Universidade de Aveiro
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Respostas ecofisiológicas e transcriptómicas de duas espécies de moluscos intertidais ao stress térmico

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha Aplicada, realizada sob a orientação científica do Doutor Dany Domínguez Pérez, Investigador Assistente no Centro Interdisciplinar de Investigação Marinha e Ambiental CIIMAR e coorientação da Doutora Adília O. Pires, Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro

The research work was supported by the Project SEEINGSHORE (NORTE-01-0145-FEDER-031893) co-financed by NORTE 2020, Portugal 2020, and the European Union through the ERDF

This work was also supported by the project MOREBIVALVES (PTDC/ASP-PES/31762/2017) and by the Strategic Funding UIDB/04423/2020 and UIDP/04423/2020 through national funds provided by FCT and ERDF, in the framework of the program PT2020.

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Agradecimentos

Gostaria de dedicar este trabalho a todos os que fizeram com que sua realização fosse possível. Primeiramente e de forma muito especial a minha mãe Idalgisa, quem foi ao mesmo tempo amiga, colega, guia e a minha inspiração.

Agradeço ao projeto SEEINGSHORE, coordenado pelo investigador PhD. Francisco Arenas Parra (Paco), Benthic Ecology Team Leader do CIIMAR, uma vez que os custos do trablho de investigação foram financiados por este projeto. E também por tornar possível esta colaboração com a Universidade de Aveiro. Um agradecimento muito especial aos meus orientadores PhD. Dany Domínguez e PhD. Adília Pires por todo trabalho realizado durante o último ano, pelo apoio e os conhecimentos que me deram durante o caminho, muito obrigado, de todo coração. Também agradeço aos membros do projeto MOREBIVALVES, coordenado pelo investigador PhD. Alexandre Campos, Blue Biotechnology and Ecotoxicology Team Leader do CIIMAR, por facilitar o uso do software licenciado OmicsBox, e também pelo o assessoramento nos trabalhos de transcriptomica.

Agradeço a todos os que me acompanharam durante a parte experimental, especialmente a equipa do BET - Bentic Ecology Team: Hugo, Óscar, Giulia, Sónia, Luís, Axel, Marta, Arianna, João e um agradecimento muito especial Noemi. Agradeço aos membros do EEB: Julia e Tiago, e do CB a Silvia. Durante este último ano alem de colegas forma também minha segunda família. Um agradecimento muito grande ao staff do BOGA (Hugo, Ricardo, Olga e Sr. Manuel).

Por último, mas não esquecendo quero agradecer a meus amigos e a todos aqueles que a distância e o tempo não lhes permitiram estar presente, mas que estiveram comigo em todo momento.

palavras-chave

Nucella lapillus, *Mytilus galloprovincialis*, Alterações climáticas, Fitness, RNAseq.

resumo

As alterações climáticas estão associadas ao aquecimento global, que em grande parte se deve à acumulação de gases com efeito de estufa resultantes da atividade humana. No **Capítulo 1** é abordado a sensibilidade do ecossistema intertidal face aos impactos das alterações climáticas, uma vez que as taxas de sobrevivência das espécies que habitam nesta zona são influenciadas em grande parte pelo nível do mar para manter o seu estilo de vida. Por este motivo, foram escolhidas duas espécies de moluscos representativos da costa portuguesa, *Nucella lapillus* e *Mytilus galloprovincialis*.

No **Capítulo 2**, foi projetado o sistema experimental de mesocosmos para manter os indivíduos de *N. lapillus* e de mexilhão *M. galloprovincialis*, ambos colhidos no intertidal rochoso de Belinho-Mar, Esposende, Portugal.

No **Capítulo 3** avaliaram-se as **Respostas Fisiológicas de *N. lapillus* e *M. galloprovincialis* face ao stress térmico**. Após a aclimatação dos indivíduos às condições laboratoriais, estes foram expostos a stress térmico que variava entre os 7 e os 28°C, durante quatro semanas num sistema idêntico ao banho-maria. Com este estudo pretendia-se avaliar o comportamento e as respostas relacionadas com o fitness fisiológico destas duas espécies-chave. No final da experiência foram quantificadas e modeladas as taxas metabólicas para ambas as espécies. A mortalidade, a interação trófica e o esforço reprodutivo de *N. lapillus* foram também quantificados.

Para explorar o mecanismo molecular subjacente à resistência térmica, no **Capítulo 4** foi traçado o perfil das **Respostas transcriptómicas das brânquias do mexilhão *M. galloprovincialis* sob stress térmico**. O RNA total das brânquias de *M. galloprovincialis* após a exposição a um curto período de stress térmico, foi sequenciado usando a plataforma Illumina NovaSeq 6000 150bp (pares de bases) e posteriormente foi analisado com as ferramentas incluídas no programa OmicsBox v1.4.11. O transcriptoma anotado de *M. galloprovincialis* revelou 1411 Genes Diferencialmente Expressos (DEGs) dentro da gama de temperaturas. Alguns biomarcadores de stress térmico foram identificados entre estes DEGs, incluindo proteínas de choque térmico.

A **Discussão Geral** no **Capítulo 5** levou às **Conclusões Gerais** no **Capítulo 6** em que, ambas as espécies mostraram tolerância ao stress térmico aplicado, porém encontramos o limite superior de tolerância térmica para *N. lapillus* acima de 28°C. Adicionalmente foi encontrada para esta espécie uma relação dependente da temperatura na Predação (máxima a 19°C) e na Reprodução (10-22°C, máximo a 16°C), ambas sem significância estatística. A resposta molecular ao stress térmico incluiu 46 DEGs com homologia a biomarcadores de defesa ao stress térmico, nomeadamente proteínas de choque térmico (Hsp70 e Hsp90) e outras chaperonas para a temperatura máxima testada (28°C).

keywords *Nucella lapillus*, *Mytilus galloprovincialis*, Climate change, Fitness, RNAseq.

abstract

Climate change is currently associated with an increased rate of global warming, largely due to greenhouse gases produced by human activities. Thus, in the **Chapter 1** is addressed how intertidal ecosystems are especially fragile to the expected impacts derived from climate change, since survival rates of the species inhabiting in this tight zone are influenced in great part by the sea level to maintain their lifestyle. Therefore, were targeted two mollusc species from Portugal coasts, *Nucella lapillus* and *Mytilus galloprovincialis*.

Then, in the **Chapter 2**, the mesocosms experimental system was designed to hold individuals of the predator snail *N. lapillus* and the mussel *M. galloprovincialis* collected from the rocky shore Belinho-Mar Beach, Esposende, Portugal.

Specifically, in **Chapter 3**, were assessed the **Physiological Responses of *N. lapillus* and *M. galloprovincialis* to thermal stress**. After specimen's acclimation to laboratorial conditions, some individuals were exposed to thermal stress ranging from 7 up to 28°C for four weeks in a bath-based system. This study aimed to assess the behaviour, physiological fitness-related responses of these two keystone species. At the end of the experiments, the metabolic and mortality rates for both species were quantified and modelled, as well as the trophic interaction and the reproductive effort in *N. lapillus*.

To explore the molecular mechanism underlying the thermal resistance, in the **Chapter 4** was profiled **The transcriptomic responses of the gills from the Mediterranean mussel *M. galloprovincialis* under thermal stress**. The whole RNA sequencing of the gills from *M. galloprovincialis* was sequenced after the specimen's exposure to an acute thermal stress period using an Illumina NovaSeq 6000 150bp (base-pair) platform, further analysed with the tools included in the software OmicsBox v1.4.11. The annotated transcriptome of *M. galloprovincialis* revealed 1411 Expressed Genes (DEGs) within the temperature range. Some heat biomarkers were identified among DEGs, including heat shock proteins.

A **General Discussion** in **Chapter 5** led to the **General Conclusions** given in **Chapter 6** that, in general, both species showed to be tolerant to the thermic stress applied. Remarkably, we found the upper thermal tolerance limit for *N. lapillus* over 28°C, showing a Temperature-dependent tendency in Predation (maximum at 19°C) and Reproductive output range (10-22°C, maxed at 16°C), both without statistical significance. The molecular response to thermal stress included 46 DEGs with homology to heat defensive biomarkers, namely heat shock proteins (Hsp70 and Hsp90) and other chaperone proteins at the maximum tested (28°C).

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Chapter 1. General Introduction

1.1. Impacts of Climate Change: The global warming in marine environment.

Climate change is probably the greatest environmental concern that threats biodiversity, ecosystems, and human life as well. Climate change is the long-term response of the Earth as a dynamic system from the crescent emissions of carbon, originally stored, in the past decades. These emissions in the form of CO_2 combined with other gases with green-house effect resulted in the warming tendency that we know as Global Warming (NASA, 2021).

By thermodynamics principle, the heat trapped in the atmosphere then is dissipated to the water bodies until it reaches the thermal balance.

The Intergovernmental Panel on Climate Change (IPCC), the world organization dedicated to study climate change, estimates a warming between 0.6 up to 2°C in the first 100m of the oceans and between 0.3 and 0.6°C in the first kilometre, until the end of this century (Krinner et al., 2013).

Global warming has multiple impacts on Oceanic systems, which interact with each other, ending on synergetic responses. Among the most notorious effect there is a reduction of glaciers mass (Houston, 2013), and thus, the subsequent rise in the global mean sea level by the expansion of the volume of water bodies (Bindoff et al., 2019) and the input of fresh water from cryosphere reservoirs (Kovats et al., 2015). This also brings fluctuations in salinity overall (Kovats et al., 2015). On the other hand, heat exchange is weakening the internal oceanic circulation (Kovats et al., 2015).

These changes are expected to have a major impact on coastal and intertidal habitats, which are dynamic ecosystems by themselves, dominated by ectothermic organisms. Intertidal communities play an important role in nutrients cycle and host some organisms of ecological and economic relevance (Krinner et al., 2013).

For instance, kelp forests present in these ecosystems play an important role in carbon sink or blue carbon. In marine ecosystems, this translates the remotion of atmospheric CO_2 and its storage as biomass.

In coastal systems, large amounts per m^2 can be stored above ground as part of the biomass (Bindoff et al., 2019; NOAA, 2021). Global warming also will increase the stratification of the ocean surface layer, making it thinner and less dense and reducing the amount of light that reaches deeper layers and nutrients that can recirculate from the bottom. This is expected to have repercussions in primary productivity and later in the food webs (H. O. Pörtner et al., 2014).

It is expected a diminution on the water column pH by the increment of atmospheric CO_2. Combined with global warming, it will affect the uptake of calcium carbonate for calcifying organisms. Since it has a higher energetic cost, it can reduce the number of new recruits (Callaway et al., 2012; H. O. Pörtner et al., 2014). For species like mussels, oysters, and cockles, largely harvested, from an economic point of view, this represents a negative impact on the revenues (Callaway et al., 2012).

With temperature ranges increasing and altered environmental variables as oxygen concentration, food, or nutrient availability, among other factors, species can cope through their ecology or phonologic plasticity (Hillebrand et al., 2018). The first response is for species to look for spatial refugees where the conditions are stable, beyond plasticity limits (acclimatization). Organisms react by changing their distribution range in latitude or bathymetry through generation dispersal or by migrations (Hillebrand et al., 2018; Pinsky et al., 2020b). The arrival of new species to a previously established ecosystem only grant success if the species is capable to compete, replace an existent species or use an empty niche (Pinsky et al., 2020b).

1.2. Effects of global warming on intertidal ecosystems

Portuguese mainland coast extends around 940 km, which includes several areas of Rocky intertidal, Sandy beaches and high cliffs (Ramos-Pereira et al., 2005). In the past years, the Portuguese Government has been giving special attention to the stabilizations of the coastline, deploying engineering structures to prevent erosion by the ocean's advance (see Fig. 1).

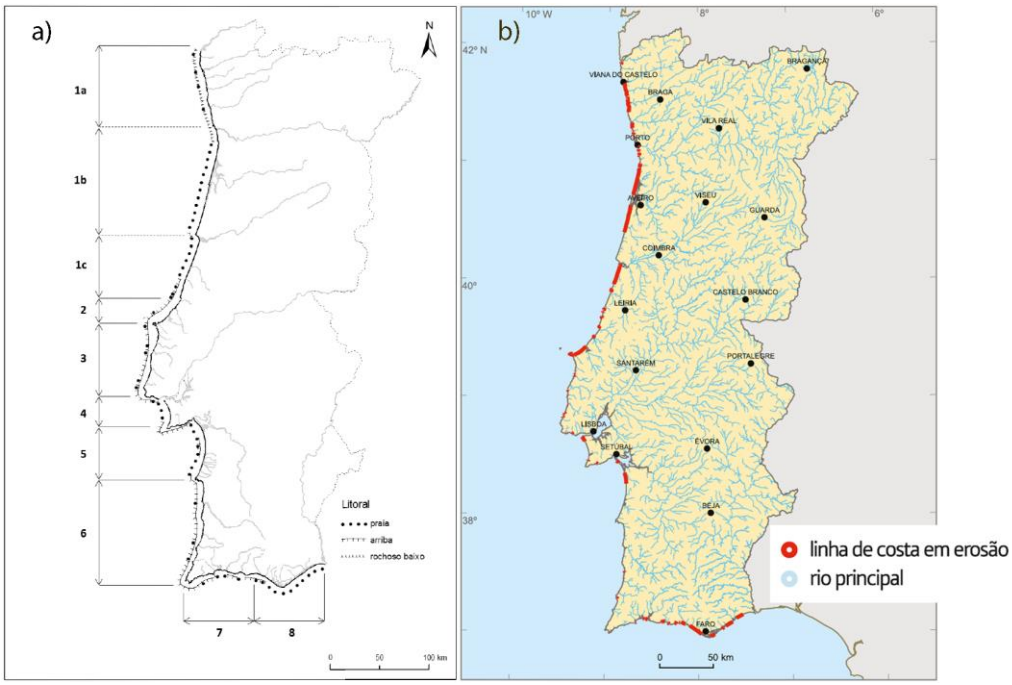

Fig. 1. Characterization of the Portuguese Littoral and the most affected areas by erosion: a) Simplified Geomorphology of the Portuguese Littoral, adapted from (Santos et al., 2017). b) Coastline in erosion between 1958-2018, adapted from (Guerra et al., 2019).

Erosion results mostly from the intense activity of the wave action, mainly due to the mean sea level rise. Models fitted for Portugal estimates a rise between 1.15 and 1.60m until the end of this century, when the NOAA model estimates a rise between 2.13 and 2.63 m above the actual relative sea level (Antunes et al., 2019).

For species that inhabit in a tight zone as the sea level rapidly rises in the rocky intertidal, they must advance toward shore and rise to maintain their lifestyle. The question brought by urbanized areas is the constrains imposed by manmade structures and leisure areas (Kaplanis et al., 2020). In addition to this squeezing effect in steep shores or loss of hard substrate for sessile species ecosystem engineers attach.

Changes in the area will affect the intertidal community structure and functionality, with consequences in all provided Ecosystem Services, including species of commercial interest.

For instance, the two species of *Mytilus* that occur in Portuguese coasts being exploited commercially over the world had their maximum global production in 2003 for *Mytilus galloprovincialis* with 147 468 tons and *Mytilus edulis* with 240 238 tons in 1999 reported by the Food and Agriculture Organization (FAO) (FAO, 2021). Despite the

large numbers, in some areas like Galicia (Spain), aquaculture still relays on wild mussels to provide the next generation of juvenile mussels (FAO, 2021).

1.3. In-situ study methods and controlled environment trials

Different approaches have been employed to study the warming effects and the resulting impacts over coastal ecosystems. Among them, should be mentioned some generic field methods widely used in Ecology to study coastal communities (e.g., intertidal habitats):

Q**uadrants:** Squares of variable dimensions, quadrants serve as a unit of survey to characterize and survey coverage, diversity, and representability in intertidal habitats. Sampling can be made randomly, on transects, by zonation on the intertidal (Murray et al., 2006).

Sampling: Sampling a specific target also can be used to study it directly (Gomes et al., 2016) or afterwards to replicate and test conditions in a controlled environment (microcosms and mesocosms trials) (Gestoso et al., 2016).

Cages: when species to test on the field are mobile, the objectives require to keep the subjects in an area isolated or semi-isolated. The use of cages allows testing the interaction with environmental factors with the subjects, although limited (Giltrap et al., 2013).

Tags: Release and recapture methods are less frequent on intertidal or coastal communities yet can be used if the biology of the target species allows identifying the marked individual later. Tagging can be done in several ways, from physical tags to paint marks, ideally, tags should not be invasive. In our case, paper tags and glue worked well on both species to track the sample evolution. On other species that grows through moulting, tags can be lost in the process. For soft body species, this process can be invasive or not even possible (Murray et al., 2006).

Overall, In-situ methods give us a high grade of realism but low replicability across locations.

Mesocosms experiments: In alternative to field observations, experiments under controlled conditions makes possible to exclude factors influencing the results. Also is

possible to test conditions and gradients simultaneously. This can be achieved through a closed or open system, depending on the experiments' design and/or objective. The principal argument against lab trials is that replicability sacrifices realism and cannot be extrapolated to the field, and this can be overcome with a balance between design complexity and posterior analysis (Widdicombe et al., 2010).

1.4. General aims

Considering the continuous increasing rates of the global temperatures as a threat for coastal ecosystems, this project aims to build causal links between temperature as one climate drive to understand and predict the impact of climate change on intertidal habitats. For this purpose, we assessed physiological responses of keystone intertidal species using a custom system in controlled conditions, to determine their thermal resistance threshold and to identify relevant and reliable stress indicators using both, standard eco-physiological and transcriptomic approaches to validate and compare stress responses.

1.5. Role of *Nucella lapillus* and *Mytilus galloprovincialis* on the rocky shore

Both intertidal species used in this study were chosen following the functionality criteria, as previously reported:

de Visser et al., 2012 resumes “Keystone species are thus species that have large effects on communities and ecosystems through many different processes such as trophic interactions, pollination, or habitat modification” (p. 60). In this category fits the gastropod *Nucella lapillus.*

As for an Ecosystem Engineer de Visser et al., 2012 stated that “organisms that directly or indirectly modulate the availability of resources (other than themselves) to other species by causing physical state changes in biotic or abiotic materials. In so doing, they modify, maintain and/or create habitats” (p. 61).

These species have a large role in the structure of intertidal communities and may be affected by climate change. Any alteration on this species abundance or functionality

reflects directly on the predator-prey relationship. It also may trigger a cascading effect over the intertidal community with deep impacts on the rocky shore assemblages.

Mussel beds of *Mytilus galloprovincialis* in rocky intertidal are known for providing shelter and food to a large community of invertebrates. On the other side, *Nucella lapillus* plays a role as a predator over calcifying organisms (Crothers, 1985), keeping under control populations and making room for new individuals to settle (de Visser et al., 2012).

1.6. Target species description and ecology

1.6.1. *Nucella lapillus* (Linnaeus, 1758)

Popularly known as Dogwhelk, *Nucella lapillus,* (Fig. 2) is a gastropod from the Family Muricidae, which contains many specialized predators. This species is from Ovoid-conic shaped shell, with a short spire in proportion to the body whorl or last whorl that compromises about 80% of shell total height. The siphonal canal is short, partially closed.

This species does not tolerate low salinity and excessive algal coverage (Hayward & Ryland, 2017).

N. lapillus feds on the intertidal by perforating the calcareous shell of its prey when there is no opening available "gape method" (MarLin, 2021b). *Nucella* perorate shells aided by the mechanical activity of the radula and enzymes produced by the Accessory boring organ (ABO) alternately. Once the shell is perforated, it injects its prey with narcotic and digestive enzymes. This species has a large selection of prey, including mussels, oysters, barnacles, and other gastropods (Crothers, 1985).

N. lapillus are abundant, easily identifiable, and easy to keep in laboratory conditions. All these traits make them excellent subjects (Crothers, 1985).

This species is the only member of the *Nucella* Genus that lives on the Atlantic coasts. It inhabits the Mid-low intertidal on rocky shores, both protected and exposed beaches (Crothers, 1985). This species can be found on all west European shores, from the Arctic Ocean to the Mediterranean Sea, Greenland, Canada and USA (MarLin, 2021b; WoRMS, 2021b).

This species shows strong preferences in their prey selection but also has the capacity to adapt itself to the available preys if their abundance changes (Hughes & De, 1984; MarLin, 2021b).

They feed during high tide and depend on the handling time during that period. In the high tide, *N. lapillus* must decide if they proceed to feed themself or remain protected in crevices. The presence of predators also triggers complex behavioural and physiological responses (Hughes & De, 1984; Sr Vadas et al., 1994).

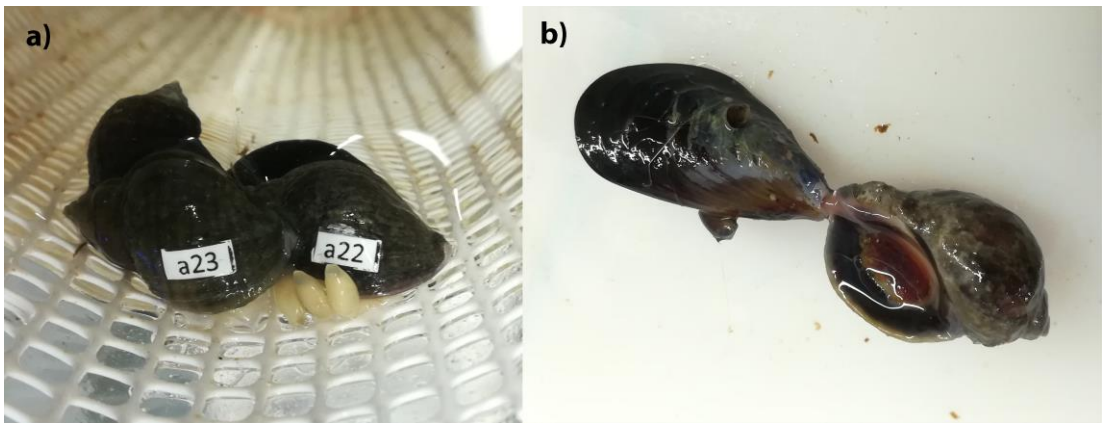

Fig. 2. *Nucella lapillus*, a carnivore gastropod from the Muricidae family. Widely common on rocky shores of the East North Atlantic and a keystone species capable of transform the local community through their predatory behaviour. On the figure is shown a) *N. lapillus* gregarious egg posture. b) *N. lapillus* feeding on a perforated *M. galloprovincialis*.

After feeding, they may enter into a quiescent stage that allows them to digest the consumed flesh and economize energy, time when they seek shelter (MarLin, 2021b).

Their life span is around six years, reaching maturity around the 3rd year. Also, they express sexual dimorphism but is not clearly visible (Crothers, 1985) with improvement in the images provided by (Smith, 2014).

As a rule, large females produce a higher number of eggs. Egg size depends on the latitude, food availability and ratio of fertilized and nursery eggs, although they can hatch in brackish water, the max hatching ratio is recorded when submerged in seawater (MarLin, 2021a).

Hatching is temperature dependent and ranges from 4 months in colder / north Atlantic waters to 2 months in warmer / Mediterranean waters (MarLin, 2021b).

Nucella lay their eggs in a vase shaped capsules cemented to the substrate by the stalk, the chamber contains eggs, and in the opposite the temporary “plug” that seals the

chamber. Larval development occurs inside the capsules, and hatched larvae use their reserves at first and later feed on the unfertilized "nurse" eggs (around 94% of the 600 eggs that can contain). Juveniles resemble the adult version, when they are ready, bore the plug and leave it open for the following. Although this process ensures the hatching probability reduces the larvae dispersal (MarLin, 2021b; Rawlings, 1989).

This species grows by adding calcareous material to the lip. Their growth is not always continuous and can be recorded in their shell (colour, banding, texture, thickness, and morphology). There is also a correlation between shell characteristics and environmental conditions (wave action, temperature) and diet (Crothers, 1985).

Two ecotypes are recognized in literature distinguished by the body, shell morphology and thickness, exposed beach, and sheltered beach ecotype. Although the name is given to the beach they inhabit, sometimes both ecotypes coexist in the area (Rolán et al., 2004).

1.6.2. *Mytilus galloprovincialis* (Lamarck, 1819)

Being a commercial species and one of the most harvested bivalves in the world, *M. galloprovincialis* Fig. 3 is commonly mistaken with *Mytilus edulis* (FAO, 2021). In Portugal the exploitation of this species is under the jurisdiction of the Instituto Português do Mar e da Atmosfera (IPMA), and it was defined five main areas, with its respective subdivisions, where this mussel can be caught by hand (North, Centre, Lisbon, and Tagus Valley, Alentejo and Algarve) and establishes that maximum of 3kg/day per fisherman. IPMA through the Sistema Nacional de Monitorzação de Moluscos Bivalves (SNMB) also provides a report of the areas where is allowed caught mussels without prejudice for human health, giving special attention to the presence of Biotoxins, Harmful Algal Blooms, Heavy Metals, and faecal contamination (IPMA, 2021).

M. galloprovincialis is common in the North Atlantic and is also present in the Mediterranean Sea, Indian Ocean, South Africa, Coastal Waters of Southeast Alaska and British Columbia, United States, Bass Strait, Chile, Argentina, South Australia, New Zealand, Indian Ocean, Japan and South China Sea (WoRMS, 2021a).

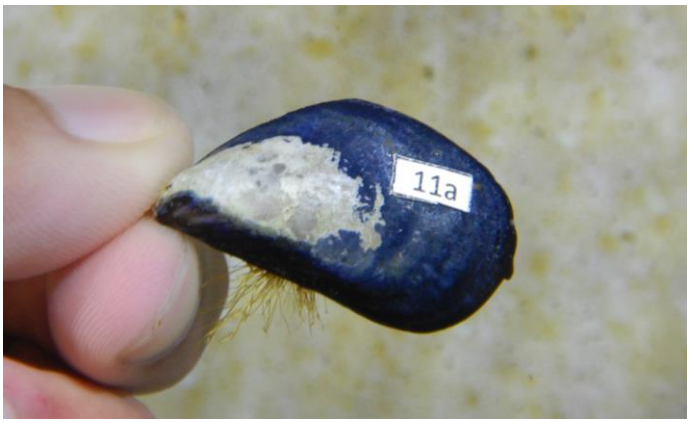

Fig. 3. *Mytilus galloprovincialis,* a gregarious mussel from Mytilidae family native to the Mediterranean Sea, despite it has spread globally by man mediated actions, including aquaculture. In the natural environment, *M. galloprovincialis* creates biogenic reefs hosting numerous species therefore, it is considered an ecosystem engineer.

Mytilus are gregarious species in rocky shores that form large beds, also called the mussel fringe in the intertidal zonation. Less dense aggregations may also occur in the subtidal. *Mytilus* are non-selective filter feeders being able to retain microorganisms, algae, detritus, and contaminants (MarLin, 2021a).

The capacity to retain harmful compounds or microorganisms makes them a good sentinel of the environmental status and quality being a validated species according to European guidelines for water quality (Moschino et al., 2011).

Mytilus are the prey for several species besides *N. lapillus.* Some crabs can break the shell (*Carcinus maenas*, *Cancer pagurus*), starfishes can protrude their stomachs to engulf isolated mussels (*Marthastherias glacialis*, *Asterias rubens*). As defence from *N. lapillus* predation, mussels use byssus threads to bind and immobilize them. More dense mussel assemblages can produce more threads resulting in a more effective defence. There are reports that, on some occasions, the threads can flip *N. lapillus* shell, resulting in their predator's death (MarLin, 2021a).

Mussels, as sessile organisms, opt for external fertilization, where the new recruits go through a pelagic larval cycle before the juvenile settles as a miniature version of the adult mussel (FAO, 2021).

It is known that *M. edulis* and *M. galloprovincialis* when occur together at the same locations, may trigger hybridization between the two species. The hybridization differs from site to site. A proposed way to this species avoid hybridization is to have different reproductive periods or with a small overlapping (Coustau et al., 1991).

Tracking the chemical signature of juvenile *M. galloprovincialis* shells across sites showed the large dispersal range that this species can achieve by travelling in the coastal currents. For a sessile species, this has an important role in the gene flow and in the diversity between marine protected areas and rocky shores without protection status (Gomes et al., 2016).

1.7. Aggravated treats by temperature rise

1.7.1. Imposex by Tributyltin (TBT)

In the decade of the 80's tributyltin was a component used in antifouling paints. This component in low concentrations induced the production of testosterone in female *N. lapillus*, causing them to develop male characteristics such as a penis- and vas deferent-like structure. This droves the females to be unable of laying eggs, and later, become sterile. The degree of masculinization, called imposex, was a measurable bioindicator of the TBT concentration to which the *N. lapillus* were exposed. In harbour areas where the concentration of TBT was naturally higher, the resident populations of *N. lapillus* became extinct. Thus, TBT favoured the colonisation of fucoid algae due to the decline of *N. lapillus* as a predator (Spence et al., 1990). Areas affected by TBT in the past indicate that imposex by TBT is persistent, however the degree of masculinisation is temperature-dependent but is not enough to cause infertility (Galante-Oliveira et al., 2010).

1.7.2. Multiple drivers

The interaction between Multiple Ambiental Drivers has been extensively studied and shown to have a greater effect when acting together than separately. As for *M. galloprovincialis* lower pH values increased the area where the periostracum is loss in the oldest part of the shell with time (Gazeau et al., 2014). When confronted with a warmer water species *Xenostrobus securis*, the Atlantic *M. galloprovincialis* showed higher mortality both with lower pH and higher temperatures in assemblages of a and mixed and single species (Gestoso et al., 2016).

In this work, particular attention will be given to temperature as a single driver and the impact in the trophic interaction.

1.7.3. Pollution as Bioaccumulation and Biomagnification sources

Mussels as filter feeder organisms have the capacity to retain compounds available in their surrounding environment. When they cannot be metabolized and discarded by the organism, depending on the nature of the compounds, they may be incorporated in the formation of a new shell or stored in the lipid reserve. Common reference organs that tend to accumulate metals in short term expositions are the gills, the hepatopancreas and the gonads. Related to chronic expositions may be the presence of these elements in the abductor muscle. Seasonal variation on heavy metals detected on a population of *M. galloprovincialis* was assigned to several environmental factors, among which was the temperature (Mejdoub et al., 2018).

Studies on this species with toxins from Harmful Algal Blooms (HABs) at different conditions of Temperature and pH showed that the elimination process becomes slower when exposed to high temperature and acidic conditions (Braga et al., 2018).

1.8. Thesis outline

Given the climatic conditions and their impact on the intertidal, the focus of this master's thesis was to assess the effect of temperature rising on the selected species, for the better understanding of its consequences upon behaviour, physiology, molecular response, and species fitness, in general. To achieve this objective, we applied a thermal stress to keystone intertidal species in a controlled environment. In this way, we selected the factors and variables that would intervene in climate disruption scenarios, such as variations in maximum and minimum temperatures and heat wave situations.

In order to achieve the general objective, this work was designed to assess the physiological responses of keystone intertidal species using a custom system in controlled conditions, (i) to determine their thermal resistance threshold of two intertidal mollusc species from Portugal coasts, (ii) in order to identify relevant and reliable stress indicators using, standard eco-physiological and transcriptomic approaches to (iii) to explore and validate the genes or biomarkers underlying the molecular responses of mussels under thermal stress.

This thesis is structured in seven major parts with the following structure:

1. This chapter provides a general introduction in which the theme of the thesis, the general concepts associated with it, the problematic targeted and the general aims of this work are presented.

2. This chapter provides a description of the two main experimental systems where the experiments were carried out.

3. This Chapter provides the results of the first experiment exposing *N. lapillus* and *M. galloprovincialis* to thermal stress in the systems described above.

4. This Chapter provides the results of the second experiment, which explore the transcriptomic profile of the gills from the mussel *M. galloprovincialis* under thermal stress .

5. This Chapter provides a general discussion where the results of both experiments are integrated and discussed together.

6. This Chapter comprises the General Conclusions drawn from both experiments combined with the information gathered from the General Introduction and General Discussion.

7. This Chapter provides our Recommendations based on the conclusions, finding and work limitations, as well as our Future Perspectives on how it can be improved and complemented in future studies.

Chapter 2. General Experimental System

2.1. Tank system

Eight experimental levels of increasing temperatures were established from 7 up to 28°C. The temperature in each tank was shifted around 1°C Day^{-1} from the initial 16°C to the desired temperatures of 7, 10, 13, 16, 19, 22, 25, and 28°C.

Our experimental setting consisted in eight PVC seawater baths with the capacity to hold up to 144 L. In each bath, we placed six independent cylinders (2.35 L capacity) containing the experimental specimens. Each cylinder was aerated by a compressed air hose connected to a PVC diffuser. A detailed scheme of the experimental setting is available in the Fig. 4 with the tank arrangement in Fig. 5. Water temperature was controlled in each tank by using chillers and heaters (Aqua Medic® GmbH, Bissendorf, Germany), which were regulated by digital controllers and individual temperature probes (STC1000 Elitch®).

Water renewal was controlled by a microcomputer (Arduino®) and motorized valves. The valves filled the cylinders with new water for 1 minute every 30 minutes. The seawater passed by a serpentine inside every seawater bath before reach the cylinder to guarantee stable temperatures.

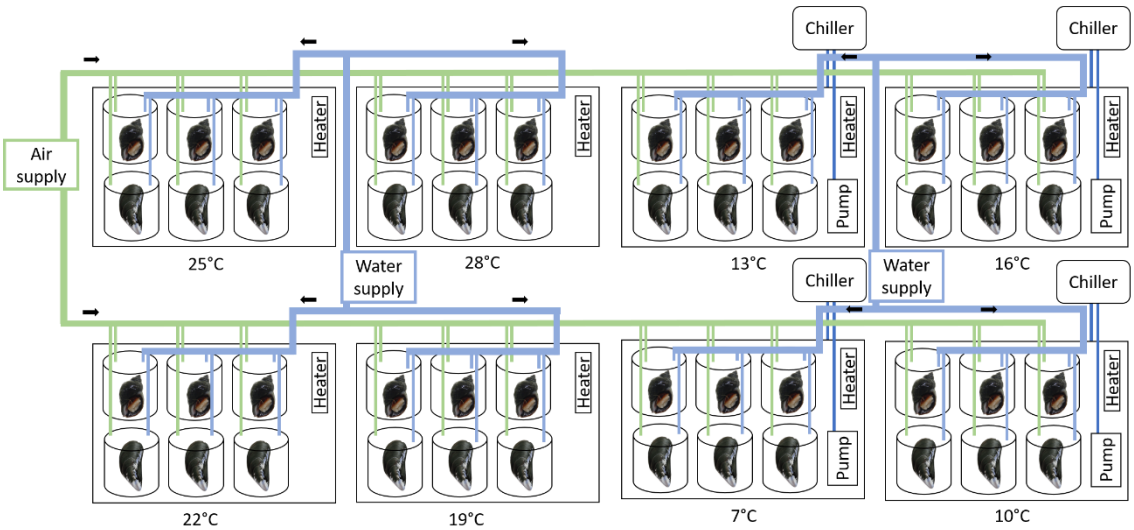

Fig. 4. 2D Diagram describing the thermal controlled system arrangement. Eight PVC tanks corresponding to the experimental temperature range (7 to 28°C). On the figure, the green lines represent the aeration circuit, and blue lines depict the seawater circuit. Black arrows indicate the flow direction, while the boxes show the relative position of the equipment regulating temperature. Each miniature of *N. lapillus* and *M. galloprovincialis* correspond to ten individuals on each cylinder.

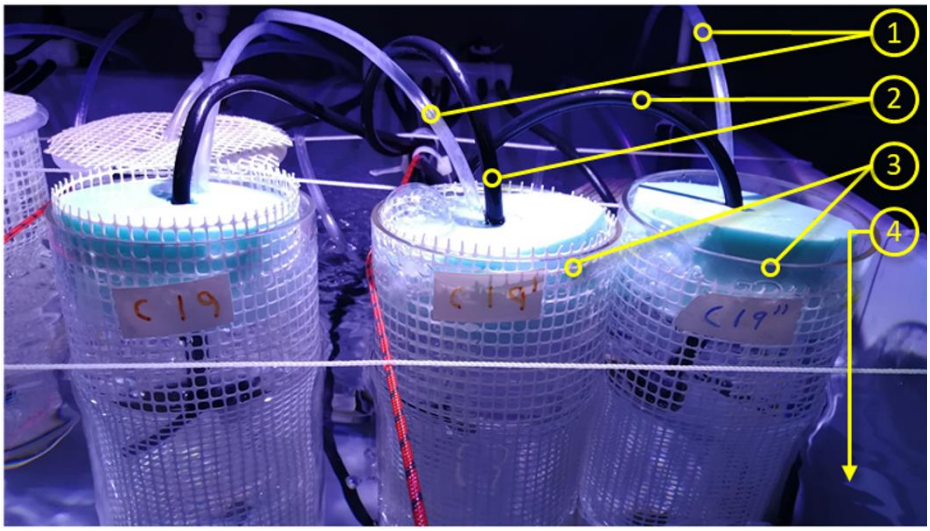

Fig. 5. Picture describing an experimental unit of the Tank system. 1 - Aeration hose, 2 - Water renewal hose, 3 - Containers, 4 - Temperature controlled bath

2.2. Respirometry system

Respiration rates were estimated by measuring oxygen fluxes inside a sealed incubation chamber. The incubation chambers consisted of four 0.07 L acrylic containers submersed in a larger glass tank (15 L capacity) with water pumped from a thermostatically controlled bath to grant a stable temperature. A thermostat TCM-1000 controlled simultaneously a chiller (Aqua Medic Titan 2000) and two titanium heaters to keep the target temperatures. Water movement inside the incubation chamber was maintained through magnetic stirrers which were separated from the animals by a perforated platform. The stirrers were driven by four 12 V electrical motors with magnets placed under the glass tank, see Fig. 6.

All the incubations were performed using filtered seawater (Aqua Medic UV sterilizer (Aqua Medic Helix Max 2.0) and a U/F membrane filter) which was kept at the target temperatures using the same setting described above.

Oxygen concentration variations were measured using a luminescent dissolved oxygen probe connected to a data-logger (PreSens PSt7-10 Oxygen Sensors connected to an OXY- 4 ST device, controlled by PreSens Measurement Studio 2 software version 3.0.3.1653 see Fig. 6, that registered a new measurement every 20 seconds and was continuously monitored. For the estimation of metabolic rates, we selected stable intervals of 30 minutes of raw O_2 consumption and corrected them using a blank measurement.

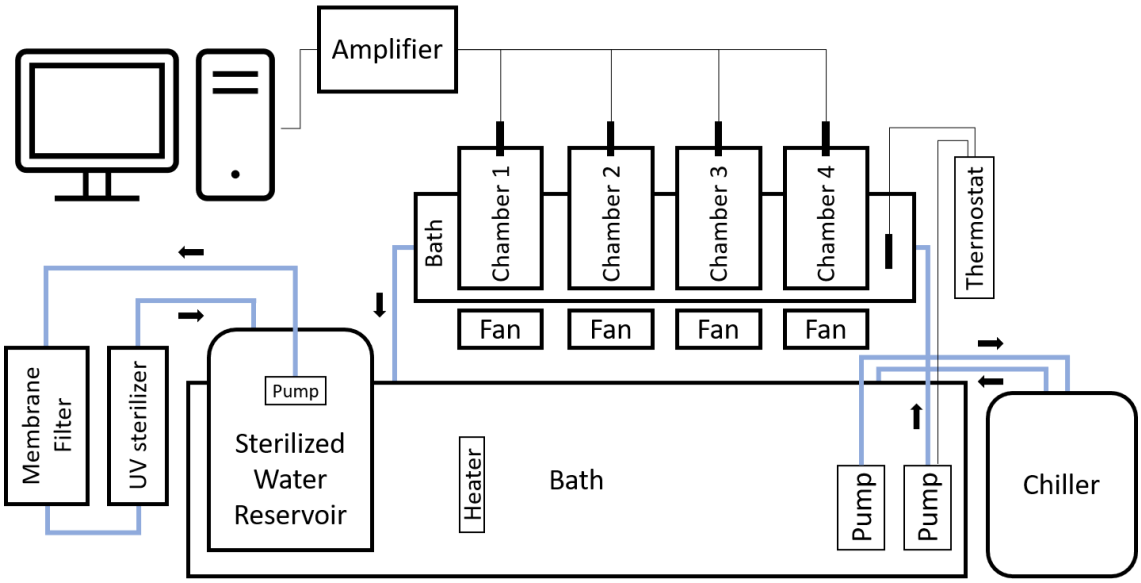

Fig. 6. Diagram describing the arrangement of the respirometry system.The system was divided into three subunits. First, the adjustable water reservoir (7 to 28°C) which supplies water to the second subunit. Secondly, the sub-unit containing the respirometry chambers and the components necessary to homogenize the water in the chambers and the temperature of the bath. Finally, the unit responsible for reading and interpreting the PreSens data. In the picture the blue lines represent the water flow inside the system and the arrow its direction, the black lines are the relevant wire connections. The boxes indicate the relative position of the equipment that make up the system.

Chapter 3. Responses of *Nucella lapillus* and *Mytilus galloprovincialis* to thermal stress

3.1. Introduction

Seawater and air temperatures in coastal areas increased notably in the last decades and are expected to continue warming due to the rising emissions of greenhouse effect gases (Christopher D. G. Harley et al., 2006; Hoegh-Guldberg et al., 2014). At best, future scenarios estimated by Climate Change Models point to a warming between +1.6 and +2.0°C in SST by the mid-century if the current trends of emissions continue (IPCC, 2019). Pervasive ocean warming is impacting the very dynamic and biodiversity-rich coastal habitats, with important effects on their ecological functioning and the services they provide (Harley et al., 2006). After decades of research on climate-related research, the basic relationships between temperature and species performance are reasonably well understood (H. O. Pörtner et al., 2014). The large experimental effort done in the last 20 years on the biological responses to marine climate change allowed to establish mechanistic understanding on the effect of ocean warming on marine organisms (Bass et al., 2021). At larger scales, macroecological climate research has focused on potential shifts in distribution and abundance of species driven directly by temperature (Harley et al., 2006). Hence, there is a large body of literature recording extensive replacement of benthic organisms by warmer species, i.e. tropicalization (Encarnação et al., 2019; Vergés et al., 2014), or poleward expansions of species distribution limits (Booth, 2020; Masselink et al., 2020).

Marine species inhabiting intertidal areas undergo strong environmental gradients at very short spatial and temporal scales and have been extensively used as indicator species in climate change research (Lima et al., 2007). Those intertidal communities, dominated by ectothermic organisms, play an important role in nutrients cycle and carbon storage and host several species of ecological and economical relevance (Bindoff et al., 2019). Particularly, rocky intertidal shores are a productive food source and an important nursery area for many fish and crustacean species (Hawkins et al., 2019; Pinsky et al., 2020a). Many of these species like to shelter in rocky shores, in areas where seaweed beds break the waves' power and reduce emersion stress(Hawkins et al., 2019; Lima et al., 2007).

Portuguese mainland intertidal shores extend from North to South around 940 km and are divided by the Nazaré submarine Canyon, which somehow sets the main areas of Atlantic (North) and Mediterranean influence (South) (Lima et al., 2007; Tuya et al., 2012). In this open coastal stretch, exposed to the strong action of waves, large sandy beaches alternate with rocky seashores (Fernandez-Arcaya et al., 2017; Ramos-Pereira et al., 2005). Mid intertidal reefs in rocky shores are often dominated by the Mediterranean mussel *Mytilus galloprovincialis* (Lamarck 1819). This species is edible, and there is a large aquaculture production in Europe (maximum global production occurred in 2003 with 147,468 tons (FAO, 2021) and nearly 96,511 tons on 2019 from Mediterranean countries (OECD Stat, 2021). In many areas like Galicia (Spain), aquaculture still relies on wild mussels to provide the next generation of juvenile mussels (FAO, 2021). Although adult individuals can survive at high temperatures (Anestis et al., 2007), like in other marine organisms, thermal stress may have large impacts on the species metabolism, abundance and large scale distribution patterns (Yao & Somero, 2012). Recently, a record-breaking heat wave affecting the Pacific Northwest and parts of Canada has killed millions of mussels, clams and other intertidal organisms (Cecco, 2021).

Coexisting with mussels in the intertidal reefs, the Atlantic dogwhelk *Nucella lapillus* is found often intermixed within the *Mytilus* beds. *N. lapillus* is a predatory gastropod with no commercial interest but has an important role in the balance of the ecosystem (Hunt & Scheibling, 1998). It is an active predator that feeds on *M. galloprovincialis,* usually by perforating its shell through enzymatic and mechanical activity (Crothers, 1985). This gastropod is an abundant and well-known species across the Atlantic European coasts. It has been extensively studied on predation trials and as a bioindicator of pollution by Tributyltin harbour areas (Galante-Oliveira et al., 2010; Spence et al., 1990). *N. lapillus* lays large capsules containing a standard number of eggs (Crothers, 1985), and this trait makes them a species easy to monitor on controlled conditions. Some reports show negative effects of rising temperatures on survival and predation rates of this gastropod (Bayne & Scullard, 1978; Crothers, 1985).

Seawater temperature is one of the most relevant environmental driver of coastal ecosystems due to its key role for chemical and biological processes (Angilletta Jr., 2009; Gestoso et al., 2016; Zippay & Helmuth, 2012) but species interactions are also sensitive to environmental change (Montoya & Raffaelli, 2010; Sampaio et al., 2017;

Zarnetske et al., 2012). Climate change linked alterations in prey-predator interactions have been outlined as fundamental structuring mussel beds (Harley, 2011). Most studies about temperature effects in a controlled environment only include a limited range of temperatures (Anestis et al., 2007; Bayne & Scullard, 1978; Gestoso et al., 2016) and neglect species interactions (Bass et al., 2021). In our study, we aimed to assess the effect of temperature as a climate driver, using a standard eco-physiological approach, to compare responses to thermal stress in a broad temperature range for the two target species, the mussel *M. galloprovincialis* and its predator *N. lapillus*. Predator-prey interactions are powerful regulators of community dynamics (Paine, 1966). Thus our second objective was to explore how much ocean warming could modify the interaction predator–prey of these two species, revealing potential shifts in the strength of the interaction, which may greatly affect community dynamics and ecosystem functioning (Hawkins et al., 2019; Tollefson, 2020).

3.2. Materials and Methods

3.2.1. Sampling

240 individuals of the two target species *Nucella lapillus* and *Mytilus galloprovincialis* were collected at the mid rocky intertidal of Belinho-Mar Beach, Esposende, Portugal (41°35'03"N 8°48'17"W) during low tide. Collection dates were the 7th of October 2020 and 3rd November 2020. Specimens were transferred to acclimation tanks at the laboratory facilities (BOGA-CIIMAR), and left acclimate for 2 days in seawater (35 ppt) at 16°C in a controlled temperature room (CT) under continuous aeration. To grant the food supply to *N. lapillus* we sampled extra mussels and keep them on an independent tank. Food supply for the *M. galloprovincialis* consisted in a stock solution of dried *Tetraselmis* sp. (60 mg L^{-1}) and *Isochrysis* sp. (40 mg L^{-1}) Phyto Bloom by Necton S.A.

Once the experimental mesocosms reached the target temperatures (7, 10, 12, 16, 19, 22, 25, 28°C) 30 individuals per treatment were tagged and measured. Tags were made of water-resistant paper and glued to the shell of the specimens. Mussels were measured with a Digital Calliper (Parkside), and the gastropods with the software Digimizer 5.4.6 after photographed. To take the pictures, all individuals were immobilized and photographed with a digital camera (Nikon Coolpix W300) at the same distance using a support tripod, dorsum and ventrally positioned with a reference scale. Size ranged

between 18-30 mm in *N. lapillus* and 16-43 mm in *M. galloprovincialis*. Then, animals were weighted in a ENTRIS6202l-1S balance from Sartorius Lab instruments GmbH & Co. All data was recorded to keep track of the individuals.

3.2.2. Fitness condition monitoring

To keep track of the condition of the individuals, each cylinder was checked every 48-72 hours. Animals were counted and any dead individual or mussel carcass was removed. Weekly, cylinders were cleaned and filled again. Mortality was calculated as a percentage (100 x No death/No total). In the case of *N. lapillus,* the number of egg capsules laid was also recorded.

3.2.3. Predation rate

Predation data was collected from the tanks containing individuals of *N. lapillus*. Except at 28°C and to a lesser extent at 25°C, mortality was negligible for most of the temperature conditions. Hence predation rate was calculated as the total number of successfully drilled mussels or eaten by the "gap method". *N. lapillus* was feed in a ratio 1:1 with the replacement of the eaten mussels on the surveys.

3.2.4. Metabolic rates

In heterotrophs, metabolic rate is determined using as a proxy the rate of oxygen consumption through respirometry essays (Killen et al., 2021). For these essays, we selected one individual from each replicated cylinder and 48 h after the target temperatures were reached and under 48-hour starvation condition, we performed the first respirometry to determine the basal metabolic rate. Once the measurement ended, we fed all the individuals in the cylinders, and after another 48 hours with food ad libitum, we performed a second respirometry using different individuals.

3.2.5. Statistical analysis

All statistical analyses were performed with a set of libraries using R software version 4.0.4 (R Core Team, 2021). Specifically for monitoring fitness responses were used the packages agricolae (de Mendiburu, 2021), tidyverse (Hadley Wickham & RStudio, 2021), and ggplot2 (Hadley Wickham & RStudio, 2021). To analyse respirometry were used the packages agricolae (de Mendiburu, 2021), tidyverse (Hadley Wickham & RStudio, 2021), respirometry (Birk, 2021), rTPC (Padfield et al., 2021), broom (D.

Robinson et al., 2021), nls.multistart (Padfield & Matheson, 2020) and ggplot2 (Hadley Wickham & RStudio, 2021).

Besides w used the utility packages xlsx, readxl, imputeTS, data.table, RColorBrewer, and ggpubr.

To determinate normality (homoscedasticity) of the data, we applied the Shapiro-Wilks test to each parameter studied. Predation was the only variable with significant heteroscedasticity, in this case Kruskal–Wallis test was applied. To detect significant differences between experimental conditions, we performed One-Way ANOVA, with a significance p-value of 0.05, and post-hoc Student-Neuman-Keuls (SNK) if necessary. At last, to find any possible statistical interaction between experimental conditions, we performed Two-Way ANOVA and post-hoc SNK when appropriate.

To calculate metabolic rate, we used the respirometry package (Birk, 2021), we run the script for all measurements in triplicated plus a blank for control. Note that metabolic rate from respirometry packages takes in account duration of the measurement and volume calculated manually plus Temperature, salinity, and pressure, provided by PreSens. With respirometry package, determine Metabolic Scaling was not possible due to the reduced body mass, instead we present Raw Metabolic Rates for both species.

Thermal performance curves were estimated in R using the package rTPC (Padfield et al., 2021) we select and fitted 17 models including: beta 2012, briere2 1999, delong 2017, flinn 1991, gaussian 1987, hinshelwood 1947, joehnk 2008, kamykowski 1985, lactin2 1995, oneill 1972, quadratic 2008, sharpeschoolhigh 1981, spain 1982, thomas 2012, thomas 2017, weibull 1995, and rezende 2019; (for more details see Padfield et al., 2021). For each one was calculated the Akaike information criterion (AIC) to understand how well the models fitted our data, then selected the one with the lowest value for each species. Two models were finally selected, the Flinn model (Equation 1) and the Gaussian model (Equation 2)

Equation 1. Flinn curve 1991

$$Metabolic\ Rate = \frac{1}{1 + a + b \cdot temp + c \cdot temp^2}$$

Equation 2. Gaussian curve 1987

$$Metabolic\ Rate = r_{max} \cdot exp^{\left(-0.5 \cdot \left(\frac{|temp - t_{opt}|}{a}\right)^2\right)}$$

3.3. Results

3.3.1. Thermal stress and mortality

During the experiment, both species showed certain tolerance to the thermal treatments applied, however dogwhelks (i.e., *N. lapillus)* showed higher mortality than mussels (*M. galloprovincialis*) at the most extreme temperatures. Although dogwhelks were able to survive under most of the experimental temperatures, at 28°C mortality rates reached 100% after 5 experimental days, suggesting that their upper critical thermal limit was already exceeded (Fig. 7a). We found significant differences on *N. lapillus* mortality due to the high mortality at 28°C, as confirmed by the SNK test, which found two groups of treatments: 7-25°C and 28°C. In the case of *M. galloprovincialis*, showed high heat tolerance, and did not record mortality between 13°C and 25°C, in its species the peak of mortality for this species occurred at 7°C with a percentage of 13% (Fig. 7b). Unlike *N. lapillus*, this species only revealed 3% of mortality at 28°C. Hence, we concluded that the selected range of temperatures was not enough to reach critical thermal limits of *M. galloprovincialis*.

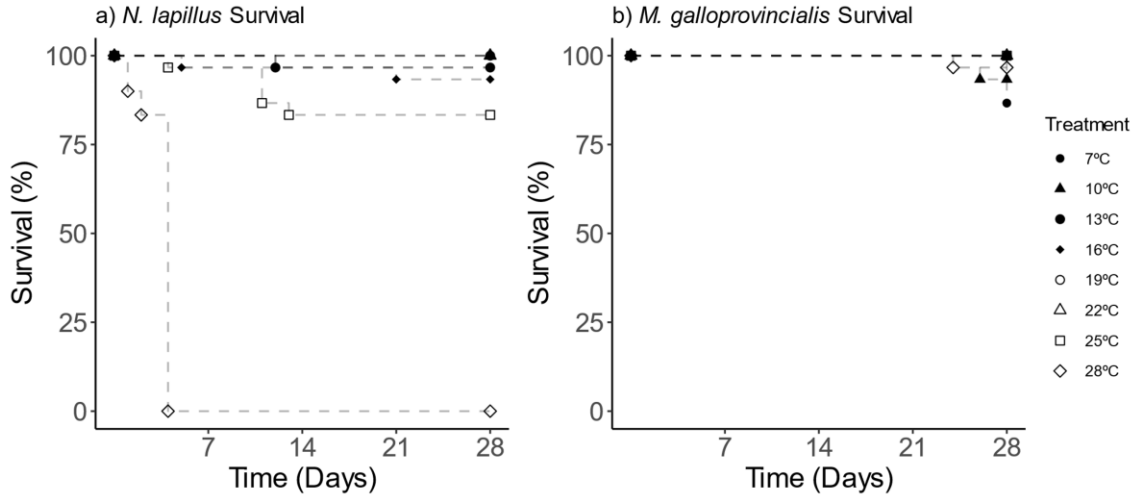

Fig. 7. Percentages of survivorship for two intertidal species against thermal stress from 7-28°C. The figure shows time in days on the x axis plotted against the percentage of survival in the y axis. Statistical analysis is depicted as line plots over time in a) *N. lapillus* (n=3); b) *M. galloprovincialis* (n=3).

3.3.2. Predatory and Reproductive behaviour in *N. lapillus*

Under the experimental conditions (different temperatures and removing tide and wave action), *N. lapillus* exhibited both predatory and reproductive behaviour with a clear

temperature-dependent tendency, as can be observed (Fig. 8). Because of large intra-treatment variability, no significant differences were found for both predation and reproduction, (p-value= 0.2159) for Kruskal-Wallis test on Predation and (p-value= 0.285) for One-Way ANOVA on Reproduction.

Visually, temperature effects on predation can be subdivided into two groups, the first increasing slowly from 7°C until reaching their maximum at 19°C, then sharply fall up to 25°C. At 28°C *N. lapillus* was fed on the 2nd day after the first respirometry, yet they did not feed.

Reproductive output increased with temperature starting at 10°C, with a peak at 16°C, and decreased sharply until 22°C. Beyond these limits, this reproductive behaviour was suppressed, however, no significant differences were found among groups (p-value=0.285). In this species, reproduction started on the second week, regardless of the temperature, in all treatments it occurred.

Statistical interactions between reproduction and mortality detected only at 28°C influenced by the high mortality.

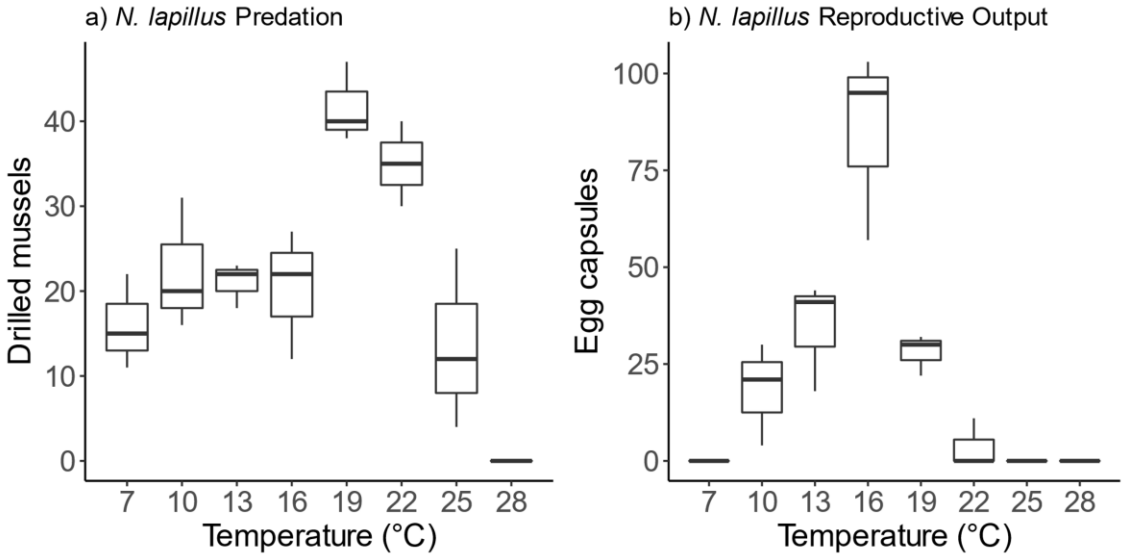

Fig. 8. Percentages of the behavioural responses of *Nucella lapillus* against thermal stress from 7-28°C. The figure shows statistical analysis as boxplots indicating temperature range on the x axis, while on the y axis the amount of a) Mussels drilled or eaten by the "gap method" (n=3); b) Egg capsules laid during the experiment (n=3). On boxplots error bars indicates min and max values, box indicates upper and lower quantile, enclosing the median.

3.3.3. Metabolic rates

When testing for significant differences between Temperatures and conditions, we verified the occurrence of significant differences between Temperatures for *N. lapillus* (p-value = 0.0149) and for *M. galloprovincialis* (p-value = 0.002), and differences

between feeding treatments were not significant, (see Fig. 9). Therefore, we grouped both treatments (Feed and Starved) for more robust results.

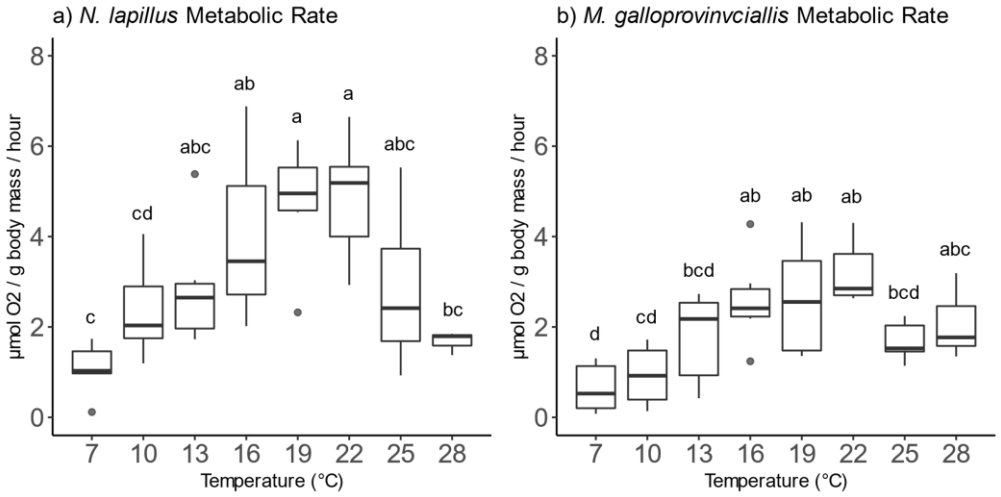

Fig. 9. Figure shows mean metabolic rates of the two intertidal species under thermal stress ranging from 7-28°C as boxplots with the outgroups from the Student-Neuman-Keuls test. On this figure, the x axis represents the temperature range in Celsius degrees while the y axis is representing the metabolic rate as oxygen consumption. a) Represents the metabolic rate of *N. lapillus* across temperatures b) Represents the metabolic rate of *M. galloprovincialis* across temperatures. On boxplots error bars indicates min and max values, points show outlier values, box indicates upper and lower quantile, enclosing the median.

Once calculated the parameters to fit the models with the *Akaike* information *criterion* (AIC) value from the rTPC selected models were Flinn 1991 for *N. lapillus* and Gaussian 1987 for *M. galloprovincialis* with scores 156.2428 and 126.8393, respectively.

Summarizing the Flinn 1991 model for Thermal Performance Curve we obtained the parameters that describe the curve: a = 0.6163781, b = -0.1466071, c = 0.0037961. Optimizing them on the Equation 1 (Padfield et al., 2021) the Maximum Metabolic rate occur at 19.31°C.

Summarizing the Gaussian 1987 model for Thermal Performance Curve we obtained the parameters that describe the curve: r_{max} = 2.8444, t_{opt} = 20.2898, a = 7.4796. Optimizing on the Equation 2 (Padfield et al., 2021) the Maximum Metabolic rate occur at 20.29°C.

Looking at metabolic rate from respirometry analysis, both species seems to tolerate a wide temperature range. As expected, *N. lapillus*, as an active predator, have greater metabolic rate than its prey, (see Fig. 10a). *M. galloprovincialis* being a sessile species compensate their lack of mobility with lower metabolic rate, (see Fig. 10b). Finally,

beyond the studied Temperature range, the Gaussian projection suggests a larger Temperature tolerance for *M. galloprovincialis*.

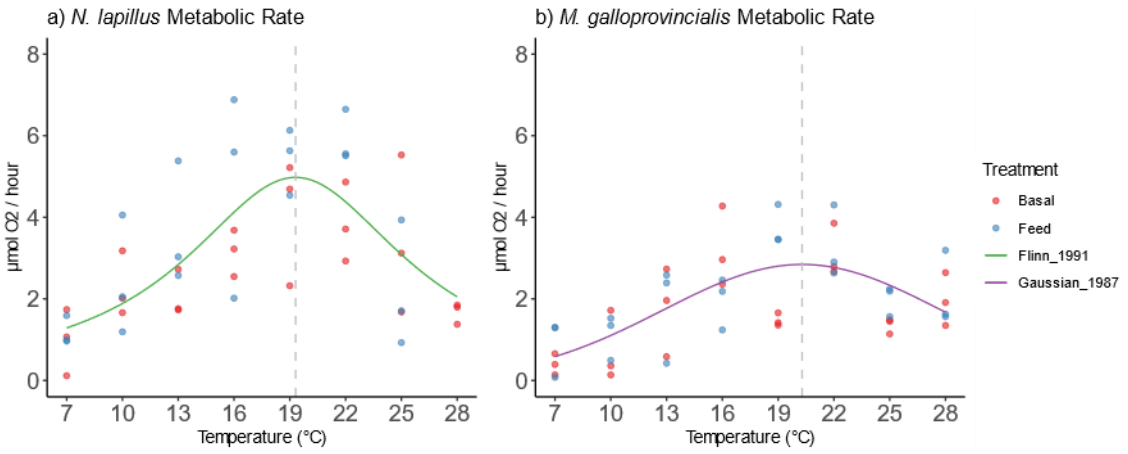

Fig. 10. Scatter plot containing metabolic rates of two intertidal species under thermal stress ranging from 7-28°C. For both species, Basal metabolic rate was measured under Starved conditions and Feed. On x axis the temperature range in Celsius degrees while on the y axis is represented the metabolic rate as oxygen consumption. a) Represents the metabolic rate of *N. lapillus* across temperatures, the dashed line = 19.31°C represents the highest rate predicted by Flinn 1991 model. b) Represents the metabolic rate of *M. galloprovincialis* across temperatures, the dashed line = 20.29°C represents the highest rate predicted by Gaussian 1987 model.

3.4. Discussion

Invertebrate responses to thermal stress at individual level are diverse, starting at the behavioural level, passing through physiological changes and ending in gene expression (Anestis et al., 2007; Chu et al., 2014; Hillebrand et al., 2018). Mortality is the result when none of these responses are able to overcome the thermal stress received (Tsuchiya, 1983).

In the case of *N. lapillus*, 28°C was the upper lethal limit for this species in our experimental conditions as no individual survived even for a week under this condition. This temperature is not far to the 28.9°C and 30°C found as median upper lethal temperature under normoxic (normal [O_2]) seawater conditions in two experiments with this species (Davenport & Davenport, 2007). Differences in the experimental settings or local adaptation of populations could explain inconsistencies across studies. For example, our experiment was longer in duration for both the acclimation and the experiment itself periods. In our experiment, we kept the species always submerged, restricting this way their mobility, otherwise the thermal stress would not by effectively applied. We aimed to find the critical thermal limit of the species for seawater

temperature, and thus our experiment is like those ramping experiments used to measure thermal resistance in invertebrates (Overgaard et al., 2012). In their natural habitat, intertidal species are alternatively emerged and submerged, and species undergo consecutive and variable levels of stress derived from air and seawater physical conditions. For example, when emerged *N. lapillus* is able to move from hot and dry sites and seek for shaded and more humid spatial refuges (Hillebrand et al., 2018). This seeking behaviour is the first defence against temperatures increasing, but it was not feasible to assess its effects on species survival through our experimental setting.

On the contrary, *M. galloprovincialis* mortality was negligible for most of the experimental temperatures. This low mortality is well below other experiments where mortality of the same species at 28°C was 30% in the first seven days and reached 60% by day ten (Bateman et al., 2021; Georgoulis et al., 2021). Intertidal *M. galloprovincialis* performance does not seem to be compromised by continuous submergence (Andrade et al., 2021), thus the experimental approach probably did not affect the survivorship in this case. In their habitats, intertidal populations of *Mytilus* spp. are often subjected to extreme conditions, reaching body temperatures at low tide above 40°C (Helmuth et al., 2016) using different strategies to cope high temperatures. Mussels are sessile species, they only move through larval dispersal, thus to cope with heat stress, the species prompt several physiological responses (Anestis et al., 2007; Gomes et al., 2016). Among those responses there are reports about bivalve species that can close the valves and induce metabolic depression to avoid thermal stress (Hui et al., 2020). This mechanism has been confirmed to occur in *Mytilus* when exposed to high temperatures (Anestis et al., 2007). During metabolic depression, *Mytilus* close the valves and recur to anaerobic metabolism consuming their energetic reserves to cover the ATP demand. In prolonged stress situations, bivalves recur to a brief opening of the valves, "called gapping", to avoid the high cost of metabolic depression (Hui et al., 2020). While other species may reduce their heartbeat rate, but this variation has been discarded in *M. galloprovincialis* (Anestis et al., 2007; Hui et al., 2020). Metabolic depression seemed to occur also in our experimental mussels, with an apparent reduction of metabolic rates at 25°C. Thus, resorting to metabolic depression and burning reserves using anaerobic pathways to cover the ATP demand (Anestis et al., 2007) can be a strategy to ensure survival in stressful environmental conditions. A similar strategy to combat thermal stress in *N. lapillus* was reproted by Leung et al.,

2000. This authors found metabolic depression on *N. lapillus* induced with thermal stress combined with cadmium exposure. However, metabolic depression was not apparent in our experimental *N. lapillus*, and we cannot confirm its existence.

In normoxic conditions, i.e. normal concentrations of oxygen in the water, the oxygen concentration decreases with the temperature increment, eventually becoming a limited resource (Rubalcaba et al., 2020), however linearity of the responses found in our experiments suggest that oxygen was not limiting the respiration rate of our experimental individuals. As expected, metabolic rates were highly temperature dependent in this experiment, with the expected bell shape pattern across the temperature range. In both species, the metabolic rates followed the anticipated thermal performance model where the consumption of O_2 increases and reach a peak before the uptake starts decreasing at higher temperatures. At low temperatures, biological processes occur at slow rates (L. P. Miller, 2013) , while at high temperatures, both species must promptly respond to grant their survival. When temperature increase beyond organism's optimum limits (at pejus temperatures) and according to the OCLTT hypothesis (H.-O. Pörtner, 2021), a mismatch occurs between energy demand and energy supply from aerobic pathways, leading to a compensatory shift to partial anaerobiosis, and energy deficiency. This forces the organisms to lower their metabolic rates and their oxygen consumption, showing similar results to those at colder temperatures.

The responses of metabolic rates and aerobic performances to temperatures are important for the Darwinian fitness of animals (Thyrring et al., 2015). Maximum metabolic rates in our experiment were close for both species, around 19-20°C. Beyond those temperatures both species were under suboptimal conditions and probably a set of thermal protection responses were launched. In these situations, the energy available may be redirected to synthesize defensive proteins against thermal stress; while in this process, the energy available to other activities is reduced (Roberts et al., 1997). In fact, both species can express a wide range of heat shock proteins and chaperone proteins under thermal stress conditions (Anestis et al., 2007; Chu et al., 2014). In the case of *M. galloprovincialis* a process of "thermal hardening" was recently described. This hardening allows the species to increase its thermal tolerance under the effect of elevated temperatures and it is triggered by brief exposures to sub-lethal temperatures (Georgoulis et al., 2021). All these capacities to respond promptly are remarkable and

may be considered adaptations to the variable temperatures in this habitat (Chu et al., 2014).

A downside for our initial approach to examine the metabolic rates of these species is that the starvation period probably was not long enough to difference basal and feed oxygen consumption on both species. Miller, 2013 used a period of 5 days to certificate that the digestive track was empty. However, this made possible modelling the metabolic rate in both species more accurate once we had twice many observations.

Regarding predation rates, in this study we took advantage of *N. lapillus* drilling habits to monitor their predatory behaviour. Besides, we maximized handling time on *M. galloprovincialis* by removing the influence of tide and wave action (Crothers, 1985). Overall, *N. lapillus* showed low feeding rates as other studies already reported (Crothers, 1985), possibly related to their feeding method. Previous studies suggested variable predation rates and a possible relation with temperature across seasons (Crothers, 1985). This supports our observations of what occurs at low temperatures and when Predation reach their peak, which is the equivalent of measurements in winter and spring or summer. Previous studies on temperature dependence of predation rates in *N. lapillus* tested a limited range of temperatures. A previous study approaching the effect of temperature on predation revealed that between 10 and 15°C the ingestion period shortens when the temperature rise (L. P. Miller, 2013), fitting well our results of increasing predation rate with increasing temperature. Stickle et al., 1985 used three experimental temperatures (5, 15 and 20°C) and found maximum predation rates at 15°C for *N. lapillus* in South England populations. In our experience, maximum predation rates for the species were observed at the same temperatures as the maximum metabolic rates. Other factors that may influence the predation process is the age / size of the individual and the experience with the type of prey offered (Crothers, 1985; L. P. Miller, 2013) or salinity (Stickle et al., 1985). *N. lapillus* collected in our study were adapted to prey mussels since it was the most abundant item, at mid intertidal, where they inhabit. Occasionally we observed more than one snail preying upon the same mussel, as well as scars of abandoned attempts of boring.

Regarding the reproductive output of *N. lapillus*, our individuals were collected in autumn, when seawater start cooling down after the maximum temperatures in late summer. In their natural environment, it would be expected that the end breeding season ended once the gonad reserves decreases, usually during summer or early autumn

(Gonçalves & Lobo-da-cunha, 2013). However, this species can breed continuously if the conditions are favourable (Crothers, 1985; Gibson, 1970; Gonçalves & Lobo-da-cunha, 2013).

From temperature loggers in a neighbour beach, we knew that bare rock in mid intertidal could reach, at least, between 4.8 (winter) and 37°C (summer) in extreme days around 2019-2021 (authors, unpublished data). For *N. lapillus* a similar temperature fluctuation has been reported correlated with Impossex expression (Galante-Oliveira et al., 2010). Seasonal changes in gonad lipid content have been reported for this species with two annual peaks of gonad maturity starting on March until September, with some temporal differences from higher latitudes (Gonçalves & Lobo-da-cunha, 2013). Also, Crothers (1985) reported that *N. lapillus* synchronizes egg posture to have a slow development over winter and hatch in more propitious conditions. When the water temperature becomes higher and the time for larval development shortens, so the eggs laid in spring may hatch in summer, otherwise they hatch in autumn, as appointed by Miller, 2013. Supporting from literature and our experimental results we assume that temperature may be a driver that triggers reproduction in *N. lapillus* but also can inhibit it below 10°C and beyond 25°C. Maximum reproductive output was found at 16°C, a temperature slightly inferior to those recorded for the maximum metabolic rate (around 19°C).

3.5. Conclusions

In this study we were able to examine fitness linked responses to temperature of both species *N. lapillus* and *M. galloprovincialis* in controlled conditions, using a custom home-made system to reduce variability. *N. lapillus* and *M. galloprovincialis* as eurythermal species are tolerant to a wide range of temperatures, showing low mortality at mid-high Temperatures.

On the other hand, the most thermal threatened response measure was the reproduction output of *N. lapillus* which seems to have a relative narrow window of optimal temperature for reproduction. This study provides new evidence on how thermal stress can produced mortality, affect physiological parameters, metabolic rates, and reproductive behaviour in different species, not only from future global warming conditions, but also gave us a better insight into how these species respond at low temperatures or winter season. However, similar studies must be applied in the future to

a broader range of intertidal species. Besides, transcriptomic and proteogenomic studies must be addressed to understand the responses underlying the mechanism at the molecular level.

Chapter 4. The transcriptomic responses of the gills from the Mediterranean mussel *Mytilus galloprovincialis* under thermal stress

Keywords: RNAseq; Differentially Expressed Genes (DEGs); Heat shock proteins (Hsp)

4.1. Introduction

Climate change models suggest that extreme weather events as marine heatwaves may increase in frequency and intensity over this century (Oliver et al., 2019). During a few days, abnormally high temperatures reach the intertidal. This has deep impacts on the intertidal communities, being able to cause high mortalities on mussel beds (Williams, 2021), changing their composition and functionality (Weitzman et al., 2021).

Mytilus galloprovincialis is a gregarious mytilid mussel, originally from the Mediterranean Sea who has expanded globally due to human-mediated introduction, accidental or for harvesting purpose (FAO, 2021; Hockey & van Erkom Schurink, 1992). *M. galloprovincialis* forms complex bed reefs on the intertidal and or beneath in the subtidal attached to an available hard substrate (Hockey & van Erkom Schurink, 1992).

A sessile filter-feeder on the intertidal must have adequate adaptations to survive in a dynamic ecosystem like the intertidal. Mytilus anatomy allows to storage seawater between the valves, *M. galloprovincialis* can depress metabolism when exposed in low tide (Anestis et al., 2007). Also, in days with high temperatures or near lethal limits, this species briefly opens the valves to cover the tissue's oxygen demand. This process is known as "gaping" (Collins et al., 2020).

M. galloprovincialis is classified as a bioindicator species (Jarque et al., 2014), and for this reason, it has been intensively and extensively studied. It is frequent combine the factor Temperature with pollutants, pharmaceutical drugs, metals, toxins (Izagirre et al., 2014; Jarque et al., 2014; Mohamed et al., 2014; Queirós et al., 2021), which makes it a good model to study the effect of Temperature isolated.

M. galloprovincialis are also relatively more resistant to temperature rising compared with other intertidal species (Chapter 3, Lockwood et al., 2010). The higher temperature supported by *M. galloprovincialis* underly, in part, in a molecular mechanism involving

several genes (Lockwood et al., 2010). High-throughput techniques, such as transcriptomic approach, have allowed to identify the Differentially Expressed Genes (DEGs) involved in mussels' responses to high temperature. Indeed, it has been demonstrated that *M. galloprovincialis* can rapidly express several genes encoding heat defensive / folding proteins from the families of Heat shock protein (Hsp), Chaperonins and Chaperon (Lockwood et al., 2010).

The expression of Hsp70 genes distinctly from other members of its family has been extensively studied, quantified and linked to thermal stress as an inducible response (Anestis et al., 2007; Roberts et al., 1997). In *M. galloprovincialis* this expression tends to be more accentuated comparatively to its relatives from colder waters, since warmer temperatures occurred more frequently on its native range (Lockwood et al., 2010, 2015).

Thus, in this study we aim to unravel the molecular responses of *M. galloprovincialis* facing a thermal stress, using a high-throughput transcriptomics approach.

4.2. Materials and Methods

4.2.1. Sampling, RNA extraction and RNASeq

To assess the transcriptomic response of the Mediterranean mussel *M. galloprovincialis* we used the same specimens assayed in the chapter 3, to correlate the physiological responses of mussels with their molecular response, once stressed with higher temperatures. As previously described in the Chapter 3, we collected nine individuals (>2.5 cm) on the rocky intertidal of Belinho-Mar Beach, Esposende, Portugal (41°35'03"N 8°48'17"W). The collection was carried on 3 Nov 3rd, 2020, during low tide. The organisms were transferred to the laboratory to acclimate at room temperature (16°C), salinity (35 ppt), under continuous aeration for two days. During this period, we feed them every two days with a mix of *Tetraselmis* sp. (60 mg/L) and *Isochrysis* sp. (40 mg/L) Phyto Bloom by Necton S.A.

In this trial, we evaluated the mussel responses facing a temperature gradient (19, 22 and 28°C). Once acclimated, the water temperature was increased ±2°C per day until reaching the desired temperature.

After 48h at the target temperature small pieces (~1cm^3) of gills were pooled from three individuals per temperature and condition, stabilized in RNAlater^{TM} (invitrogen) solution, and stored at -80°C until used.

4.2.2. RNA extraction and illumina sequencing

Sample preparation was performed following a similar protocol used by Domínguez-Pérez et al., (2019, 2021). Briefly, preserved samples in RNAlater solution were transposed to 2 ml tubes pre-filled with ceramic beads (Precellys® Ceramic kit 1,4) and then homogenized in homogenization buffer (RLT buffer) using an automatic bead-based homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) with a custom program (speed: 6800 RPM at 0°C; 3 x 20 sec cycles; 30 interval). The supernatant was recovered and conducted for total RNA extraction using Qiagen's RNeasy Mini kit (Venlo, The Netherlands) according to the fabricant instructions.

Total RNA concentration and relative quality was determined photometrically at 260nm with DeNovix DS-11 Spectrophotometer (DeNovix Technologies, Wilmington, Delaware, USA), and RNA integrity was evaluated with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Three samples corresponding to each temperature were submitted to Macrogen, Inc. (Seoul, South Korea) for total RNA sequencing using Illumina NovaSeq Stranded Total RNA Library Construction with Ribo-Zero Gold Human/Mouse/Rat + NovaSeq 6000.

4.2.3. Transcriptome assembly and completeness assessment

Assembly and completeness were similar to the protocol used by Domínguez-Pérez et al., (2021). The RNA sequencing on the Illumina platform 150bp (base-pair) paired-end (PE) produced around 60 million of reads for each sample. Transcriptomic and downstream analyses were performed with a set of bioinformatic tools incorporated in the OmicsBox v1.4.11 (BioBam Bioinformatics, 2021). Firstly, the quality of the raw reads was assessed using the FastQC (Fast Quality Check) software v0.11.8 (Andrews, 2018). Then, the original FASTQ files were pre-processed using Trimmomatic v0.38 (Bolger et al., 2014). The adapters were trimmed for each sample individually, and low-quality bases and low-quality reads were removed. High quality reads across all samples were combined and assembled into a single *de novo* transcriptome assembly with Trinity v2.12.0 (Grabherr et al., 2011), using 15 as the minimum k-mer coverage in the strand-specific mode.

The assembly quality and completeness were assessed with the Benchmarking Universal Single-Copy Orthologs (BUSCOs, BUSCO v5), searched for metazoans orthologues in transcriptome mode with a cut-off E-Value 1.0E-3 on OrthoDB v10.

4.2.4. Open reading frame Prediction and Transcript abundance estimation

Following the previous pipeline described by Domínguez-Pérez et al., (2021), firstly, the *de novo* assembled transcriptome was submitted to clustering analyses to reduce sequence redundancy using CD-HIT 4.8.1 (Fu et al., 2012; W. Li & Godzik, 2006), setting 0.9 as the Sequence Identity Threshold. Then, the clustered transcriptome was scanned for coding regions by TransDecoder v5.5.0 (http://transdecoder.sf.net) (Haas et al., 2013; Haas & Papanicolaou, 2021), and all six-frame translations were filtered for a minimum length of 100 amino acids for open reading frames (ORFs). The resulting ORFs were scanned for homology to known proteins via Pfam searches (PFAM 32, HMMER 3.2.1) (Finn et al., 2011; Punta et al., 2012) and only the best isoform per gene was retained (Haas et al., 2013; Haas & Papanicolaou, 2021). The corresponding coding genes for the best isoforms and all their transcript isoforms were used as reference transcriptome template for the expression analyses. The original pre-processed reads (clean reads without adapters) were mapped back against the reference transcriptome on a per sample basis using Bowtie2 v2.3.4.1 (Langmead & Salzberg, 2012), followed by calculation of abundance estimates using RSEM (B. Li & Dewey, 2011). The resulting count tables obtained was used for the Pairwise Differential Expression Analysis tool, based on the Bioconductor software package NOISeq v2.30.0 (Tarazona et al., 2011, 2015), suited to compare samples from two experimental conditions without biological replicates, simulating five technical replicates per sample. The data were normalized using the trimmed mean of M-values (TMM) normalization method, which was corrected for library size and reduced RNA compositional effect (M. D. Robinson & Oshlack, 2010). The Differential Expressed Genes (DEGs) were determined between experimental conditions 16, 22 and 28°C samples from gill tissue, with a cut-off p-value of 0.01.

4.2.5. Functional annotation and enrichment

The best isoforms per coding gene obtained from the clustered transcriptome, previously analysed against Pfam, were then characterized based on sequence similarity against. These transcripts (best isoforms) were searched against the Mollusca section of

the non-redundant (nr) protein database from NCBI (nr database: ftp://ftp.ncbi.nlm.nih.gov/blast/db; accessed on Sep 14, 2021) and UniProtKB/Swiss-Prot protein database (Bateman et al., 2021; UniProt Consortium, 2021) accessed on Sep 14, 2021), using CloudBlast option with the BlastX 2.10.0+ program setting a cut-off E-Value 1.0E-3 (BioBam Bioinformatics, 2021). The transcripts without hits were retrieved and CloudBlasted against the same protein database but excluding metazoans. Protein signatures were also obtained from the InterPro member databases (i.e. Pfam, PROSITE, PRINTS, ProDom, SMART, TIGRFAMs, PIRSF, SUPERFAMILY, Gene3D, and PANTHER) using the InterProScan (v5.51-85.0) software (Mitchell et al., 2019). All annotation and the corresponding gene ontology (GO) terms were then transferred to the sequences and eventually merged with existing GO terms resulting from mapping, InterProScan, EggNOG (evolutionary genealogy of genes: non-supervised orthologous groups) annotation and KEGG pathways (Mitchell et al., 2019), obtained with eggNOG-Mapper 1.0.3 with EggNOG 5.0.0 (Huerta-Cepas et al., 2019). Enrichment analyses to determine over/under expressed pathways was performed to the categorical GO terms corresponding to the DEGs, with the Gene Set Enrichment Analyses (GSEA), applying a False Discover Rate as adjusted value (FDR <0.05).

4.2.6. Statistical analysis

Using R software version 4.0.5 (The R Foundation, 2021), and the readxl to import the data. dplyr, tidyr, stringr and ggplot2 from tidyverse package (Hadley Wickham & RStudio, 2021) for fata handling , filter, search and plot, respectively. Lastly, with ggVennDiagram package (Gao et al., 2021) we elaborated the Venn diagram depicting genes in common between experimental conditions.

4.3. Results

4.3.1. Illumina sequencing, *de novo* assembly and completeness

The total RNA from gills was extracted using Qiagen's RNeasy Mini kit, and then, total RNA samples were conducted for paired-end RNA sequencing using an Illumina NovaSeq 6000 platform. The total RNA sequencing delivered around 60 million of reads per sample with 151 bp of read length, accounting for more than 6,2 billion of bases (Table 1). Most of the sequenced reads were identified with high accuracy, with approximately 98% showing a phred quality score over 20 (Q20(%): Ratio of bases that expresses the accuracy of each nucleotide, corresponding to 99 % base call accuracy,

and around 94 % of reads with phred score over 30 (Q30(%), corresponding to 99.9 % base call accuracy (Table 1). After adapter trimming, high-quality reads were conducted for *de novo* transcriptome assembly using Trinity v2.10.0.

Around 82,71% of the pre-processed reads comprising 223,587,527 bases were aligned in the *de novo* assembly. Altogether, we obtained an assembled transcriptome with 279,284 transcripts and 142,077 genes by combining all three samples into a single transcriptome. The *de novo* assembly generated showed a contig length (N50) of 1,277 bp, average contig length of 800.57 bp, with a minimum and maximum contig length of 200 bp and 19,611 bp, respectively (Table 1). The assembly completeness revealed that 84.3% of the BUSCO groups have complete gene representation (single-copy or duplicated), while 2.42% are only partially recovered, and 13.28% are missing (Table 1). The number of duplicated BUSCOs were reduced from 31.1% to 9.41 % after clustering analyses Fig. 11, resulting in 158,863 number of clusters at 90% identity (Table 1).

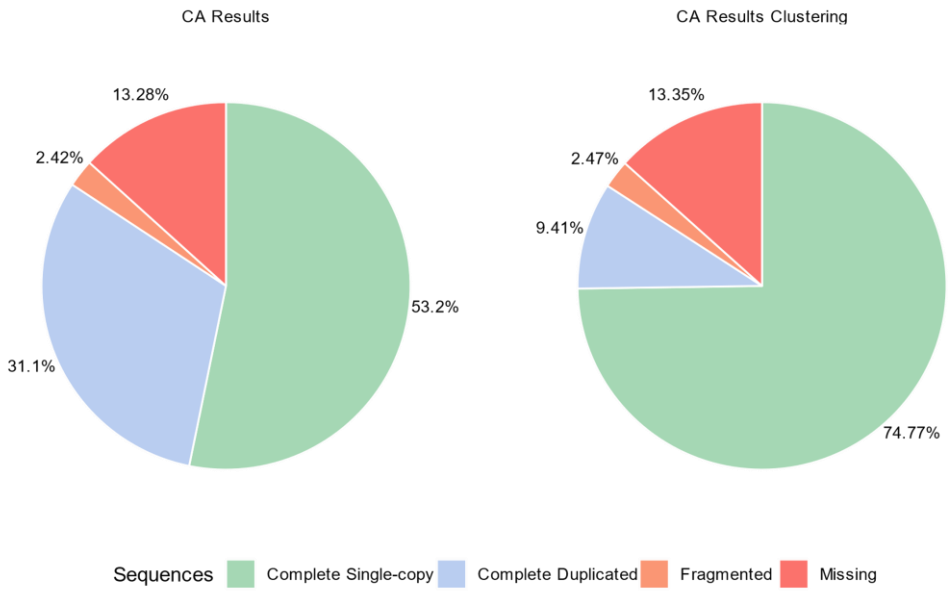

Fig. 11. Completeness assessment with the Benchmarking Universal Single-Copy Orthologs (BUSCOs), showing the number of BUSCOs. The relative percentage of Sequences shows the effects of Clustering isoform as an effective way of reducing Complete Duplicated sequences.

The transcriptomic project has been registered with the BioProject database under BioProject ID: PRJNA770496 (http://www.ncbi.nlm.nih.gov/bioproject/770496). The filtered Transcriptome Shotgun Assembly project containing 279,146 sequences has been deposited at DDBJ/EMBL/GenBank under the accession GJMF00000000.

The pre-processed raw data have been deposited at the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra), under the accessions SAMN22224041 (H_4, https://www.ncbi.nlm.nih.gov/biosample/22224041), SAMN22224042 (H_5, https://www.ncbi.nlm.nih.gov/biosample/22224042), SAMN22224043 (H_6, https://www.ncbi.nlm.nih.gov/biosample/22224043).

Table 1. Statistics summary of transcriptomic analyses performed to Mediterranean *M. galloprovincialis* exposed to thermal stress.

Summary of analyses per Sample/Condition[1]	Statistics	H_4	H_5	H_6
Raw data Statistics	Total Bases[2]	13,490,084,810	13,727,140,616	11,994,759,896
	Read Count[3]	89,338,310	90,908,216	79,435,496
	GC(%)[4]	39.94	39.46	39.72
	AT(%)[5]	60.06	60.54	60.28
	Q20(%)[6]	98.38	98.33	98.21
	Q30(%)[7]	95.04	94.82	94.21
de novo **Transcriptome Assembly** (Trinity v2.10.0)	Assembled transcripts	279,284		
	Total genes	142,077		
	Minimum/Maximum Contig length (bp: base pair)[8]	200/19,611 bp		
	Contig length (N50)[8]	1,277 bp		
	Contig length (Average)[9]	800.57 bp		
	GC(%)	35.43		
	Total assembled bases (All transcripts)	223,587,527		
	Total assembled bases (Longest isoform per gene)	89,124,628		
	Percentage of all read aligned/not aligned[10]	84.99%/15.01%	77.38%/22.62%	85.35%/14.25%
Assembly Completeness (BUSCO v5)[11]	number of BUSCOs/species	5295/22		
	Complete Single-Copy	2,817 / 53.2%		
	Complete duplicated	1,647 / 31.1%		
	Fragmented	128/2.42%		
	Missing	703/13.28%		
Assembly Clustering[12] (CD-HIT 4.8.1) and **Completeness**[11]	number of clusters at 90% identity	158,863		
	Complete Single-Copy	3,959 / 74.77%		
	Complete Duplicated	498 / 9.41%		
	Fragmented	131 / 2.47%		
	Missing	707 / 13.35%		
ORFs prediction[13] (TransDecoder v5.5.0)	Transcripts with ORF (best isoform per gene)	48,000		

Table 1. (Continued)

Summary of analyses per Sample/Condition[1]	Statistics	H_4	H_5	H_6
ORFs Annotation	Blast Hits nr/NCBI (Mollusca/Non-Mollusca)[14]	3,181/45,619		
	Overall ORFs with Blast hits/no hits[15]	105 (0,22%)/48695 (99,8%)		
	EggNOG[16]	16,179 (33,7 %)		
	KEGG Pathways/KO[17]	27,543 (56,4%) / 34,866 (71,4%)		
	IPS/GO annotation (%)[18]	34,422 (70,5%)/ 21,723 (44,5%)		
	Gene ontology (ORFs with GO annotation)[19]	32,560 (66,73%)		

[1] Sample ID/Condition of gills samples from *M. galloprovincialis*: H_4 (16℃), H_5 (22℃), H_6 (28℃).
[2] Total read bases : Total number of bases sequenced.
[3] Total reads : Total number of reads, corresponding to the sum of read 1 (Upstream Files Pattern: _1) and read 2 (Downstream Files Pattern: _2), obtained from Illumina paired-end sequencing.
[4] GC(%) : GC content.
[5] AT(%) : AT content.
[6] Q20(%): Ratio of bases that have phred quality score (expresses the accuracy of each nucleotide) of over 20 (99% base call accuracy).
[7] Q30(%): Ratio of bases that have phred quality score (expresses the accuracy of each nucleotide) of over 30 (99.9% Base call accuracy).
[8] N50: is defined as the sequence length of the shortest contig at 50% of the total transcriptome/genome length.
[9] Average contigs length
[10] RNAseq Read Representation corresponding to the percentage of the overall alignment (reads aligned/not aligned).
[11] The *de novo* assembly quality and completeness assessment with the Benchmarking Universal Single-Copy Orthologs (BUSCOs), showing the number of BUSCOs/relative representation (percentage) of Complete Single-Copy, Complete Duplicated, Fragmented and Missing. BUSCO v5 run in mode: Transcriptome, Mollusca section, e-value 1.0E-3.
[12] Sequence redundancy of the *de novo* assembled transcriptome was reduced using CD-HIT 4.8.1, setting 0.9 as the Sequence Identity Threshold.
[13] Protein Coding Sequences (CDS) obtained by six-frame translation with TransDecoder v5.5.0., considering a minimum length of 100 amino acids for open reading frames (ORFs), homology to known proteins via Pfam searches, and the best/longest isoform per gene.
[14] Blast hits of the 48800 CDS (best isoforms)obtained from the transcriptome assembly searched against the non-redundant (nr) protein database from NCBI and UniProtKB/Swiss-Prot, using BlastX program, filtering by the taxonomic section Mollusca/non-Mollusca (accessed on Sep 14, 2021), setting a cut-off E-Value 1.0E-3.
[15] Summary of Blast hits/no hits found against both databases searched, and their relative representation (percentage) within all (48,800) predicted ORFs.
[16] Functionally annotated ORFs according to orthologous groups (OGs: i.e., COGs, arCOGs, ENOGs, KOGs), using the eggNOG Mapper 1.0.3 with EggNOG 5.0.0, and its relative representation (percentage) within all (48,800) predicted ORFs.
[17] Functionally annotated ORFs according to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and KEGG Orthology (KO), and its relative representation (percentage) within all (48,800) predicted ORFs.
[18] Functionally annotated ORFs according to Gene Ontolgy (GO) and known proteins domains using InterProScan (IPS) v5.51-85.0, searched against InterPro member databases (i.e., Pfam, PROSITE, PRINTS, ProDom, SMART, TIGRFAMs, PIRSF, SUPERFAMILY, Gene3D, and PANTHER), as well as its relative representation (percentage) within all (48,800) predicted ORFs

[19] Overall functionally annotated ORFs according to Gene Ontology (GO) and its relative representation (percentage) within all (48,800) predicted ORFs.

4.3.2. Open Reading Frames (ORF) Prediction and Annotation

The resulting 158,863 clusters with 0.9 of sequence identity obtained were used for the open reading frames (ORFs) prediction using the TransDecoder v5.5.0., considering a minimum length of 100 amino acids. Overall, 48,800 Protein Coding Sequences (CDS) were identified with homology to known proteins via Pfam searches as the best/longest isoform per gene (Table 1). A total of 15,414 CDS were complete proteins, corresponding to 31.59%, whereas around 32.2% were partial sequences and 36.21% were internal, as can be observed in Fig. 12.

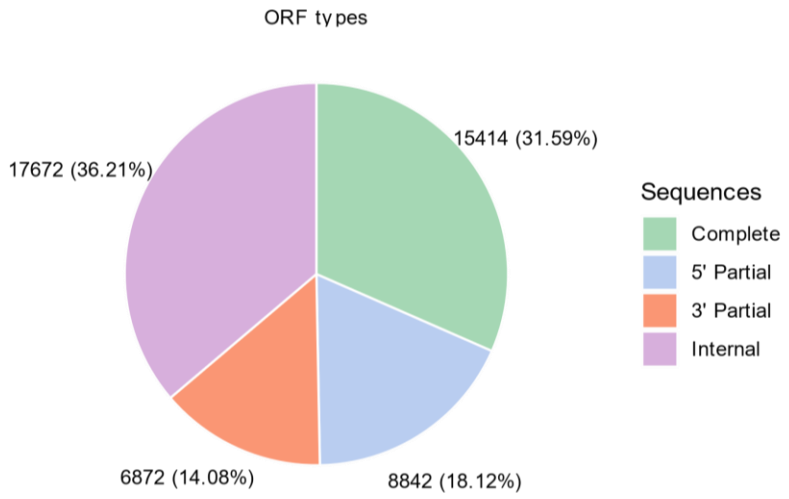

Fig. 12. Predicted relative percentages of Protein Coding Sequences (CDS) given by the characterization of the 48800 Open Reading Frames (ORFs)

These 48,800 ORFs were CloudBlasted against the Mollusca section of the non-redundant (nr) protein database from NCBI and UniProtKB/Swiss-Prot protein database (Bateman et al., 2021), using CloudBlast option with the BlastX program setting a cut-off E-Value 1.0E-3 (BioBam Bioinformatics, 2021). A total of 3181 transcripts had Mollusca hits within the database. Overall, 105 transcript sequences comprising the 0,22% had blast hits, whereas 48,695 remain without hits corresponding to the 99,8% (Table 1).

Besides, 16,179 (33,7 %) of these 48,800 ORFs were also annotated with eggNOG-Mapper 1.0.3 with EggNOG 5.0.0 (Huerta-Cepas et al., 2019). Moreover, the

corresponding GO annotation was performed by combining the existing GO terms (Blast hits mapping/GO annotation) and EggNOG annotations. In this respect, 32,560 (66,73%) transcripts were functionally annotated by the Gene Ontology, being 16,179 annotated with EggNOG (33,7 %) and 34,866 (71,4%) with KEGG pathway. For more details, see (Table 1).

It is noteworthy that our samples were retrieved from the wild, thus, putative contaminants were filtered out before pairwise differential expression analyses, once automatically detected by the NCBI/TSA submission system. The remaining ORFs encoded genes were then used for differential expression analyses. The list of these ORFs, as well as the list of putative contaminants with their corresponding annotation can be found in the Supplementary Tables S1 and S3, respectively.

Table 2. List of Differentially Expressed Genes (DEGs) related to markers involved in the gill's response to thermal stress. The Table list all candidate gene/transcript among DEGs related to known molecular markers involved in the *M. galloprovincialis* heating response.

Functional[1] **Categories**	**Gene/transcript**[2]	**Gene/transcript** Description[3]	**Gene/transcript**[4] GO/IP description	Organism[5] (**Accession**)	DEGs Subset[6] UP-regulated (red) / Down-regulated(green) LFC[7] **unique 16°C**	**common 16°C/22°C**	**unique 22°C**	**common 22°C/28°C**	**unique 28°C**	**common 16°C/28°C**	**common 16°C/22°C/28°C**
Heat Defensive	TRINITY_DN361_c0_g1		chaperone binding								-5.2 -2.7 -7.9
Response	TRINITY_DN9228_c0_g1				3.5 4.5						
	TRINITY_DN59925_c0_g3						5.9 -3.3				
	TRINITY_DN3581_c0_g1								-8.3 -5.0		
	TRINITY_DN1043_c0_g1	Hsp70 protein binding response to heat	Hsp70 protein binding						-4.2 -5.1		
	TRINITY_DN10920_c0_g1	ATP binding ATP hydrolysis activity	protein folding	Aplysia californica Q16956					-5.1 -8.3		
	TRINITY_DN1502_c0_g1	ATP binding ATP hydrolysis activity	protein folding						-2.7 -3.2		
	TRINITY_DN1820_c0_g1	ATP binding ATP hydrolysis activity	'*de novo*' protein folding						-3.7 -3.9		
	TRINITY_DN2305_c0_g1	ATP binding ATP hydrolysis activity	'*de novo*' protein folding						-3.0 -4.5		

Table 2. (Continued)

Functional[1] **Categories**	**Gene/transcript**[2]	**Gene/transcript** Description[3]	**Gene/transcript**[4] GO/IP description	Organism[5] (**Accession**)	DEGs Subset[6] UP-regulated (red) / Down-regulated(green) LFC[7]						
					unique 16°C	**common 16°C/22°C**	**unique 22°C**	**common 22°C/28°C**	**unique 28°C**	**common 16°C/28°C**	**common 16°C/22°C/28°C**
	TRINITY_DN2480_c1_g1	protein folding	Chaperone cofactor-dependent protein refolding						-3.7		
		unfolded protein binding							-5.4		
	TRINITY_DN2504_c0_g1	NuA4 histone Acetyltransferase complex	protein folding						-3.1		
		R2TP complex							-3.8		
	TRINITY_DN2651_c0_g1	ATP hydrolysis activity	'*de novo*' protein folding						-3.2		
		protein folding							-3.2		
	TRINITY_DN29242_c0_g2	chaperone binding	protein folding						-5.5		
		Hsp90 protein binding							-4.5		
	TRINITY_DN300_c0_g1	ATP binding	protein folding	Aplysia californica Q16956					-4.7		
		ATP hydrolysis activity							-6.2		
	TRINITY_DN3572_c0_g1	ATP binding	heat shock-mediated polytene chromosome puffing	Aplysia californica Q16956					-6.9		
		ATP hydrolysis activity							-6.9		
	TRINITY_DN3871_c0_g1		response to heat						-6.1		
									-7.4		
	TRINITY_DN4028_c0_g1	ATP binding	regulation of protein folding in endoplasmic reticulum	Aplysia californica Q16956					-3.7		
		ATP hydrolysis activity							-5.3		

Table 2. (Continued)

Functional[1] **Categories**	**Gene/transcript**[2]	**Gene/transcript** Description[3]	**Gene/transcript**[4] GO/IP description	Organism[5] (**Accession**)	DEGs Subset[6] UP-regulated (red) / Down-regulated(green) LFC[7] **unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**
	TRINITY_DN4170_c0_g1	ATP binding ATP hydrolysis activity	chaperone cofactor-dependent protein refolding	Aplysia californica Q16956					-4.8 -3.9		
	TRINITY_DN421_c0_g1		protein folding						-4.5 -4.5		
	TRINITY_DN4811_c0_g1	ATP binding ATP hydrolysis activity	regulation of protein folding in endoplasmic reticulum	Aplysia californica Q16956					-5.7 -5.7		
	TRINITY_DN5286_c0_g1		protein refolding						-3.8 -5.9		
	TRINITY_DN833_c0_g2		protein folding						-3.2 -4.7		
	TRINITY_DN3164_c0_g1	protein dimerization activity	cellular response to heat			-3.0					
	TRINITY_DN31152_c0_g1		response to heat			3.9					
	TRINITY_DN4821_c0_g1	Histone deacetylation Histone deacetylase activity	heat shock protein binding			4.2					

Table 2. (Continued)

Functional[1] **Categories**	**Gene/transcript**[2]	**Gene/transcript** Description[3]	**Gene/transcript**[4] GO/IP description	Organism[5] (**Accession**)	DEGs Subset[6] UP-regulated (red) / Down-regulated(green) LFC[7]						
					unique 16°C	**unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**
	TRINITY_DN21_c0_g1		protein folding							-4.5	
	TRINITY_DN29815_c0_g1	protein folding	protein folding							-4.7	
	TRINITY_DN61_c0_g1		protein folding							-3.0	
	TRINITY_DN557_c0_g2	protein binding	response to heat							-3.0	
	TRINITY_DN2729_c0_g1		protein folding in endoplasmic reticulum							-4.4	
	TRINITY_DN11597_c0_g1	unfolded protein binding chaperonin-containing T-complex	Chaperone mediated protein folding independent of cofactor							-4.3	
	TRINITY_DN6654_c0_g1		heat shock protein binding							-3.5	
	TRINITY_DN1347_c0_g1		Hsp70 protein binding							4.9	
	TRINITY_DN2896_c0_g1		chaperone-mediated protein folding							-5.0	

Table 2. (Continued)

Functional[1] **Categories**	**Gene/transcript**[2]	**Gene/transcript** Description[3]	**Gene/transcript**[4] GO/IP description	Organism[5] (**Accession**)	DEGs Subset[6] UP-regulated (red) / Down-regulated(green) LFC[7]						
					unique 16°C	**unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**
	TRINITY_DN4895_c0_g2	protein binding	heat shock protein binding							-4.2	
	TRINITY_DN2026_c0_g1	ATP hydrolysis activity unfolded protein binding	regulation of protein folding in endoplasmic reticulum	Aplysia californica Q16956						-5.2	
	TRINITY_DN1204_c0_g1									-3.7	
	TRINITY_DN1331_c0_g1	ATP hydrolysis activity unfolded protein binding	'*de novo*' protein folding					-3.1			
	TRINITY_DN57497_c0_g1		'*de novo*' protein folding					-3.2			
	TRINITY_DN3862_c0_g1	ATP hydrolysis activity unfolded protein binding	protein folding	Aplysia californica Q16956				-2.9			
	TRINITY_DN2965_c0_g1	protein kinase binding	HSP90-CDC37 chaperone complex					-4.1			
	TRINITY_DN8071_c0_g1		Hsp90 protein binding					4.4			
	TRINITY_DN644_c0_g1		chaperone-mediated protein folding					-3.0			

Table 2. (Continued)

Functional[1] **Categories**	**Gene/transcript**[2]	**Gene/transcript** Description[3]	**Gene/transcript**[4] GO/IP description	Organism[5] (**Accession**)	DEGs Subset[6] UP-regulated (red) / Down-regulated(green) LFC[7] **unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**	**unique 16°C**
	TRINITY_DN1136_c0_g1							-3.9			
	TRINITY_DN1909_c0_g1							-4.7			
	TRINITY_DN48677_c0_g1					3.9					

[1] Major Functional Categories related to known proteins/biomarkers involved in heat defensive response / protein refolding.

[2] Sequence name of the DEGs identified within the corresponding Major Functional Category.

[3] Blast top-hit description of the corresponding DEGs.

[4] Gene Ontology (GO)/InterProScan (IPS) description of the corresponding DEG (Gene/transcript).

[5] Organism and accession number of the corresponding Blast top-hit obtained for each DEGs.

[6] Subsets of unique and overlapped DEGs (used as test set in the enrichment analyses), identified in the gills of the mussel *M. galloprovincialis*, where contrasting

[7] The log-fold change (M) and the absolute value of the difference in expression obtained with NOISeq R/Bioc package between experimental conditions 16, 22 and 28°C

4.3.3. Differentially Expressed Genes and Functional analysis

Overall, 1411 unique Differentially Expressed Genes (DEGs), of which 641 DEGs resulted when comparing 16 *versus* 22℃, 633 DEGs in 22 *versus* 28℃ and 654 DEGs in 16 *versus* 28° (Fig. 13-15, Table S3 and Table S4). Only three DEGs were identified as overlapped among comparisons (Fig. 15 a). On the contrary, the number of unique DEGs identified for each pairwise comparison accounted 511 DEGs corresponding to 36.2 % of the total, being 150 DEGs excusive for 16℃, 186 DEGs for 22℃ and 175 for 28℃ (Fig. 15 a).

The pattern of expression corresponding to the up or down-regulation of DEGs from each pairwise comparison can be observed in Fig. 13 and 14. This pattern of regulation across temperatures did not show a clear tendency. The percentages of up regulation were 40% for 16 vs 22℃, 57% for 22 vs 28℃ and 52% for 16 vs 28℃ on the overlapped DEGs. After filtering total up regulated DEGs greatly decreased to only 13% of up regulation.

Among the GO names obtained were observed in their descriptions 23 relevant distinct terms (e.g., heat shock proteins, cellular defence to heat, chaperone, and refolding proteins). In addition, 13 of these GO names corresponded to the major category Biological Process, sin to Cellular component and four to Molecular function.

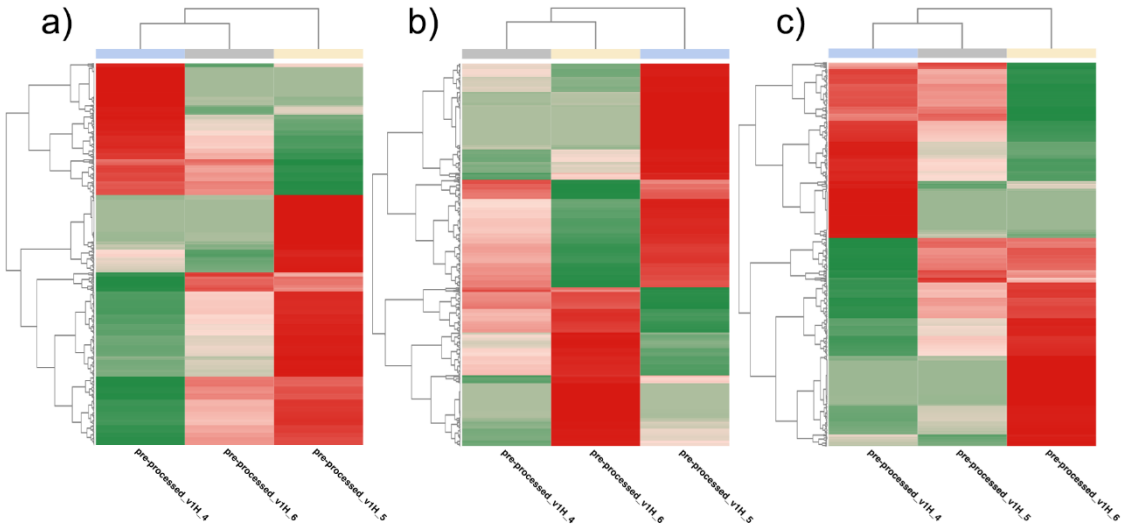

Fig. 13. The heat map depicts the corresponding expression (Z-score values) of the Differentially Expressed Genes (DEGs) for each pair of condition tested: a) 16 vs 22℃ b) 22 vs 28℃ c) 16 vs 28℃

Remarkably, we found the highest value of DEGs between mussels from 16 and 28℃ we found the highest value of DEGs, corresponding to a total of 45 out of 46 filtered

DEGs. Also, the individuals exposed to the highest temperature expressed 39 DEGs, 19 of those DEGs were unique to 28°C Fig. 15 b). A unique chaperone DEG was shared between all three experimental conditions (TRINITY_DN361_c0_g1), and the remaining 24 were shared DEGs between the remaining experimental conditions pairs.

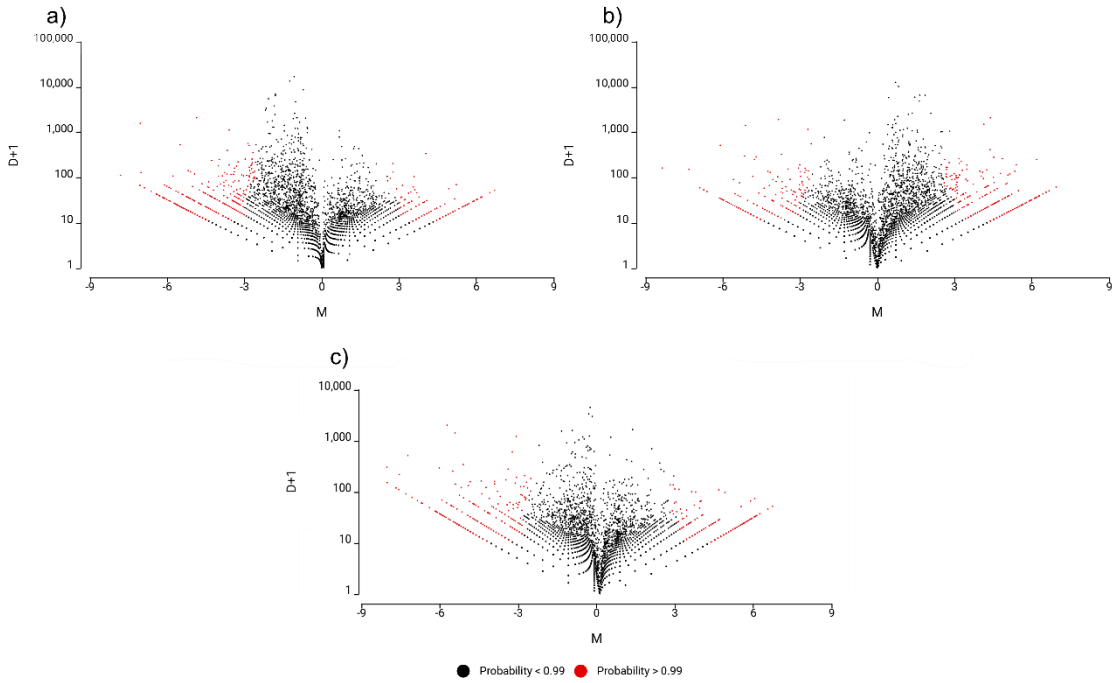

Fig. 14. The figure displays a composite scatter plot corresponding to the Pairwise Differential Expression performed among conditions. On the x-axis is shown the log-fold change (M) and the y-axis the absolute value of the difference in expression between conditions (D), in a logarithmic scale. The Differentially Expressed Genes (DEGs) are depicted as red dots for each condition tested a) 16 vs 22°C b) 22 vs 28°C c) 16 vs 28°C

In this study, we found molecular markers of Hsp90, the constant form of Heat shock proteins and Hsp70, its inducible form (Anestis et al., 2007). The number of GO names associated with Hsp90 was lower than its inducible form, being one DEG at 16°C and two at 28°C. On the other hand, Hsp70 markers were higher in all three conditions (see Fig. 15 c). Moreover, the DEGs at 16 and 22°C were common to those recorded at 28°C. As expected, the highest number of Hsp DEGs were observed at the highest temperature. The fact of observing DEGs concentrated on specific temperature subset, suggest a temperature-dependent relationship.

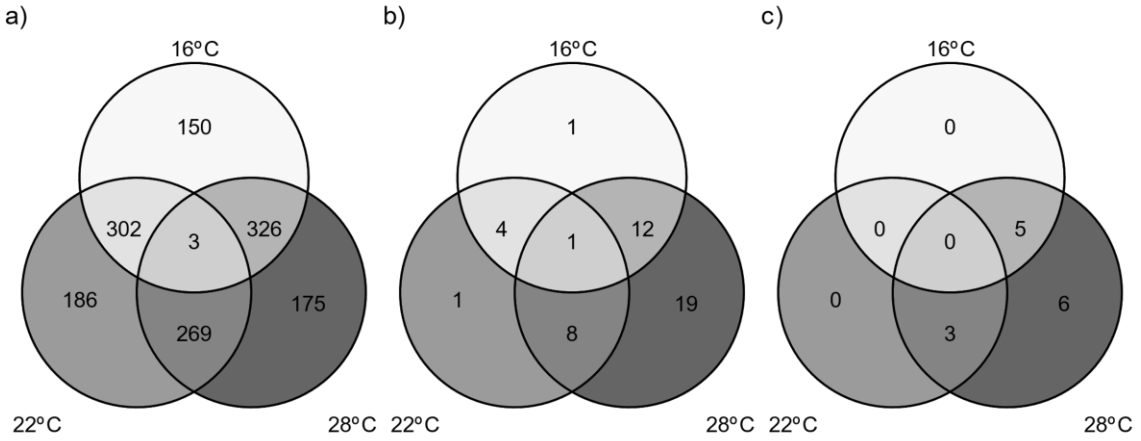

Fig. 15. Distribution of Differentially Expressed Genes (DEGs) obtained among pairwise comparisons in the transcriptomic profile of the gills from *M. galloprovincialis* exposed to thermal stress. The image depicts a Venn Diagram comprising DEGs among three experimental conditions at the temperatures 16, 22 and 28°C, showing the interactions and overlapped DEGs between a) All identified DEGs; b) Filtered DEGs involved in heat defensive functions; c) DEGs encoding Heat shock proteins.

Besides the above-mentioned proteins, we also found molecular markers of another set of proteins that work together in the thermal stress response, namely protein folding, refolding and degradation (MacLean & Picard, 2003; Qiu et al., 2006). This is the case of the complex cdc37 + Hsp90, the dnaJ and its homologous Hsp40 which both coerce a regulatory function of the Hsp70 proteins (Walsh et al., 2004). This association has been exhaustively described in the literature and model organism (MacLean & Picard, 2003; Qiu et al., 2006).

4.4. Discussion

M. galloprovincialis is a species tolerant to heat, which has been previously reported by other authors and corroborated by us (non-published) in controlled conditions using a custom heating system (Chapter 3). This resistance has been known for long-time tanks to controlled environment experiments like survival, respirometry, biomarkers, and heat shock protein quantification (Anestis et al., 2007; Dowd & Somero, 2012; Izagirre et al., 2014).

In total, we could obtain 1,411 unique/non-redundant DEGs in pairwise comparisons, of which three DEGs were common to all temperatures exposed 16, 22 and 28°C. Additionally, 897 DEGs were shared by pairs of experimental conditions. The

remaining 511 were unique DEGs found in the gills exclusive for each temperature (Fig. 15 a).

DEGs involved in folding activities were found in a higher number on the highest temperature subset, probably linked to a greater need of heat defensive proteins at 28°C than below. The same applies to protein turnover by proteasome activity and ubiquitination (data not shown). For further details, see Table S3.

Among the 46 selected DEGs, only five were up-regulated, one unique to 16°C and four shared between pairs of experimental conditions. Two of those DEGs were related to Hsp binding proteins, while the other was responsible for cellular response to heat. Upregulation, in this case, could be interpreted as the response to chaperon demand by the gills. Also, the presence of Hsp markers evidenced thermal stress. The closer to its thermal tolerance limit, the greater the need to express refolding proteins (Kroeker et al., 2014; Lockwood et al., 2010). From among the remaining DEGs, 36 down-regulated DEGs was possible to find most GO terms associated with thermal stress response at 28°C and 16°C, most of them in common Fig. 15 b).

Noteworthy, there was not detected a response to thermal stress so notorious at 22°C, as was detected at 28°C and 16°C. In this case, there may be a replacement of the protein folding subset with another set of DEGs in response to the stress applied.

Besides the selected set of DEGs, the pairwise GSEA Enrichment Analysis confirmed our hypothesis revealing high percentage of overrepresentation of protein folding and '*de novo*' protein folding but also included several GO terms related to cilium and flagellum movement, structures present on mussels' gill responsible for the pump-filter (Riisgård et al., 2015) which respond when the temperature at organism is exposed varies (Jørgensen et al., 1990).

Other authors have reported different subsets of proteins in response to thermal stress on *M. galloprovincialis* acting isolated or combined with other factors. However, in most cases, only heat shock proteins from 70 and 90 kDa match with our findings (Lockwood et al., 2010; Mohamed et al., 2014; Negri et al., 2013) (see Table 3).

Table 3. Different Heat defensive markers reported on *Mytilus* in response to thermal stress alone or combined with other stressors factors.

Group	Markers	Authors
Gene (protein folding GO:0006457)	Dnaj B4; Chaperonin TCP1 β; Chaperonin TCP1 γ-2; Dnaj A2; Chaperonin TCP1 β; heatshock protein 90 (Hsp90); Chaperonin TCP1 δ; Chaperonin TCP1 η; Chaperonin TCP1 γ-1; heatshock protein 90 (Hsp90); Chaperonin TCP1 ε; Chaperonin TCP1 ς-2; heatshock protein 60 (Hsp60); Chaperonin TCP1 ς-1; Calreticulin; Peptidyl-prolyl cis-trans isomerase B precursor; Peptidyl-prolyl cis-trans isomerase G-like; Bardet-Biedl syndrome 10 protein; Novel protein (DnaJ domain-containing) [Danio rerio]; Hypothetical protein BRAFLDRAFT_276314 [Branchiostoma floridae]; Von Hippel-Lindau binding protein 1; Peptidylprolyl isomerase domain and WD repeat-containing protein 1	Lockwood et al., 2010
Cluster 1	elongation factor - 1 alpha; heterogeneous nuclear ribonucleoprotein r; pnn protein; small heatshock protein p26; eukaryotic translation initiation factorsubnunit 911kda; dead (asp-glu-ala-asp) box polypeptide 17; eukaryotic translation elongation factor 1 alpha 1; col protein; 90-kda heatshock protein; heterogenous nuclear ribonucleoprotein r; mgc81857 prtein; heatshock protein 70; calreticulin; ubiquitinisoform cra_a; nadh dehydrogenase subunit 6; dual specificity phosphatase 6; eukaryotic translation initiation factorisoform 1 isoform 2; mitochondrial phosphatase carrier protein; nascent polypeptide-associated complex alpha polypeptyde; upstream of nras; loc562304; eukaryotic translation initiatiation factorsubunit 6 48kda; at5 autophagy related 5 homolog; proin precursor; protocadherin gamma subfamily9	Negri et al., 2013
Protein Folding	Small heatshock proteinp26; Calreticulin; 90kda heatshock protein; fk506-binding protein; Heatshock protein 27kda protein 1	Mohamed et al., 2014

Despite the name of heat shock proteins, this large family of proteins engulfs more functions than respond to thermal stress. However, this is the most common association described in the current literature (Anestis et al., 2007; Lockwood et al., 2010; D. J. Miller & Fort, 2018). Hsp proteins are a group of proteins highly conserved on the phylogenetic scale (D. J. Miller & Fort, 2018; Qiu et al., 2006). Starting with the dnaJ, a highly conserved protein and considered to be homologous of the Hsp40 both have been recognized by exerting a regulatory activity in the functioning of the chaperone Hsp70 (Qiu et al., 2006). This protein, in particular, has been associated with the functions of protein translation, folding, unfolding, translocation, and degradation (Qiu et al., 2006). As mentioned before, was one of the markers found by Lockwood et al., (2010) when comparing the transcriptomic response of *M. galloprovincialis* with *M. trossulus.*

The dnaJ / Hsp40 defines the function of Hsp70 by binding and stabilizing their interactions. This binding through the J domain promotes ATP degradation by the Hsp70, which is naturally low (Qiu et al., 2006). Besides that, the dnaJ is known for translocating misfolded protein signalling and facilitating the degradation process (Qiu et al., 2006). The degradation process was heavily marked in this study despite was not treated here.

Another interesting element is the expression of the complex cdc37 + Hsp90. Although both can operate separately, the cdc37, not a well-conservated protein, binds the Hsp90 forming a chaperone proteic complex (MacLean & Picard, 2003). Different from what occurs in the Hsp70, the cdc37 initially inhibits the ATPase activity from the Hsp90. This association improves the complex activity in protein folding, maturation or stability, if not all (MacLean & Picard, 2003). It is important to mention that cdc37 is not specific for Hsp90 and can be found associated with different proteins, also as a chaperone (MacLean & Picard, 2003). Additionally, this marker also was detected in the Pacific Oyster *Crassostrea giga*, where it was revealed to be part of the physiological response to acidification (Timmins-Schiffman et al., 2014).

4.5. Conclusions

Despite *M. galloprovincialis* is considered as a model species among molluscan and metazoans, the number of studies approaching the transcriptomic response toward rising temperature is still scarce. This study and the data generated constitutes a valuable resource to unravel the effect of higher temperature in *M. galloprovincialis*. Indeed, we were able to identify some DEGs previously reported to be involved in the first line of defence against heat stress. Among these DEGs could be mentioned Hsp70 protein binding (TRINITY_DN1347_c0_g1), being up-regulated in 28°C, which corresponded to the higher temperature tested. Herein, we only performed the differential expression analyses to coding transcripts, thus, the data generated could be used in future works to study the non-coding transcripts expression and their putative role in responses to heat stress.

Chapter 5. General Discussion

In this work, we assessed the physiological and molecular response of two intertidal species facing a gradient of temperature. To accomplish that goal, firstly, we explored how the temperature influenced the base physiology of the studied species. With this objective cleared, we could advance and understand how the temperature modulated their interaction as predator-prey.

Thus, we planned and executed two experiments. The first experience was performed with *N. lapillus* and *M. galloprovincialis* facing a temperature gradient while isolated and interacting as they would in the natural environment. *M. galloprovincialis* showed more tolerance along the whole temperature gradient compared to *N. lapillus*, especially at the higher temperature tested, in which the latest had greater fluctuations in the physiological parameters, showing a narrower window for predation and reproductive behaviour.

Considering these findings, we can expect that the reproductive behaviour may be the most affected by temperature increase soon, with a negative implication for the population. Indeed, in this experiment, we noted that the reproductive output of *N. lapillus* had a notable decrease in the number of egg capsules recorded as temperature increased from 16°C (see Fig. 8).

In addition, we found that the metabolic rates showed a temperature-dependent relationship as well. As expected from two ectotherms, temperature caused variation in their metabolic rate. Remarkably the models showed on *N. lapillus* the highest amplitude while the *M. galloprovincialis* skewed to the right for higher temperatures. On the other hand, predation rates also showed a temperature-dependent behaviour through more inconsistent than the other parameters mentioned before.

At last, we found that temperature would not impact as pronounced as expected on mortality, as shown in section 3.3.1. Although *N. lapillus* reached its thermal limit at 28°C, such conditions are scarce in nature, and they still can find spatial refuges on the rocky intertidal. Nonetheless, higher temperatures might negatively affect the reproduction of this species in a future climate change scenario.

In the case of the mussels, we identified the main set of genes and proteins that allowed *M. galloprovincialis* to cope with higher temperatures facing a temperature gradient with quick increment simulating a heatwave. Afterwards, we resort to a RNAseq and transcriptomic analysis.

In this experiment, *M. galloprovincialis* had the upper hand one is model species. This species is well represented in the literature and had its genome sequenced and annotated on NCBI. With these factors in favour, we successfully detected markers of thermal stress such as HSPs, over the temperature gradient with a notable number of DEGs at 16 and 28°C (see Fig. 15).

Although we focused on DEGs with defensive heat functions, there were also other categories represented such as protein folding, and some DEGs associated with protein turnover. Also, the high number of non-aligned sequences may suggest that part of the *M. galloprovincialis* transcriptome may be associated with non-coding genes as it was suggested to be present in this species (Pereiro et al., 2021).

Chapter 6. General Conclusions

Considering both experiments, on the first one, we focused on the performance of two intertidal species exposed to thermal stress, especially on *N. lapillus*, while on the second experiment, we delved into the molecular response of *M. galloprovincialis*. Overall, both experiments delivered satisfactory results. First, we were able to determine the upper thermal limit of *N. lapillus*, and we could link temperature as a factor influencing their reproductive output. Both species revealed to be tolerant to thermal stress, being *M. galloprovincialis* more resistant than *N. lapillus* to the higher temperature assessed. This data was then supported by transcriptomic analysis and corroborate though the differential expression of genes involved in heat defence. In this sense, we successfully detected markers of heat shock protein in the transcriptomic profile of the gills from *M. galloprovincialis*, as a molecular mechanism involved in the highest temperatures tolerance.

The transcriptome of *M. galloprovincialis* provides a large dataset containing the DEGs, known biomarkers, and annotated genes involved in heat defence. The raw data and supplementary materials publicly provided, constitute a valuable information for future studies once the literature available on this species related to thermal stress remains scarce. There are still subsets of data to explore and link to the thermal stress response of the Mediterranean mussel, as for example, the protein turnover and the long non-coding mRNA.

Chapter 7. Recommendations and Future Perspective

In future studies, the custom system should be improved to simulate the intertidal systems as better as possible, since the air temperature and desiccation could influence the thermal stress tolerance in the studied species. Besides, special attention should be given to the timing between fast and feeding to determine metabolic rates adjusted to each species ingestion periods.

Considering the lack of biological replicates in the transcriptomic analyses, the inclusion of a higher number of samples per condition should be considered in future studies. Although we observed a high percentage of sequenced reads that did not match coding transcripts when mapping, we only performed the differential expression analyses to coding transcripts. Hence, the data generated could be used as a reference in future works to study the non-coding transcripts expression and their role in regulating different genes/proteins involved in the heating responses. In future works, it would also be interesting to profile the *N. lapillus* transcriptomic response to temperature increment.

The use of a different approach is also encouraged, for instance, the use of climate models to predict future changes in spatial distributions of these two species with a basis on their thermal tolerance/performance.

Supplementary Material

Supplementary data can be found within the supplementary file deposited at Mendeley data storage (DOI: 10.17632/8vt6hy3hnj.1).

Supplementary_tables_Mytilus_transcriptomics.xlsx contain relevant information mentioned in the Tables S1-S5 but not included in the text due its length. Additionally, **omicsbox_assembly.fasta** file with *de novo* assembly of the transcriptome using Trinity v2.12.0 included in OmicsBox v1.4.11 using 15 as minimum k-mer coverage in strand-specific mode. While the processed samples sequences are stored at NCBI database under BioProject ID: PRJNA770496 (http://www.ncbi.nlm.nih.gov/bioproject/770496). At last, **NCBI_filtered_sequences.xlsx** with the file with the name of the discarded sequences when submitting the assembly to the NCBI database.

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