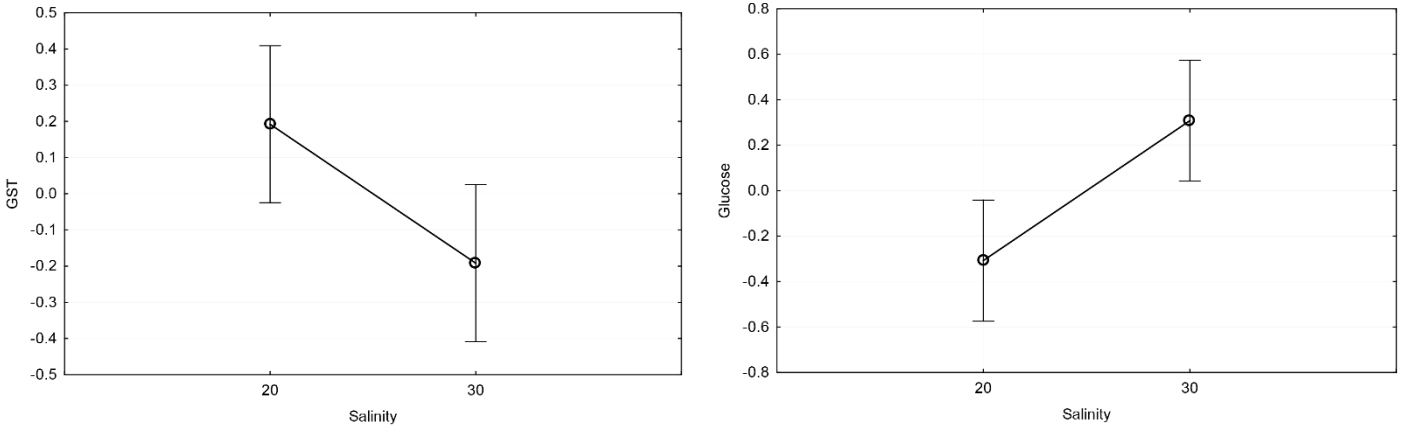


Figure 7.2 - Graphical representation of patterns of biomarkers that were significant in factorial ANOVAs for the factor Salinity.

7.1.3 Day

Table 7.3 - Tukey's post-hocs tests for the biomarkers that were significant in factorial ANOVAs for the factor Day. Significant results are marked in bold (≤ 0.05).

	Biomarkers						
Groups	Hsp70	CAT	GST	SOD	TAC	LPO	Glucose
Day	*p*						
14 *vs* 28	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

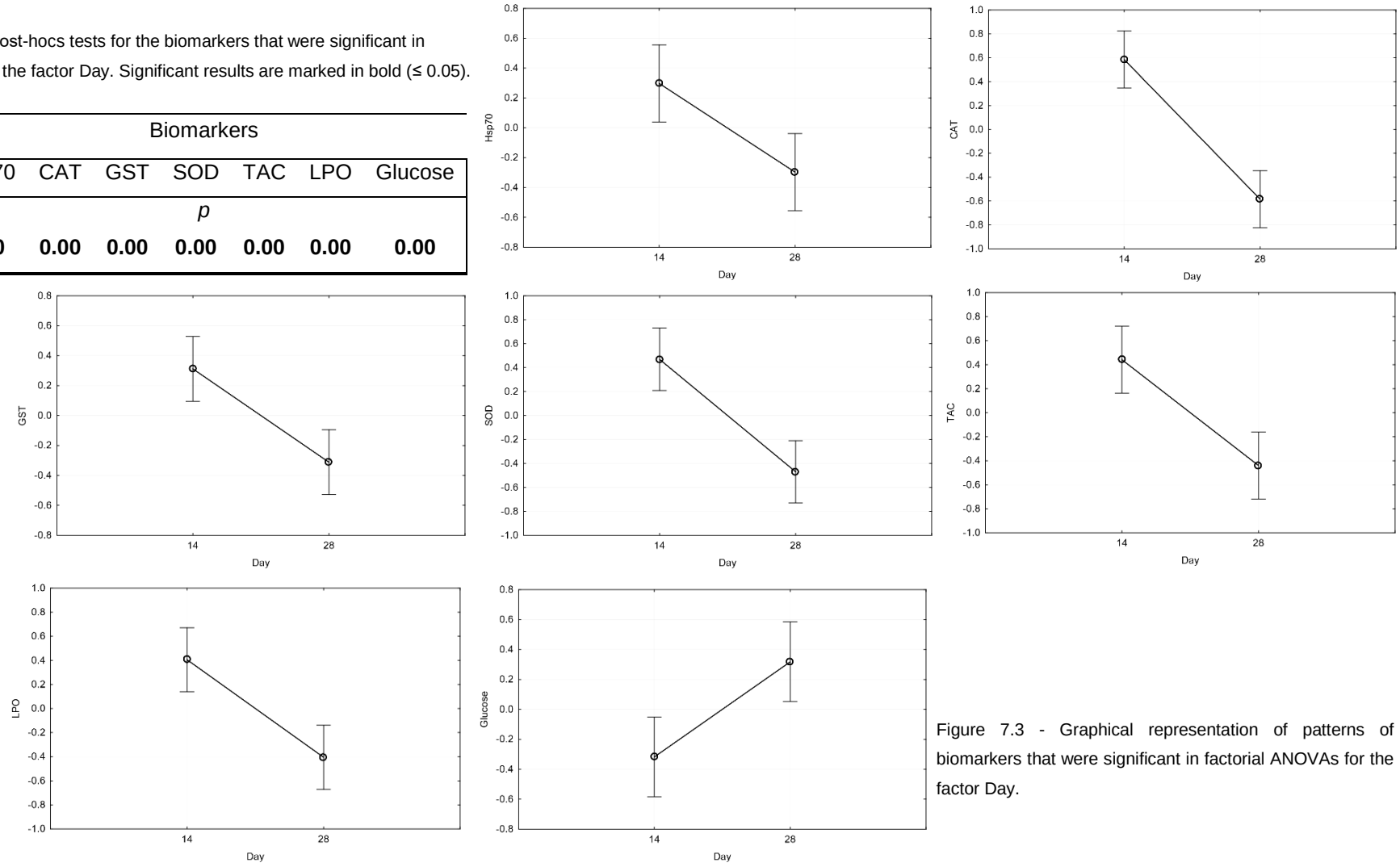


Figure 7.3 - Graphical representation of patterns of biomarkers that were significant in factorial ANOVAs for the factor Day.

7.1.4 Temperature x Salinity

Table 7.4 - Tukey's post-hocs tests for the biomarkers that were significant in factorial ANOVAs for the interaction between Temperature and Salinity. Significant results are marked in bold (≤ 0.05).

	Biomarkers	
Groups	SOD	LPO
Temperature x Salinity	*p*	
T24S20 vs T24S30	0.90	0.98
T24S20 vs T27S20	0.90	**0.03**
T24S20 vs T27S30	0.99	0.98
T24S20 vs T30S20	0.94	0.99
T24S20 vs T30S30	0.66	0.99
T24S30 vs T27S20	1.00	0.17
T24S30 vs T27S30	0.75	1.00
T24S30 vs T30S20	0.36	0.85
T24S30 vs T30S30	0.99	0.99
T27S20 vs T27S30	0.76	0.16
T27S20 vs T30S20	0.37	**0.01**
T27S20 vs T30S30	0.99	0.13
T27S30 vs T30S20	0.99	0.85
T27S30 vs T30S30	0.46	0.99
T30S20 vs T30S30	0.16	0.90

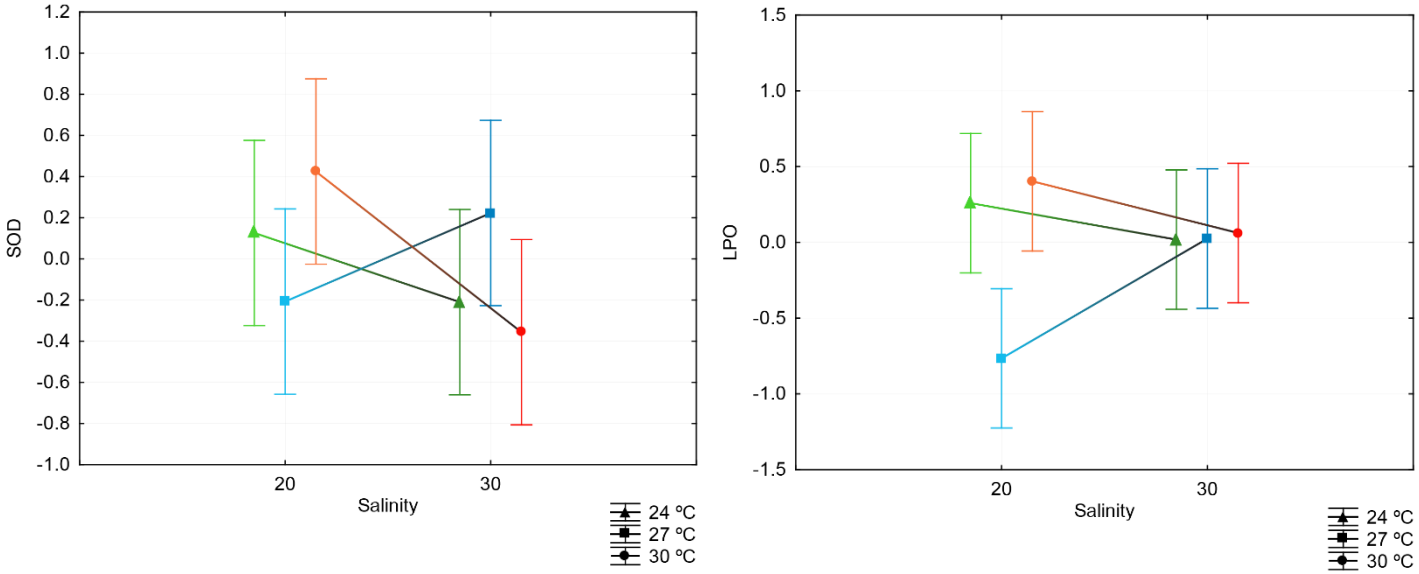
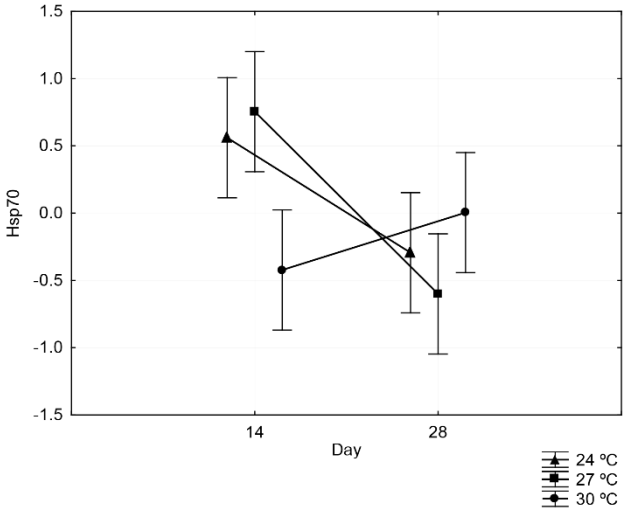


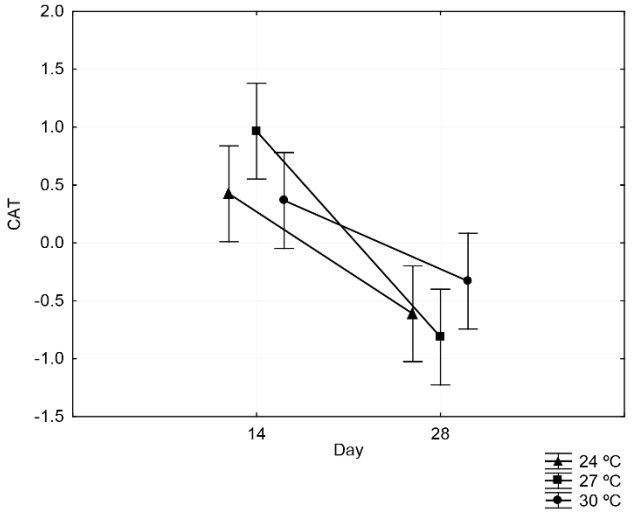
Figure 7.4 - Graphical representation of patterns of biomarkers that were significant in factorial ANOVAs for the interaction between Temperature and Salinity.

7.1.5 Temperature x Day

Table 7.5 - Tukey's post-hocs tests for the biomarkers that were significant in factorial ANOVAs for the interaction between Temperature and Day. Significant results are marked in bold (≤ 0.05).

	Biomarkers		
Groups	Hsp70	CAT	GST
Temperature x Day		*p*	
T24D14 *vs* T24D28	0.09	**0.01**	0.99
T24D14 *vs* T27D14	0.98	0.45	**0.00**
T24D14 *vs* T27D28	**0.01**	**0.01**	0.99
T24D14 *vs* T30D14	**0.03**	0.99	**0.00**
T24D14 *vs* T30D28	0.50	0.12	**0.01**
T24D28 *vs* T27D14	**0.02**	**0.00**	**0.00**
T24D28 *vs* T27D28	0.93	0.98	0.99
T24D28 *vs* T30D14	0.99	**0.02**	**0.00**
T24D28 *vs* T30D28	0.93	0.93	**0.01**
T27D14 *vs* T27D28	**0.00**	**0.00**	**0.00**
T27D14 *vs* T30D14	**0.01**	0.33	0.16
T27D14 *vs* T30D28	0.18	**0.00**	0.98
T27D28 *vs* T30D14	0.99	**0.00**	**0.00**
T27D28 *vs* T30D28	0.41	0.57	**0.02**
T30D14 *vs* T30D28	0.76	0.18	**0.03**





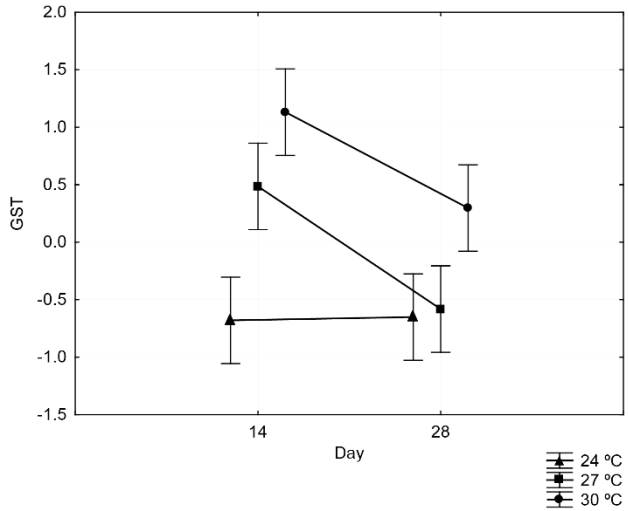


Figure 7.5 - Graphical representation of patterns of biomarkers that were significant in factorial ANOVAs for the interaction between Temperature and Day.

7.1.6 Temperature x Salinity x Day

Table 7.6 - Tukey's post-hocs tests for the biomarkers that were significant in factorial ANOVAs for the interaction between Temperature, Salinity and Day. Significant results are marked in bold (≤ 0.05).

	Groups										
Biomarkers	T24S20D14 *vs* T24S20D28	T24S20D14 *vs* T24S30D14	T24S20D14 *vs* T24S30D28	T24S20D14 *vs* T27S20D14	T24S20D14 *vs* T27S20D28	T24S20D14 *vs* T27S30D14	T24S20D14 *vs* T27S30D28	T24S20D14 *vs* T30S20D14	T24S20D14 *vs* T30S20D28	T24S20D14 *vs* T30S30D14	T24S20D14 *vs* T30S30D28
	p										
Hsp70	**0.03**	0.60	0.43	0.84	**0.03**	0.99	**0.02**	0.61	0.54	**0.00**	0.43
Ub	0.14	0.16	0.97	0.96	0.36	1.00	0.18	0.32	0.53	0.23	0.18
CAT	**0.01**	0.70	0.27	1.00	**0.02**	0.99	**0.00**	0.99	0.56	0.93	**0.04**

	Groups									
Biomarkers	T24S20D28 *vs* T24S30D14	T24S20D28 *vs* T24S30D28	T24S20D28 *vs* T27S20D14	T24S20D28 *vs* T27S20D28	T24S20D28 *vs* T27S30D14	T24S20D28 *vs* T27S30D28	T24S20D28 *vs* T30S20D14	T24S20D28 *vs* T30S20D28	T24S20D28 *vs* T30S30D14	T24S20D28 *vs* T30S30D28
	p									
Hsp70	0.95	0.99	0.80	1.00	**0.01**	1.00	0.95	0.97	0.99	0.99
Ub	1.00	0.91	0.92	0.99	0.27	1.00	1.00	0.99	1.00	1.00
CAT	0.46	0.88	**0.01**	0.99	**0.00**	1.00	**0.03**	0.60	0.20	0.99

	Groups								
Biomarkers	T24S30D14 *vs* T24S30D28	T24S30D14 *vs* T27S20D14	T24S30D14 *vs* T27S20D28	T24S30D14 *vs* T27S30D14	T24S30D14 *vs* T27S30D28	T24S30D14 *vs* T30S20D14	T24S30D14 *vs* T30S20D28	T24S30D14 *vs* T30S30D14	T24S30D14 *vs* T30S30D28
	p								
Hsp70	1.00	1.00	0.95	0.29	0.91	1.00	1.00	0.51	1.00
Ub	0.93	0.94	1.00	0.30	1.00	1.00	0.99	1.00	1.00
CAT	0.99	0.85	0.83	0.18	0.55	0.98	1.00	0.99	0.95

	Groups							
Biomarkers	T24S30D28 *vs* T27S20D14	T24S30D28 *vs* T27S20D28	T24S30D28 *vs* T27S30D14	T24S30D28 *vs* T27S30D28	T24S30D28 *vs* T30S20D14	T24S30D28 *vs* T30S20D28	T24S30D28 *vs* T30S30D14	T24S30D28 *vs* T30S30D28
	p							
Hsp70	0.99	0.99	0.18	0.97	1.00	1.00	0.68	1.00
Ub	1.00	0.99	0.99	0.94	0.99	0.99	0.96	0.94
CAT	0.42	0.99	**0.03**	0.93	0.75	0.99	0.99	0.99

	Groups						
Biomarkers	T27S20D14 *vs* T27S20D28	T27S20D14 *vs* T27S30D14	T27S20D14 *vs* T27S30D28	T27S20D14 *vs* T30S20D14	T27S20D14 *vs* T30S20D28	T27S20D14 *vs* T30S30D14	T27S20D14 *vs* T30S30D28
	p						
Hsp70	0.79	0.53	0.71	1.00	0.99	0.28	0.99
Ub	0.99	0.99	0.95	0.99	0.99	0.97	0.95
CAT	**0.03**	0.99	**0.01**	0.99	0.73	0.98	0.08

	Groups										
Biomarkers	T27S20D28 *vs* T27S30D14	T27S20D28 *vs* T27S30D28	T27S20D28 *vs* T30S20D14	T27S20D28 *vs* T30S20D28	T27S20D28 *vs* T30S30D14	T27S20D28 *vs* T30S30D28	T27S30D14 *vs* T27S30D28	T27S30D14 *vs* T30S20D14	T27S30D14 *vs* T30S20D28	T27S30D14 *vs* T30S30D14	T27S30D14 *vs* T30S30D28
	p										
Hsp70	**0.01**	1.00	0.94	0.97	0.99	0.99	**0.00**	0.29	0.24	**0.00**	0.17
Ub	0.57	1.00	1.00	1.00	1.00	1.00	0.33	0.52	0.74	0.40	0.33
CAT	**0.00**	0.99	0.12	0.91	0.53	1.00	**0.00**	0.90	0.11	0.42	**0.00**

Biomarkers	Groups									
	T27S30D28 *vs* T30S20D14	T27S30D28 *vs* T30S20D28	T27S30D28 *vs* T30S30D14	T27S30D28 *vs* T30S30D28	T30S20D14 *vs* T30S20D28	T30S20D14 *vs* T30S30D14	T30S20D14 *vs* T30S30D28	T30S20D28 *vs* T30S30D14	T30S20D28 *vs* T30S30D28	T30S30D14 *vs* T30S30D28
	p									
Hsp70	0.90	0.93	0.99	0.97	1.00	0.51	1.00	0.58	1.00	0.69
Ub	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.99	0.99	1.00
CAT	**0.04**	0.70	0.26	0.99	0.95	0.99	0.25	0.99	0.98	0.75

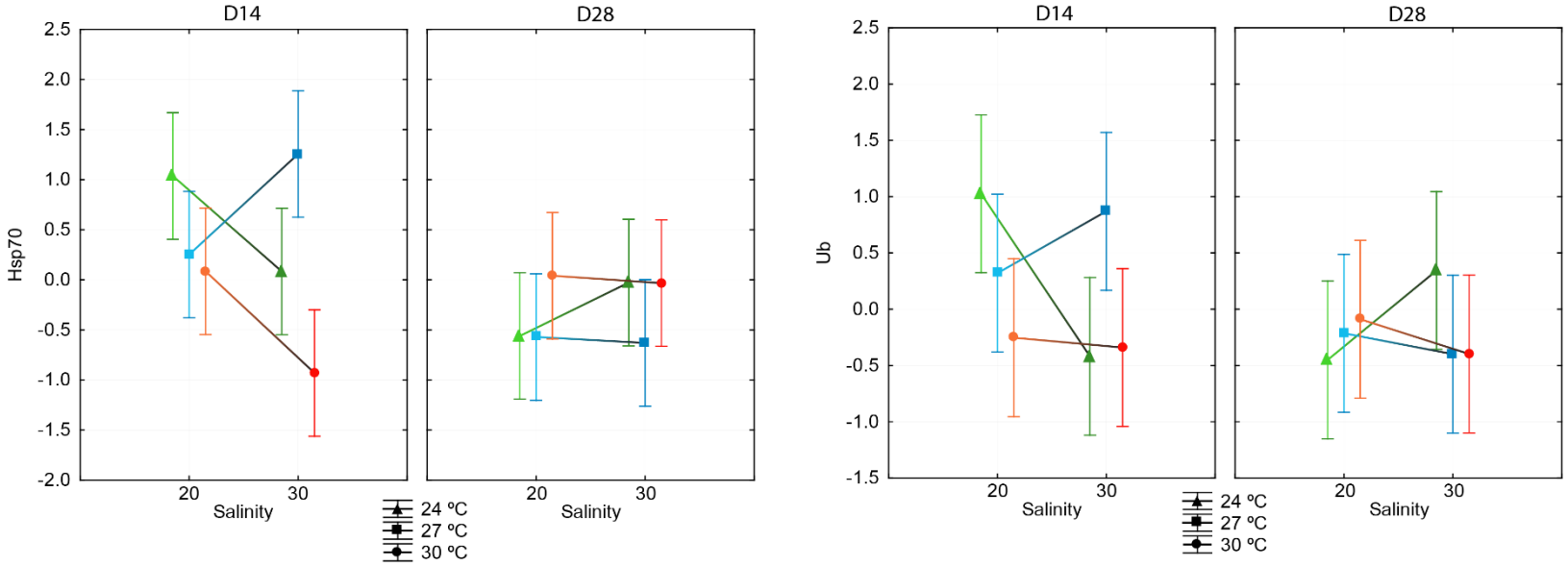


Figure 7.6 - Graphical representation of patterns of biomarkers that were significant in factorial ANOVAs for the interaction between Temperature, Salinity and Day.

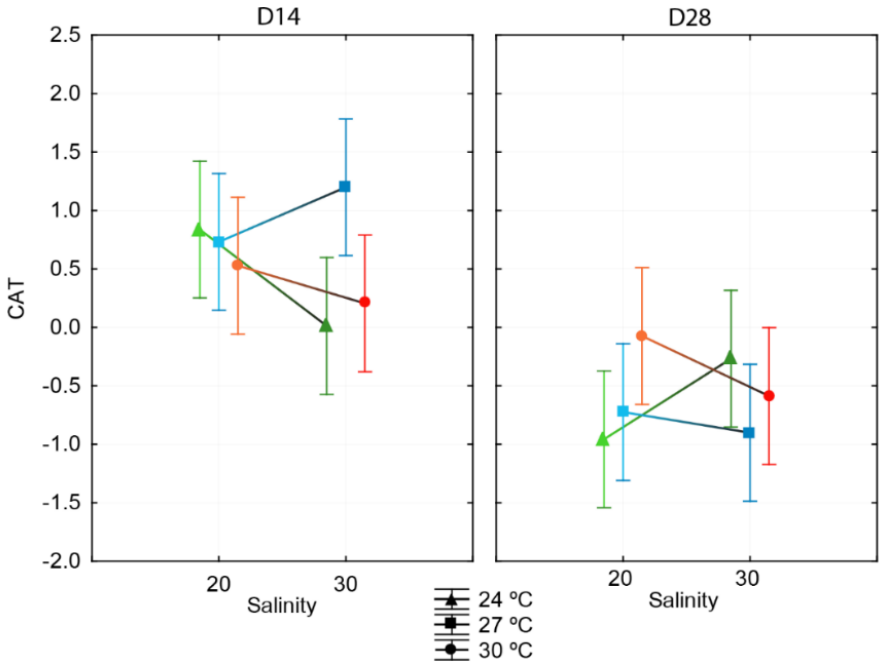


Figure 7.7 – (cont.) Graphical representation of patterns of biomarkers that were significant in factorial ANOVAs for the interaction between Temperature, Salinity and Day

7.2 Tukey's post- hocs tests (*n*-3 PUFA, *n*-6 PUFA, Total *n*-3/*n*-6, ARA, EPA and DHA)

7.2.1 Temperature

Table 7.7 - Tukey's post-hocs tests for the variables that were significant in factorial ANOVAs for the factor Temperature. Significant results are marked in bold (≤ 0.05).

	Variables				
Groups	∑*n*-3 PUFA	∑*n*-6 PUFA	20:4 *n*-6 (ARA)	20:5 *n*-3 (EPA)	22:6 *n*-3 (DHA)
Temperature	*p*				
24 ºC *vs* 27 ºC	**0.00**	**0.00**	**0.00**	**0.00**	**0.02**
24 ºC *vs* 30 ºC	0.99	0.93	0.97	0.92	0.80
27 ºC *vs* 30 ºC	**0.00**	**0.00**	**0.00**	**0.00**	0.08

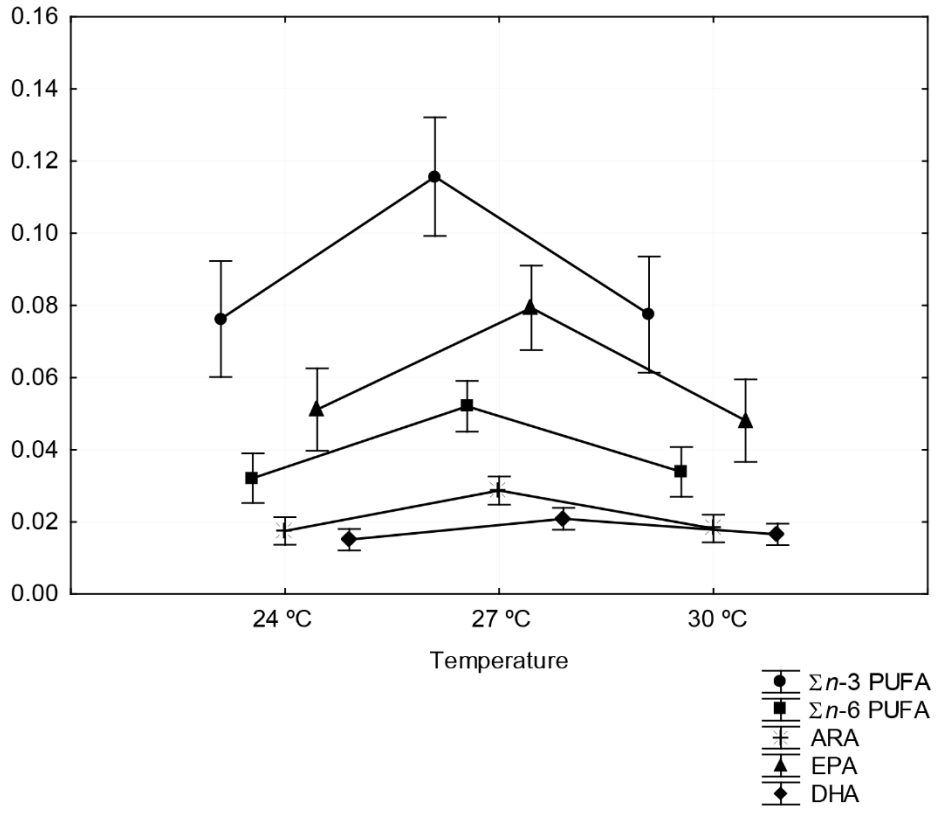
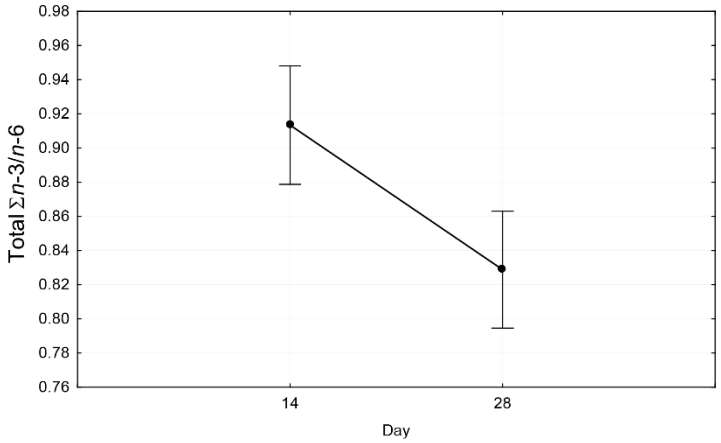


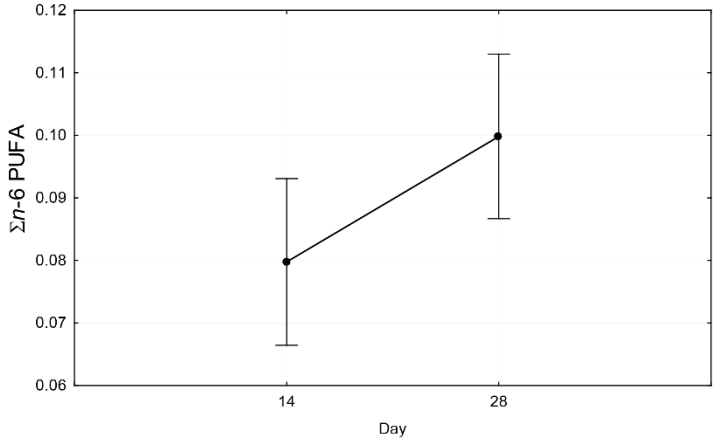
Figure 7.8 - Graphical representation of patterns of variables that were significant in factorial ANOVAs for the factor Temperature.

7.2.2 Day

Table 7.8 - Tukey's post-hocs tests for the variables that were significant in factorial ANOVAs for the factor Day. Significant results are marked in bold (≤ 0.05).

	Variables			
Groups	∑*n*-3 PUFA	Total ∑*n*-3/*n*-6	20:4 *n*-6 (ARA)	22:6 *n*-3 (DHA)
Day	*p*			
14 *vs* 28	**0.04**	**0.00**	**0.04**	**0.01**





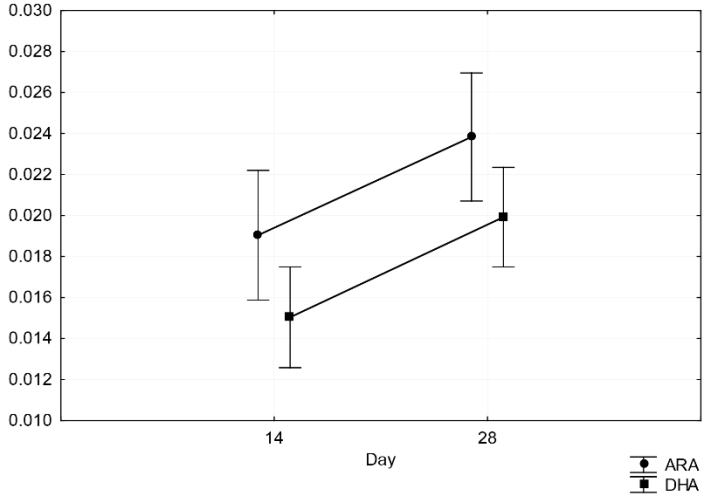


Figure 7.9 - Graphical representation of patterns of variables that were significant in factorial ANOVAs for the factor Day.

7.2.3 Temperature x Salinity

Table 7.9 - Tukey's post-hocs tests for the variables that were significant in factorial ANOVAs for the interaction between Temperature and Salinity. Significant results are marked in bold (≤ 0.05).

	Variables				
Groups	∑*n*-3 PUFA	∑*n*-6 PUFA	20:4 *n*-6 (ARA)	20:5 *n*-3 (EPA)	22:6 *n*-3 (DHA)
Temperature x Salinity	*p*				
T24S20 vs T24S30	0.12	**0.05**	0.07	0.12	0.27
T24S20 vs T27S20	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
T24S20 vs T27S30	0.09	**0.02**	**0.02**	0.10	0.26
T24S20 vs T30S20	0.77	0.69	0.77	0.92	0.72
T24S20 vs T30S30	0.72	0.35	0.47	0.90	0.52
T24S30 vs T27S20	0.21	0.12	0.11	0.19	0.48
T24S30 vs T27S30	0.99	0.99	0.99	0.99	1.00
T24S30 vs T30S20	0.81	0.66	0.69	0.61	0.97
T24S30 vs T30S30	0.85	0.93	0.92	0.65	0.99
T27S20 vs T27S30	0.30	0.27	0.35	0.27	0.54
T27S20 vs T30S20	**0.01**	**0.00**	**0.00**	**0.00**	0.13
T27S20 vs T30S30	**0.01**	**0.01**	**0.01**	**0.00**	0.24
T27S30 vs T30S20	0.74	0.46	0.36	0.55	0.97
T27S30 vs T30S30	0.79	0.79	0.65	0.59	0.99
T30S20 vs T30S30	0.99	0.99	0.99	1.00	0.99

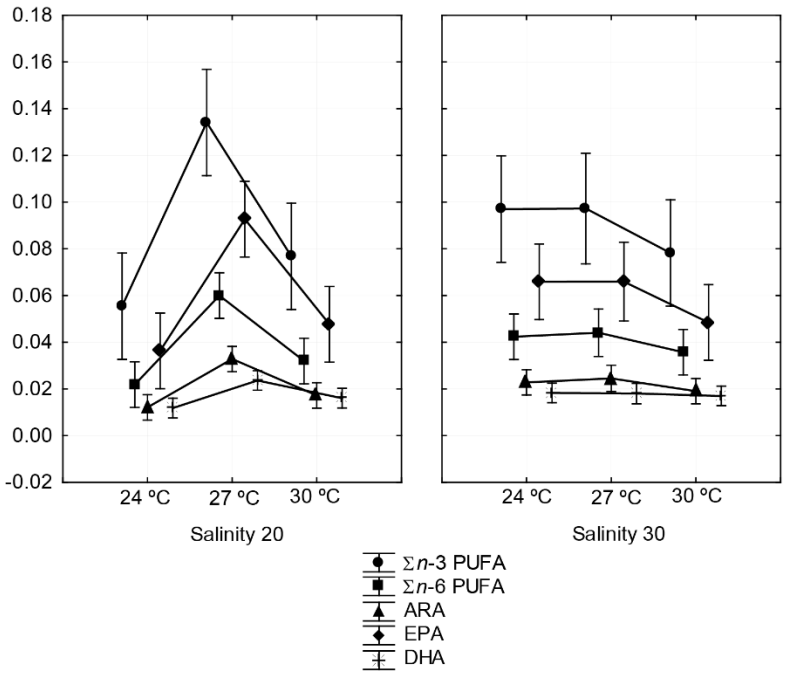


Figure 7.10 - Graphical representation of patterns of variables that were significant in factorial ANOVAs for the interaction between Temperature and Salinity.

7.2.4 Salinity x Day

Table 7.10 - Tukey's post-hocs tests for the variables that were significant in factorial ANOVAs for the interaction between Salinity and Day. Significant results are marked in bold (≤ 0.05).

	Variables				
Groups	∑*n*-3 PUFA	∑*n*-6 PUFA	20:4 *n*-6 (ARA)	20:5 *n*-3 (EPA)	22:6 *n*-3 (DHA)
Salinity x Day			*p*		
S20D14 *vs* S20D28	0.65	0.52	0.77	0.61	0.93
S20D14 *vs* S30D14	0.06	0.21	0.17	0.07	0.10
S20D14 *vs* S30D28	0.34	0.40	0.21	0.42	0.12
S20D28 *vs* S30D14	0.52	0.93	0.69	0.57	0.31
S20D28 *vs* S30D28	**0.03**	**0.02**	**0.02**	**0.04**	**0.03**
S30D14 *vs* S30D28	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

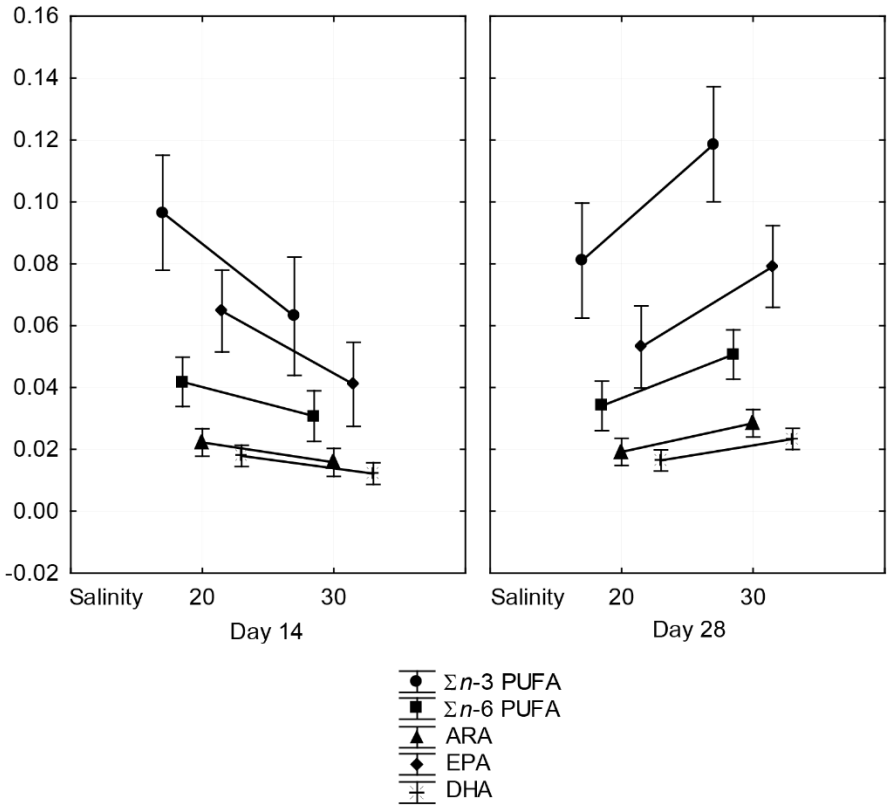


Figure 7.11 - Graphical representation of patterns of variables that were significant in factorial ANOVAs for the interaction between Salinity and Day.