NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

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BSc in Biochemistry

GENE NETWORKS MODULATING HEAT ACCLIMATIZATION IN COMMON GOBIES: INFLUENCE OF LATITUDE AND SEASON IN FISH METABOLISM AND HEALTH

MASTER IN BIOTECHNOLOGY

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ABSTRACT

Global change is shifting coastal regimes towards warmer climates with increasing seasonal thermal variation. This thesis aimed at uncovering plasticity, from genes to phenotypes, contributing to acclimatization and adaptation of the intertidal fish *Pomatoschistus microps* to seasonal warming in two locations (Ria de Aveiro - RA and Tróia - TR). Overall, environmental data showed that throughout the year, TR displayed higher mean and maximum water temperatures and 2× the temperature amplitude when compared to RA. Transcriptomics results showed 22 differentially expressed genes (DEGs) in fish livers between spring vs. summer: 19 underexpressed and 3 overexpressed. Fish from RA displayed higher number of DEGs (18 vs. 5 found in fish from TR) and only 1 DEG was common to both locations. DEGs between seasons were related with energy metabolism, transcription regulation and immune responses whereas differences between latitudes were related with protein ubiquitination and apoptosis (in TR) vs immune response, transcription regulation and energy metabolism (in RA). PPI networks showed 2 groups of interrelated genes jointly contributing to the regulation of gene expression and energy metabolism. Biomarkers (i.e., glycogen, glucose and proteins), histopathological index and Fulton's K showed significant differences between locations in summer, with TR displaying higher IBR index values in comparison to RA. Results suggest that *P. microps* exhibited energy-saving mechanisms during periods of seasonal warming by downregulating cellular metabolism, at the expense of other biological processes including liver function (RA), detoxification and disease resistance (both RA and TR). Additionally, *P. microps* populations utilize energy reserves differently in summer indicating either dietary differences between locations or asynchronous development between populations in each season. The findings show that thermal environment modulates physiological plasticity at the molecular level, resulting in different phenotypes with specific adaptations to cope with different environmental regimes and seasonal fluctuations.

Keywords: intertidal gobies, fish populations, thermal biology, transcriptomics, biomarkers, histology

RESUMO

As alterações globais estão a alterar regimes costeiros para climas mais quentes e variáveis. Esta tese visou descobrir a plasticidade (genes-fenótipos), importante na adaptação dos peixes *Pomatoschistus microps* ao aquecimento sazonal em duas latitudes (Ria de Aveiro - RA e Tróia - TR). Os peixes foram recolhidos na primavera vs. verão nos dois locais e dados de temperatura foram obtidos com sondas. Os fígados dos peixes foram usados em análises de trancriptómica, biomarcadores, histologia e condição. Os dados ambientais mostraram que TR apresentou temperaturas médias e máximas da água mais elevadas comparada com RA. O transcriptoma mostrou 22 genes diferencialmente expressos (GDEs) entre a primavera e o verão: 19 subexpressos e 3 sobreexpressos. Os peixes de RA mostraram um número mais elevado de GDEs. Os GDEs entre estações estavam relacionados com metabolismo energético, regulação de transcrição, e respostas imunitárias, enquanto as diferenças entre latitudes estavam relacionadas com ubiquitinação de proteínas, apoptose (TR), vs resposta imunitária, regulação de transcrição, metabolismo energético (RA). As redes PPI mostraram 2 grupos de genes importantes na regulação da transcrição e metabolismo energético. Os biomarcadores (glucose e proteínas), índice histopatológico e K de Fulton mostraram diferenças significativas entre locais no Verão, e TR teve valores mais elevados do índice IBR em comparação com RA. Os resultados sugerem que os *P. microps* exibiram mecanismos de poupança de energia durante os períodos de aquecimento sazonal pela subexpressão do metabolismo celular, à custa da função hepática (RA), destoxificação, e resistência a doenças (RA e TR). As populações de *P. microps* utilizam diferentes reservas energéticas no Verão, indicando diferenças alimentares ou desenvolvimento assíncrono entre populações em cada estação. Os resultados mostram que o ambiente térmico modula a plasticidade fisiológica a nível molecular, resultando em diferentes fenótipos com adaptações específicas para lidar com diferentes regimes ambientais e flutuações sazonais.

Palavras chave: cabozes intertidais, populações de peixes, biologia térmica, transcriptómica, biomarcadores, histologia

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ACRONYMS

BlastP	Basic Local Alignment Search Tool for Proteins.
BSA	Bovine Serum Albumin
cDNA	complementary Deoxyribonucleic Acid.
Csa	Mediterranean climate with dry hot summers.
Csb	Mediterranean climate with dry mild summers.
CTmax	Critical Thermal maximum.
CTmin	Critical Thermal minimum.
DEG	Differentially-expressed gene.
DNA	Deoxyribonucleic Acid.
DPX	Dibutylphthalate Polystyrene Xylene.
EDTA	Ethylenediamine Tetraacetic Acid.
EUMOFA	European Market Observatory for Fisheries and Aquaculture Products.
***e*-value**	Expectation value or Expect value.
FAO	Food and Agriculture Organization of the United Nations.
FDR	False Discovery Rate.
Fulton's K	Fulton's condition factor.
GHG	Greenhouse gases.
GO	Gene Ontology.
H&E	Hematoxylin and Eosin staining technique.
HI	Histopathological Index.

HSI	Hepatosomatic Index.
IBR	Integrated Biomarker Response.
IPCC	Intergovernmental Panel of Climate Change.
log_2CPM	Average log_2 counts per million.
log_2FC	log_2 Fold Change.
NGS	Next-Generation Sequencing.
NOS	National Ocean Strategy.
ORF	Open Reading Frame.
PAS	Periodic Acid Schiff's staining.
RCP	Representative Concentration Pathway.
RIN	RNA Integrity Number.
RNAseq	Ribonucleic Acid sequencing.
SDG	Sustainable Development Goals.
SG	Strategic Goals.
SG1	Fight Climate Change and Pollution and Protect and Restore the Ecosystems.
SG2	Foster Employment and a Circular and Sustainable Blue Economy.
SG7	Stimulate Scientific Knowledge, Technological Development nad Blue Innovation.
SST	Sea Surface Temperature.
Swiss-Prot	"UniProtKB/Swiss-Prot" (reviewed manually annotated)
TC	Tetachrome staining technique.
Tprotein	Total protein.
UniProt	Universal Protein Resource.
β-ME	β-mercaptoethanol.

1

INTRODUCTION

1.1 Climate change in the oceans

Despite being the largest ecosystem on Earth, the ocean has been impacted by anthropogenic activities at all levels, including those that affect climate. Indeed, the increase in greenhouse gases (GHG) emissions (namely the release of CO_2) due to burning of fossil fuels and industrialization processes have imposed the climate change '*deadly trio*' – ocean warming, acidification and deoxygenation, mainly in coastal regions, but also in the open ocean (Bijma et al., 2013; Nowicki et al., 2012). These stressors have been undermining ecological resilience in many marine habitats, and the future of marine biodiversity seems increasingly dire in the upcoming decades.

Mean temperatures in the oceans are rising at unprecedented rates since the beginning of record, with ocean warming accounting for 91% of heating of the global climate system (IPCC, 2021). The Intergovernmental Panel on Climate Change (IPCC) has reported a temperature increase of approximately 2 ˚C in global surface temperature since pre-industrial times (IPCC, 2021) and an average of 0.8 ˚C rise in global sea surface temperature (SST) was observed over the past century alone (Hansen et al., 2010). Furthermore, ocean warming patterns have shown heterogenous trends in different latitudes, with polar regions warming four times faster than the rest of the world (Voosen, 2021), followed by temperate regions; whereas tropical regions are warming at a slower pace (Serreze & Barry, 2011). In future scenarios, climatological models estimate an increase of + 0.9˚C to + 1.4 ˚C in the near-term (2031 to 2050) and an increase of + 1.0˚C to + 3.7˚C for the end of the century (2081-2100) in global mean surface air temperature, according to the Representative Concentration Pathways (RCPs) – RCP 2.6 (reduced GHG emissions) and RCP 8.5 (business as usual GHG emissions), respectively (IPCC, 2019) (Fig. 1.1). This predicted temperature increase will be a direct consequence of an uptake of heat in the oceans from 1000 to 2400 × 10^{21} Joules, according to the two scenarios, respectively (IPCC, 2019).

Thermal regimes in coastal environments are therefore being modified, including through i) shifts towards overall warmer climates, ii) increasing decadal, inter-annual and seasonal thermal variability, iii) asymmetric temperature distribution towards hot weather and iv) increasing the frequency and

magnitude of extreme events (e.g., marine heatwaves) (IPCC, 2021). Given that the equilibrium of climate sensitivity is estimated to be between temperature anomalies of 2 ˚C to 5 ˚C (IPCC, 2021), planet Earth may be facing severely disruptive climate futures.

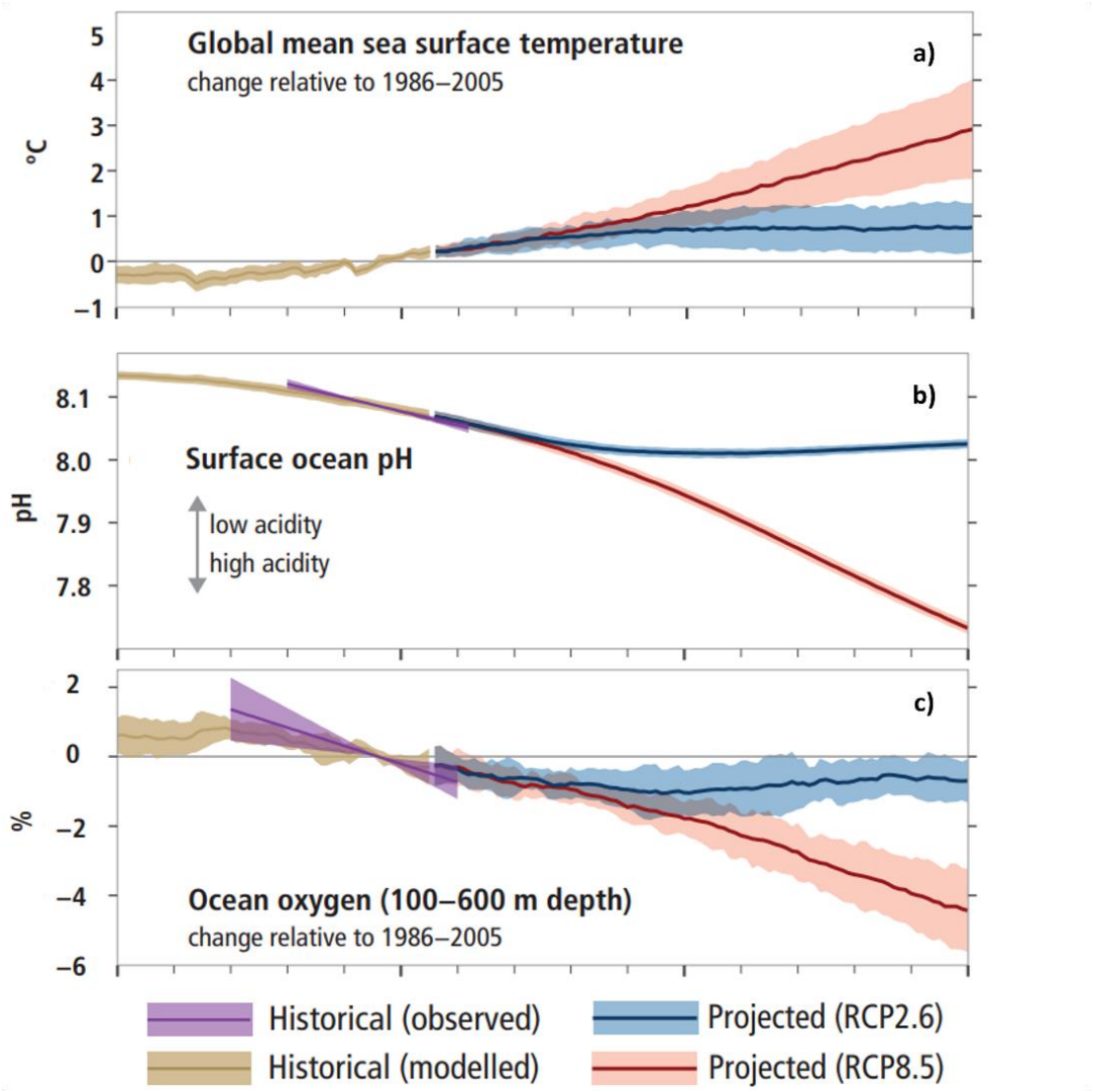

Figure 1.1 Historical (observed and modelled) and projected climatological changes under RCP 2.6 and RCP 8.5 scenarios for key ocean stressors: a) global mean sea surface temperature, b) surface ocean pH, c) ocean oxygen. Source: IPCC (2019).

Depending on geographic location, many climatological models predict that a rise in temperature also impacts the dynamics of other physical and chemical processes in the oceans, such as stratification of ocean waters, sea level rise, ocean biogeochemistry, decrease in ice cover, and salinity (Bijma et al., 2013; le Quéré et al., 2003). For instance, ocean deoxygenation is intrinsically related to temperature and is being amplified by climate change, specifically by ocean warming (Gobler & Baumann, 2016). Since oxygen is not easily dissolved in water, increasing temperatures decrease its dissolution even further, and temperature-induced circulation changes such as increased stratification that reduces sub-surface water mixing and ventilation, intensifies this effect (IPCC, 2019). In the past five decades, oxygen levels in the water column in the first 1000 m depth have decreased by 0.5-3.3%, leading to the expansion of oxygen minimum zones in the ocean by 3 to 8% (Doney et al., 2012; Stramma et al., 2008).

The most recent projections indicate oxygen declines 32-71% greater (at 100-600 m depth) when compared to the previous models in the IPCC 5th Assessment Report, showing that under current data and updated climatological modelling, the scenarios of ocean deoxygenation for the end of this century have significantly worsen under ocean warming trends (IPCC, 2021).

Lastly, the reduction of pH in ocean layers, i.e., ocean acidification, is a direct result of the excessive absorption of anthropogenic CO_2, which, once dissolved reacts with water to form carbonic acid, which then dissociates into bicarbonate and H^+ ions, while carbonate concentrations decrease, significantly impacting carbonate chemistry in seawater (Bijma et al., 2013; le Quéré et al., 2003). It is estimated that 30% of all emitted CO_2 has been uptaken by the ocean (IPCC, 2021), which resulted in an overall decrease of 0.1 pH units in the oceans since the pre-industrial period (Doney et al., 2020). pH decreasing rates have shown to be higher in surface and intermediate water layers (e.g., North Atlantic ~ -0.020 pH units per decade) when compared to deeper and bottom layers (IPCC, 2021). Future projections show that average pH in surface waters will continue to decrease throughout the century, ranging from – 0.16 to – 0.44 pH units, depending on emission scenarios (IPCC, 2021). Significant variability can also be expected in different ocean regions, as carbonate undersaturation will be more severe in areas with sea ice melt and freshwater inputs from rivers and/or increased precipitation.

These three climate factors – ocean warming, deoxygenation and acidification, both individually and combined are therefore posing harsh conditions for marine ecosystems and biological communities. How we manage, mitigate and adapt to these climatological changes in order to preserve ocean live resources and the services they provide will be the challenge of this century, as the Anthropocene may be facing an unmatched biodiversity crisis, for which we must find sustainable solutions.

1.2 The Iberian Peninsula: a climate-vulnerable EU region

Although climate change is a global phenomenon, some regions, due to their geographic location and specific regional features, are more susceptible to its impacts, as well as to the occurrence of extreme weather events, such as the Iberian Peninsula and the Mediterranean zones (Böhnisch et al., 2021; Cardoso et al., 2019; Parente et al., 2018). The Iberian Peninsula in particular, is in fact considered to be one of Europe's critically vulnerable outermost regions (Pereira et al., 2021). Typically, the Iberian Peninsula is characterized by two climate types (according to the Kõppen Climate Classification, which is based on the average monthly values of precipitation and air temperature). The Southern half of the Iberian Peninsula (approximately 40%) is dominated by a Mediterranean climate (Csa) with dry or hot summers and the northern half by a Mediterranean oceanic climate (Csb) with dry or temperate summers. In Portugal, most of the west coast is dominated by the Csb classification. The remaining southern central plateau of Portugal is characterized by a climate with dry or hot summer (Csa) (Cunha et al., 2011). This combination of dry climate with high temperatures is one of the main risk factors for climate change in this region.

Mid-century predictions for Portugal suggest environmental anomalies of + 1 ˚C in seawater temperature, especially in the southern coast, a decrease in pH of ~ -0.113 units in northern/central coast and a decrease in salinity in the northern coast, whereas an increase will occur in the southern coast (Figure 1.2). Temperature predictions for the end of the century for Portugal and the Mediterranean waters mention an increase of + 2 ˚C (Miranda et al., 2002) and 3 ˚C to 4 ˚C (Fischer & Schär, 2010) in water temperature, respectively. Moreover, extreme weather phenomena are particularly prone to occur (Laufkötter et al., 2020; Madeira et al., 2020). For instance, heatwaves in the Iberian Peninsula and Mediterranean regions are expected to increase by 200% to 500% (Laufkötter et al., 2020) with predicted air temperature increases up to 4 ˚C to 7 ˚C by 2100 (Miranda et al., 2002). For Portugal in particular, models predict that the number of days per year with maximum temperatures above the 90th percentile of historical datasets will rise from 15 days (1975-2000) to ~ 80 days by the end of the century. Moreover, heatwave amplitude will increase to unprecedented values in historical records, whereas heatwave duration will increase from 5-13 days to 22-48 days, with more than 3 events per year (Cardoso et al., 2019). Given that current water temperatures in the Portuguese coastal area range from 14 ˚C to 22 ˚C (depending on season and geographic region), future temperatures will likely range from 16 ˚C to 24 ˚C, due to gradual warming, and potentially surpassing 30 ˚C during heatwaves, especially in semi-enclosed water bodies such as estuaries and coastal lagoons.

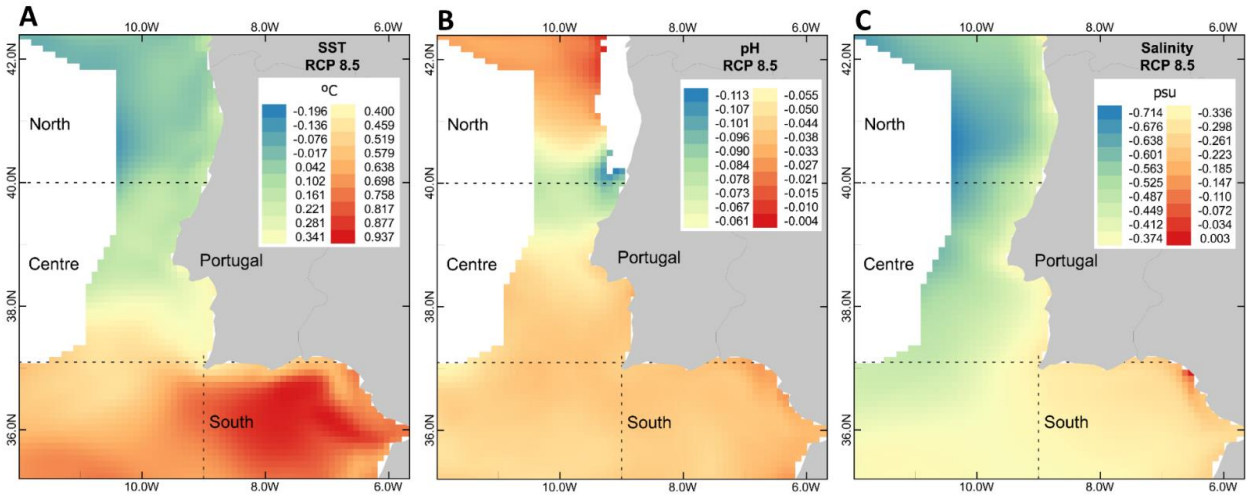

Figure 1.2. Anomalies in environmental drivers in Portuguese coastal waters for the future period 2040-2059 (in comparison with the reference period 2000-2019), based on RCP8.5 scenario: A) SST, B) pH, C) salinity (note: no data were available for oxygen). Source: adapted from (Bueno-Pardo et al., 2021)

1.3 Climate change impacts in marine live resources

As climate change continues to change our oceans' abiotic regimes, extremes and latitudinal gradients, marine live resources, especially in coastal and estuarine ecosystems, which are amongst the most productive (serving as nursery homes and as crucial food stocks, Able, 2005; Beck et al., 2001; Blaber, 2000), respond in different ways. For instance, changes in major climate variables such as temperature, pH and oxygen are known to alter the structure and availability of suitable macrohabitat as well as microhabitat refugia (Heino et al., 2009), driving organism populations distribution expansions or

contractions, and species local and regional extinction risks (Brierley & Kingsford, 2009). Changes in phenology (i.e., migration patterns), and timing of biological and key life cycle events, such as growth or the onset of reproduction have also been observed in marine fauna in response to climate stressors (Brander et al., 2003; Edwards & Richardson, 2003; Miller et al., 2015; Pankhurst & Munday, 2011; Peck et al., 2018, Dulvy et al., 2008; Edwards & Richardson, 2004; Madeira et al., 2017, 2018). Species interactions and trophic web dynamics are also being affected (Gauzens et al., 2020; Taucher & Oschlies, 2011), as, for example, regional decreases in primary productivity due to alterations in phytoplankton community can propagate across trophic webs, which may result in trophic mismatches, reduced food availability and poor nutrition for many consumer and top predator species (Blanchard et al., 2012; Hixson & Arts, 2016; Pethybridge et al., 2015). As consequence, large-scale redistributions of maximum fisheries catch potential and increased vulnerability of many fisheries to climatological phenomena can be expected, with overall changes in the availability of targeted species (Blaber, 2002; Cheung et al., 2010; Cook & Heath, 2005; Gamito et al., 2015; Lam et al., 2016; Lluch-Cota et al., 2018; Roessig & Woodley, 2004; Zhang et al., 2011). For instance, reduced biomass catch, as a result of decreased adult body sizes and fertility (Bobe, 2015; Johnston & Leggett, 2002), occurrence of mass mortalities (Madeira et al., 2016) and increased disease outbreaks (Howells et al., 2020) is now a reality for many species, leading to low population renewal rates, with serious impacts for fisheries stocks and revenues. Aquaculture productions, especially land-based ones, will also be negatively affected, due to high animal mortality rates during summer, reduced water quality (e.g., eutrophication, hypoxia or anoxia, higher salinity), propagation of parasites and other pathogenic species, toxic algal blooms, and an overall reduced quality or even unviability of the final product (Cascarano et al., 2021; Rosa et al., 2012).

As mentioned by Heath et al., (2012), some effects of climate change have already been detected for example, in a warm-water species of red mullet (*Mullus barbatus)* of which the increased catches are suggested to be related to ocean warming. Moreover, at higher latitudes (e.g. Northern Atlantic) where seawater temperatures are usually colder and expected to be more impacted than the global average waters, species like the Atlantic salmon (*Salmo salar*) which is ranked the top ten most produced through aquaculture, Greenland halibut (*Reinhardtius hippoglossoides*) and capelin (*Mallotus villosus*) will experience negative impacts due to climate change such as mass mortality events and shifts in their distribution patterns (Árnason, 2003; Calado et al., 2021; Rose, 2005), and consequently in stock productivity (Brander, 2007). Additionally, since fishing reduces geographic diversity, age and size of the populations, temperature rising will further enhance the stress on marine ecosystems (Hsieh et al., 2006).

Another potential impact of climate change on marine biodiversity is ‘tropicalization’, a phenomenon in which coastal areas undergo a gain in tropical and subtropical species, in detriment of autochthonous temperate species, which is already being observed in the Iberian Peninsula, including Portugal (Encarnação et al., 2019; Vinagre et al., 2011). Moreover, a recent study assessing climate change vulnerability of main commercial marine species in Portugal concluded that, among 74 species of fish and invertebrates, at least four were highly vulnerable (mostly fish), 11 were moderately vulnerable,

whereas the remaining species displayed high plasticity and adaptive capacity (Bueno-Pardo et al., 2021). Despite this, at least 35 species showed high or very high potential for enduring changes in their distribution, including fish species of great economic relevance (e.g., *Sardina pilchardus, Solea solea, Sparus aurata, Diplodus sargus, Trachurus trachurus*). There was also a distinct regional pattern of higher ecological vulnerability in the central region of the Portugal due to higher exposure to climate stressors (Bueno-Pardo et al., 2021). Moreover, the water temperature profile of the Portuguese coast will likely become suboptimal for many fish species produced in aquaculture, such as seabass, seabream, and sole, which in total make up ~ 43% of marine fish farming in Portugal (Instituto Nacional de Estatística, 2021).

Amongst all marine environments, the impacts of climate change are most likely to occur first in intertidal zones and shallow waters due to their lower thermal inertia and interface with land/air environment, increasing their exposure to stressors (Helmuth et al., 2006; Vinagre et al., 2016). Intertidal habitats and shallow water communities make very useful study sites, providing a natural mesocosm for thermal stress studies since they i) experience steep environmental gradients depending on tidal cycles (e.g. temperature variation of ~ 20 ˚C during an immersion/emersion cycle, (Tomanek, 2010)), ii) experience both short- (i.e., heatwaves) and longer-term fluctuations (i.e., seasonal and inter-annual) in water temperatures, iii) hold varied biological organisms, including commercially important species (such as fish, gastropods and crustaceans) (Madeira et al., 2018; Vinagre et al., 2016); iv) can be accessed easily (Nguyen et al., 2011) and vi) are an important nutrient source and are essential for cycling nutrients (Billerbeck et al., 2006). Considering that these environments are therefore subjected to multiple co-occurring fluctuating temperature regimes, they may be ecological traps for coastal organisms. In fact, intertidal areas can sometimes display maximum environmental temperatures higher than the upper thermal limits of several species, which can result in low or negative thermal safety margins and consequent thermal stress and increased local extinction risk for several marine fauna (Madeira et al., 2017; Vinagre et al., 2016). Altogether, climate change will impact not only marine communities that inhabit these ecosystems but also human communities whose livelihoods depend on these valuable resources (Árnason, 2003).

1.4 Marine live resources and sustainable development goals (SDGs)

Alterations in climate variables, especially in highly productive ecosystems, which tend to be the most susceptible to natural or anthropogenic stressors, can have a deep impact in marine fisheries and aquaculture, due to biodiversity reorganisation or loss.

The growing importance of marine organisms for food security therefore requires appropriate management practices, and concerns for sustainability are now at the forefront of policy guidelines (FAO, 2020). In fact, fish and seafood are the main source of dietary protein worldwide, and according to a study conducted by the Food and Agriculture Organization of the United Nations (FAO) in 2017,

approximately 17% comes from marine live resources globally. These resources are also an important source of essential omega-3 fatty acids, as well as micronutrients such as vitamins A, D, B and E, and minerals such as calcium, zinc, iodine, and iron. Fish and seafood are therefore crucial for human nutrition, contributing to a healthy metabolism, reduced risk of chronic diseases, and normal neurological development and function (Dinicolantonio & O'keefe, 2020; FAO, 2020).

However, reported data on per capita fish consumption provided by EUMOFA estimated that in 2019, European citizens decreased the consumption of weight fishery and aquaculture products when compared with 2018 (390 grams less) driven by the overall decrease in fishery catches. Additionally, although Portugal stands out as the major European fish consumer its consumption decreased from 2018 when compared to 2019, due to a decrease in supply (because of lower importation) and increase in exportation. Globally, per capita fish consumption is projected to reach 21.5 kg in 2030 up from 20.5 kg in 2018, of which Europe is expected one of the highest growth rates of 7%. Models predict that in 2030, most of the fish available for human consumption will derive from aquaculture production (59%) up from 52% in 2018 (FAO, 2020). In spite of the World Food Programme prediction that the number of people suffering from acute hunger due to global climatic changes will rise to 840 million by 2030, fish and seafood production seem crucial to counteract and mitigate this problem, contributing to achieve SDG 2 (zero hunger).

For instance, in Europe, total fish production is expected to increase from 18 102 million tons in 2018 to 19 290 million tons in 2030. Aquaculture is projected to be the stimuli behind this growth in global fish production, reaching 3620 million tons in 2030 in the EU (an increase of 17.7% from 2018). According to the EUMOFA (European Market Observatory for Fisheries and Aquaculture Products), in 2019 total world catches and aquaculture production was at its highest, driven by the growth in aquaculture production. Additionally, this trend of increasing growth in aquaculture is mainly led by Asian countries, where in 2019 their aquaculture production represented more than 90% of the world's total farmed production.

Given the combination of the current climate-biodiversity crisis, the investment in innovative tools and sustainable strategies to manage, harvest and produce marine fauna are an increasing necessity. Governments, institutions and policies therefore need to comply with SDG13 (climate action) and SDG14 (protection of life below water) to keep up with better adaptation policies to respond to climate change. For example, the National Ocean Strategy (NOS) program set by Portugal, organized ten different strategies for the decade of 2021-2030 (Strategic Goals – SG) to reduce the impacts of global climate change. Strategies such as Fight Climate Change and Pollution and Protect and Restore the Ecosystems (SG1), Foster Employment and a Circular and Sustainable Blue Economy (SG2) and Stimulate Scientific Knowledge, Technological Development and Blue Innovation (SG7) are amongst the actions to face the challenge of climate change.

1.5 Fish as models in climate change and blue biotechnology research

Fish species have become quite relevant in biological research as they provide several advantages as model systems, such as i) being small and easily bred to large numbers at a relatively low cost, ii) are easily collected and iii) are suitable to approaches such as NGS and omics (next-gen sequencing) (Cossins & Crawford, 2005; Schartl, 2013; Wittbrodt et al., 1998). Additionally, fish, as the most diverse group of vertebrates, share developmental pathways, physiology related mechanisms and organ systems with many other species, facilitating the extrapolation of results (Cossins & Crawford, 2005; Schartl, 2013).

Throughout recent years, effects-driven studies surrounding the thermal biology of ectothermic species have been critical to understand the impacts of ocean warming and extreme heat events on biodiversity and integrity of marine communities. As ectotherms, fish have been used as model systems in climate change physiology because they are unable to regulate their body temperature and are constantly in a process of acclimatization (e.g., through phenotypic plasticity) to the external thermal environment (Teal et al., 2015). Particularly, thermal sensitivity of fish species has been investigated through experimental biology approaches with the aim of determining relevant physiological endpoints at whole-body level, such as the critical thermal minimum and maximum (CTmin, CTmax) (Madeira et al., 2012), resting and maximum metabolic rates (Hui et al., 2020), aerobic scope (Pörtner & Knust, 2007), swimming performance (Ober et al., 2017), as well as quantification of biomarkers at the molecular level as diagnostic of animal stress status (Somero, 2010) (see Table 1.1 for examples).

Recent technological development of high throughput methodologies for global data acquisition, data storage, mathematical algorithms, and computational tools for data analysis were responsible for the advance of NGS (next-generation sequencing) technologies with severally reduced costs and time requirements. These developments have improved the assembly of gene annotation of genomes from greatly studied model fish species like zebrafish (Min et al., 2021) and medaka (Kim et al., 2016) and produced an impactful number of fish genomes from relevant species for aquaculture like rainbow trout, Atlantic cod, Atlantic salmon and common carp (Berthelot et al., 2014; Lien et al., 2016; Star et al., 2011) (see Table 1 for examples). However, a big challenge when researching non-conventional marine species is the lack of sequenced and well-annotated genomes available for further bioinformatic studies. Nevertheless, a successful and numerous amount of proteins have been identified and studied through homology-driven approaches in the absence of sequenced data (Junqueira et al., 2008). With advances in techniques like RNA-seq (where sequenced mRNA is used for the identification of non-model species) it is possible and sometimes faster and less expensive to sequence genomic data to fill up the “space” where many fish species lack sequence data available (Conesa et al., 2016). Moreover, with increasing anthropogenic pressure over aquatic environments, “omics” may provide a better understanding on how the molecular basis of production traits such as growth rate, resilience to environmental stressors (e.g., temperature, fluctuations in salinity and pH) and diseases or low oxygen levels are developed (Rise et

al., 2019). For instance, with the increasing importance of aquaculture to global population, marine omics data can offer a crucial amount of knowledge regarding the biological responses to better improve current practices (Rise et al., 2019). Therefore, environmental omics are a valuable tool in identifying changes at a molecular level, either through the transcriptome, proteome or metabolome that contribute to ecological richness and adaptation of marine life (Veldhoen et al., 2012).

Table 1.1 A selection of representative examples of recent studies (last 5 years) using different marine fish model species and research approaches to study climate change impacts and blue biotechnology tools.

	Research topic	Species	Habitat-Region	Variables studied	Reference
Experimental	Transgenerational plasticity to global warming	*Acanthochromis polyacanthus*	Coral reefs-Tropical	oxygen consumption, hepatosomatic index, body condition, gene expression	(Bernal et al., 2022)
Review	Aquaculture omics	*Dicentrarchus labrax, Callorhinchus milii, Scophthalmus maximus, Tetraodon nigroviridis, Cynoglossus semilaevis, Gadus morhua, Larimichthys polyactis, Petromyzon marinus, Fugu rubripes,*	Multiple-Multiple	reproduction, immunity, growth, development, stress toxicology, disease, microbiome	(Sundaray et al., 2022)
Review	Phenotyping and phenomics in aquaculture breeding	Multiple	Multiple-Multiple	growth traits, meat quality traits, sex-related traits, disease resistance, salinity/stress tolerance, high and low temperature tolerance, feed conversation rates, physiological/biochemical traits, body colours, gene expressions	(Fu & Yuna, 2022)
Experimental	Implications of warming on performance	*Pelates sexlineatus, Centropogon australis, Acanthopagrus australis, Monacanthus chinensis, Acanthaluteres spilomelanurus*	Estuarine-Temperate	growth rate, foraging behaviour (e.g. bite rate, time to feeding, boldness, escape response)	(O'Connor & Booth, 2021)
Field	Seasonal warming effects in stress biomarkers	*Lipophrys trigloides*	Rocky reefs-Temperate	stress biomarkers: HSP70, LPO, catalase, GST, SOD	(Vinagre et al., 2021)
Field	Local adaptation over steep environmental gradient	*Pomatoschistus minutus*	Sandy beach-Temperate	genetic variation, gene expression, sperm motility	(Leder et al., 2021)
Experimental	Fatty acid metabolism response to warming	*Amphiprion ocellaris*	Coral reefs-Tropical	total lipids, fatty acids, lipid metabolic pathways, body mass	(C. Madeira et al., 2021)
Review	Potential of genomic selection in aquaculture	*Salmo salar, Oncorhynchus mykiss, Gadus morhua, Dicentrarchus labrax, Sparus aurata, Salvelinus alpinus*	Multiple-Temperate/Polar	disease and parasite resistance, thermal and salinity tolerance, fillet quality and yield	(Boudry et al., 2021)
Meta-analysis	Thermal bottlenecks in life-cycle	694 species	Multiple-Multiple	thermal tolerance	(Dahlke et al., 2020)
Experimental	Life-stage specific sensitivity to heatwaves	*Sparus aurata*	Multiple-Temperate	stress biomarkers: HSP70, ubiquitin, antioxidant enzymes, LPO; and phenotypic measures: histopathology, condition and mortality	(D. Madeira et al., 2020b)

Review	Stress response: from molecules to evolution	Multiple	Multiple-Multiple	metabolic strategies, plastic, genetic, epigenetic mechanisms, fish survival, population persistence	(Petitjean et al., 2019)
Experimental	Metabolic responses to temperature	*Lythrypnus dalli*	Reefs-Subtropical	metabolic rate, energy consumption, thermal sensitivity	(Rangel & Johnson, 2018)
Experimental	Warming effects in stress biomarkers	*Scartella cristata, Abudefduf saxatilis*	Rocky reefs-Subtropical	Stress biomarkers: Hsp70, ubiquitin, antioxidant enzymes, LPO	(C. Madeira et al., 2017)
Perspective	Application of genomics in aquaculture breeding programs	*Salmo salar*	Pelagic-Temperate	trait heritability, growth rate, disease resistance, age at sexual maturity, feed conversion efficiency, fillet yield, flesh quality and colour	(Norris, 2017)

Abbreviations: HSP70 – heat shock protein 70 kDa, LPO – lipid peroxides, GST – glutathione-S-transferase, SOD – superoxide dismutase.

1.6 Fish thermal biology: mechanisms to deal with heat stress

1.6.1 Gene expression and physiological plasticity

Temperature can have a widespread influence in organismal physiology, performance and fitness (Poloczanska et al., 2013; Walther et al., 2002). Indeed, several relevant cellular properties, mechanisms and physiological traits are temperature sensitive (e.g., enzyme's activity, muscle functioning, aerobic respiration, metabolism) (Somero, 2012), with downstream impacts on how organisms locomote, behave and reproduce (Bicego et al., 2007; C. & A., 2001; Walther et al., 2002). For instance, Madeira et al., (2020) showed increased mortality and lack of acclimatization capacity in the larval life-stage of a demersal temperate fish (*Sparus aurata*) exposed to elevated temperatures, with severe consequences for life-cycle progression of the species.

How species and populations change with climate change is the basis of ecological genomics - an emergent field in the scientific community (Ungerer et al., 2008) . Recent studies investigating responses to climate change at the transcriptome level, for instance, offer a valuable opportunity to unravel genetic adaptation to heat (Oomen & Hutchings, 2017; Ungerer et al., 2008). By profiling the transcriptome (mRNAs, non-coding RNAs, small RNAs), scientists can study transcriptional changes induced by certain conditions (e.g., temperature stress) and identify gene regions or pathways that are impacted by these stressors (Smith et al., 2013; Valenzuela-Quiñonez, 2016), or discover specific genes or gene clusters that confer heat tolerance to organisms when over- or sub-expressed. For instance, (Long et al., 2012) identified multiple temperature-regulated genes related to the nucleosome assembly, chromatin organization and protein folding in larval zebrafish when exposed to temperature stress. Moreover, Jesus et al., (2016) identified overexpressed genes related to transcription regulation in *Squalius carolitertii* and underexpressed genes related to cell division and growth processes in *Squalius torgalensis* (in order to maintain homeostasis and energy reserves, respectively) in response to thermal stress environments. Such changes in gene expression patterns can persist over generations (via epigenetic inheritance), transmitting important information to allow phenotypic change to best respond to environmental challenges (Badyaev & Uller, 2009; J. Marshall & Uller, 2007). For instance, Donelson et al., (2012) showed that transgenerational plasticity was behind the rapid acclimatization of tropical reef fish exposed to small increases in water temperature. By analysing the transcriptomic response to temperature stress, we can better understand the mechanisms underpinning genetic and biochemical adaptation that are crucial for heat tolerance in a warming climate (Smith et al., 2013).

1.6.2 Tradeoffs during heat stress and heat acclimatization

The physiological demands to maintain homeostasis induced by environmental stressors are responsible for both plastic and evolutionary adaptations (Crain et al., 2008). These demands will be affected whenever two or more specific traits cannot be optimized, meaning a "trade-off" (Agrawal et al., 2010). By definition, a trade-off simply means a compromise between two or more incompatible qualities. In this case, these trade-offs can either occur at a genetic level or at a physiological level (Sokolova, 2013; Walsh & Blows, 2009). Briefly, at a genetic level these trade-offs arise when traits are genetically correlated and at a physiological level these trade-offs often occur through competing requirements on metabolism (see Kelly et al., 2016 for a review). Combined, these two types of trade-offs, can change how a population of organisms responds to environmental change. For instance, Kelly et al., 2016 showed that after several generations of selection of two hybridized populations of the copepod *Tigriopus californicus* (divergent for both heat and salinity tolerance), a trade-off in fecundation was observed in heat-selected lines.

Phenotypic plasticity is achieved when within a genome, various phenotypes are produced under different environments (West-Eberhard, 2003). Phenotypic responses can either be represented by continuous or discrete traits and plasticity can be either adaptive, maladaptive or nonadaptive depending on the trait and how well it matches the environment (see Ghalambor et al., 2007 for a review). "Norms of reaction" are known as the responses that an organism undergoes to face variable environmental changes, therefore plasticity can be represented as a norm of reaction. The purpose of these reaction norms is to visualize and quantify how organisms shape their responses to different environmental stimuli (Purchase et al., 2010; Roff, 1999). For instance, Purchase & Brown, (2000) showed that, geographically separated Atlantic cod (*Gadus morhua*) in the Atlantic waters, have different larval growth rates when exposed to different temperatures, suggesting variations in energy allocation within the same population. Responses can be species-specific and related to the environmental tolerance window displayed by each species. These are known to match how organisms are exposed in their habitat to environmental challenge and therefore underline the importance in understanding how individual fitness and performance are impacted.

Our study species, the common goby *Pomatoschistus microps* (Krøyer, 1838) is an epibenthic and euryhaline small fish belonging to the Gobiidae family, with a widespread distribution across Europe and along the coast of Portugal. This species inhabits soft or sandy bottoms under different climatic regimes (e.g., Csa and Csb in the Portuguese coast) and consequently, environmental conditions (e.g., temperature and salinity). Due to their abundance and wide geographical distribution (Arruda et al., 1993), small size and ease of collection and importance in the estuarine food-webs this species makes a suitable model for environmental studies. In fact, *Pomatoschistus* spp. are often used as model species in behavioral (Lindström et al., 2006), ecological (Rodrigues et al., 2006) and population genetic studies (Gysels et al., 2004). Additionally, living mostly in shallow coastal waters and estuaries, this

species is exposed to high temperature variations, highlighting its relevance for climate change research.

1.7 Thesis objectives and research questions

The main goal of this thesis was to discover potential molecular mechanisms contributing to physiological acclimatization of the goby fish *Pomatoschistus microps* to thermal stress, assess its effects and tradeoffs in fish energy metabolism, condition and health, and evaluate the species' adaptations to different climatic regimes.

More specifically, the objectives of this thesis were to compare two distinct populations of *P. microps* upon seasonal warming (spring vs. summer) and a latitudinal thermal gradient (Csb vs. Csa climatic regime):

- To identify how gene pathways may be affected/altered by climatic factors in this species;
- To understand how fish energy balance is modulated and regulated in response to or as a consequence of heat stress;
- To assess pathological aspects related with climatic phenomena and associated metabolic/molecular responses.

This work will help to understand how thermal environment shapes fish stress coping mechanisms at the molecular level, assess the role that their plasticity plays in the mediation of fish thermal vulnerability and unravel new actionable genetic targets that may be crucial in fish acclimatization and adaptation to thermally stressful environments. This study is therefore expected to provide critical new molecular tools for fish conservation, with potential extrapolations relevant for fisheries management, and aquaculture production.

2

MATERIALS AND METHODS

2.1 Ethical statement

Animal collection was authorized by competent authorities: Direção Geral de Recursos Naturais, Segurança e Serviços Marítimos (DGRM), Instituto da Conservação da Natureza e Florestas, as well as local captaincies (Porto de Setúbal e Porto da Ria de Aveiro), according to the fishing licenses for scientific purposes no. 573/2021/DRI, 715/2021/CAPT and 716/2021/CAPT.

2.2 Animal collection and pre-processing

Pomatoschistus microps (n=180 individuals) were captured in sand beaches during low tides at the warmest hours of the day (between 12.00 am – 2.00 pm) using hand nets. Collections were made in two different seasons: Spring (17th-18th May 2021), corresponding to the control group; and Summer (27th-29th July 2021), corresponding to the seasonal thermal stress group; and in two different locations of the Portuguese coast (Fig. 2.1): Praia de Biarritz in Ria de Aveiro (40° 37' 43.85'' N, 8° 44' 41.55'' W, north-western coast) and Caldeira de Tróia in Tróia Peninsula (38° 29' 06.9'' N, 8° 53' 15.1'' W, central-western coast). Forty-five individuals were captured in each season and location, weighed and measured (using a scale and an icthyometer, respectively), and from these, 35 fish were euthanized by cervical dissection, and snap frozen in liquid nitrogen for molecular analyses, including transcriptomics and quantification of energy reserves ($n_{molec.}$ = 35 specimens × 2 seasons × 2 locations = 140 specimens). The remaining ten collected fish at each site and location were euthanized by an overdose of MS222 in seawater (500 mg l^{-1}, pH 8.0) and fixed in Davidson solution (9–10% v/v formalin, 10% v/v glacial acetic acid and 30% ethanol) for further histological analysis ($n_{histol.}$ = 10 specimens × 2 seasons × 2 locations = 40 specimens). Upon arrival to the laboratory, livers were dissected from fish and snap frozen fish and were stored at -80 ˚C until further molecular analysis, whereas fixed fish were left in Davidson solution for an additional 24 h to 48 h.

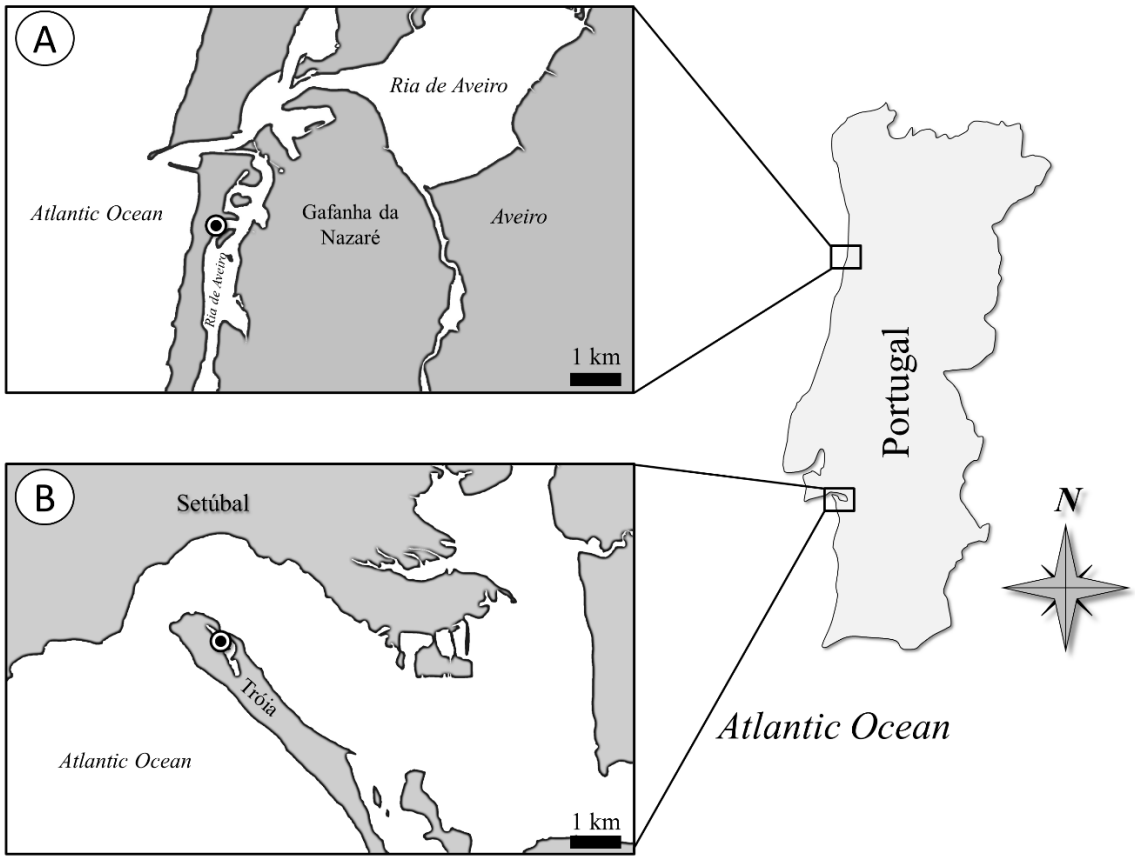

Figure 2.1 Sampling locations in the Portuguese coastal area: A) Ria de Aveiro, B) Caldeira de Tróia. According to the Köppen-Geiger climate classification, Ria de Aveiro in the north of Portugal is located in the climate normal Csb, typically characterized by a temperate Mediterranean climate with dry mild summers, whereas Caldeira de Tróia in central Portugal is located in the climate normal Csa – temperate Mediterranean climate with dry hot summers.

2.3 Environmental characterization

Field environmental data, namely water temperature, salinity, dissolved O_2 and pH were also collected from each sampling location (Ria de Aveiro and Caldeira de Tróia) and season (Spring and Summer) (Fig. 2.2). During the sampling days, seawater parameters were measured in five replicate tide pools and five replicate subtidal points (~ 30 – 70 cm depth) during low tide using a multi-parameter meter (VWR® pHenomenal®, N/A, MU 6100 H S2 portable, Germany). Moreover, seawater temperature data was recorded for over one year (from May 2021 to July 2022) using EnvLogger data loggers (27 mm EnvLogger with zip-tie mounting holes v2.4, ElectricBlue©, Portugal) deployed in the field (N=3 per sampling location, Fig. 2.2A-D). Temperature was measured every half an hour (d=0.1 ºC) and collected with the EnvLogger Viewer software application (v4.91) every 3 to 5 months.

Figure 2.2 Data loggers used for temperature data collection: A) Location of the data logger placed in Ria de Aveiro (40°37'50"N, 8°44'39"W); B) Location of the three data loggers placed in Tróia (38°29'7"N,8°53'16"W; 38°29'6"N,8°53'17"W; 38°29'5"N, 8°53'16"W, north to south, respectively) ; C) Placement of the Intertidal logger 1 marked with a star in the map; D) Closer image of the Intertidal logger 1 after deployment. *Scale bar* 10 m.

2.4 Transcriptomics

2.4.1 Nucleic acid extraction

Total DNA and RNA were simultaneously extracted from 5-15 mg of liver tissue from 3 female fish from each season and location (n=12), using the AllPrep DNA-RNA Mini kit (Qiagen, Germany) following manufacturer's instructions. Some modifications were made to the manufacturer's protocol to optimize total RNA and DNA extraction and quality parameters. Briefly, tissue samples were ground into powder in liquid nitrogen in a cryotube using a plastic pestle and were further homogenized by adding 600 µL of Buffer RLT Plus enriched with β-mercaptoethanol (β-ME) and passing the homogenates 10 to 15 times through a blunt 23-gauge needle fitted to nuclease free syringe to disrupt nuclear membranes. Homogenates were then centrifuged at 12,000 rpm for 4 minutes and supernatants were collected to new microtubes. Subsequently, for DNA extraction, supernatants were transferred to an AllPrep DNA spin column placed in a 2 mL collection tube and centrifuged for 30 seconds at 10,000 rpm.

The flow through was then collected, placed in a new microtube and 1 volume of 50% ethanol was added for later RNA purification, while the AllPrep DNA spin column was placed in a new 2 mL collection tubed. Afterwards, 500 µL of Buffer AW1 were added to the AllPrep DNA spin column and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded and the spin column was reused. 80 µL of RNase A previously prepared were added to each sample and incubated for 10-15 minutes at room temperature. 700 µL of Buffer AW2 previously prepared were added to the AllPrep DNA spin columns, centrifugated for 15 seconds at 10,000 rpm (to wash the spin column membranes) and the flow-through discarded. An extra 3.5 minute centrifugation at 10,000 rpm was performed to ensure that no residual ethanol is carried over during DNA elution. The AllPrep DNA spin columns were then placed in new 1.5 mL collection tubes and 50 µL of the provided Buffer EB (pre-heated to 70 ºC) were added and incubated at room temperature for 3 minutes. To elute DNA, the columns were then centrifugated for 1 minute at 10,000 rpm. This final step was repeated to elute further DNA and achieve higher DNA concentrations.

To perform the RNA purification, the flow-through previously collected for RNA purification was then transferred to the AllPrep RNA spin column placed in a 2mL collection tube, centrifugated for 15 seconds at 10,000 rpm and the flow-through discarded. Subsequently, 350 µL of RW1 buffer were added and then centrifugated for 15 seconds at 10000 rpm to wash the spin column membranes. Optional on-column DNase digestion was performed by adding 80 µL of DNase I mix (10 µL of DNase I stock mixed with 70 µL of Buffer RDD, supplied in the RNase-Free DNase Set kit, Qiagen) to the AllPrep RNA spin column membranes and incubated at room temperature for 15 minutes. To stop DNA digestion, 350 µL of Buffer RW1 were added and centrifugated for 15 seconds at 10,000 rpm. Afterwards, a series of successive washes and centrifugations were performed (1x 500 µL Buffer RPE for 15 seconds at 10,000 rpm and 1x 500 µL Buffer RPE for 3 minutes at 10,000 rpm) in order to wash the spin column membranes of residual ethanol. An optional centrifugation for 2 minutes at full speed (13,000 rpm) was performed to eliminate any carryover of Buffer RPE. Lastly, the AllPrep RNA spin columns were placed in new 1.5 mL collection tubes and 30 µL of RNase-free water were added and centrifugated for 1 min

at 8,000 rpm to elute RNA. Furthermore, to increase RNA yield the previous step was repeated using another 25 µL of RNase-free water.

2.4.2 RNA-seq

Initial quantification and quality assessment of the extracted RNAs were performed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) (ratios A260/280 nm and A260/230 nm ≥ 2.0). Total RNA samples (n=12) were shipped to StabVida (Caparica, Portugal) and an additional quality control step was performed using a BioAnalyzer and gel electrophoresis to confirm concentration, integrity and RNA bands. All samples met concentration and quantity requirements (≥ 100 ng/uL and ≥ 1 ug total RNA), however three samples did not meet the required RIN (≥ 7). Nevertheless, all samples were used for library construction and sequencing (Romero et al., 2014). The library construction of cDNA was carried out using the Stranded mRNA Preparation Kit. Generated libraries were then sequenced in the Illumina Novaseq platform (Illumina, San Diego, CA, USA) using 150 bp paired-end sequencing reads (40 M reads). Quality of raw sequence data was assessed using FastQC v0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) Furthermore, TrimGalore v.0.6.6 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) was used with default parameters to trim the Illumina adapter and the reads with length under 20 bp.

2.4.3 Transcriptome assembly, quality, quantification assessment and functional annotation

Transcriptomes were *de novo* assembled using Trinity v.2.6.6 (Grabherr et al., 2011; Haas et al., 2013) due to the absence of an available reference transcriptome for the study species. All samples (40M reads) were used for the *de novo* transcriptome assembly. Quality of transcriptome assembly was verified using N50, Ex90N50 and read content statistics using Trinity, Bowtie2 v.2.4.1 and Samtools v1.11 (Danecek et al., 2021; Haas et al., 2013; Langmead et al., 2019; Langmead & Salzberg, 2012). Transcriptome quantification was performed using Kalisto v0.43.0 (Bray et al., 2016).

The R packages tximport and seqnir (Charif & Lobry, 2007; Soneson et al., 2016) were used to import data for the identification of the differentially-expressed transcripts employed by the edgeR and limma packages (Ritchie et al., 2015; Robinson et al., 2010). Additionally, the normalization factors were estimated by using the edge R package. In order to filter differentially-expressed genes, cut-offs were set at $| \log_2 FC | > 1.5$ and FDR-adjusted $p < 0.05$. To identify the predicted coding regions in the assembled transcriptome, the TransDecoder v5.5.0 (Haas et al., 2013) was used. Subsequently, the predicted coding regions (ORFs) were functionally annotated by homology-matching against the SwissProt database (Release 2022_03) with BLASTP (*e*-value < 1E-5) from NCBI blast+ v2.13.0 (Camacho et al., 2009). To annotate the homology-matching sequences the R package UniprotR was used (Bateman et al., 2021; Soudy et al., 2020). For plotting, the packages gplots (Warnes et al., 2022) , RColorBrewer (Neuwirth, 2022) , ggplot2 (Wickham, 2016), cowplot (Wilke, 2020) and gridGraphics (Murrel & Wen, 2020) were employed.

Protein-protein interaction (PPI) network was predicted (with a minimum required interaction score of 0.150) based on the identified differentially-expressed genes by homology-matching against the organism Zebrafish (*Danio rerio)*, using the web-based tool STRING (Szklarczyk et al., 2011).

All statistics were performed using R 4.2.0 (Ihaka & Gentleman, 1996). R script and command line for assembly, quality, quantification, functional annotation and differential gene expression were adapted from (Moutinho Cabral et al., 2022) and are presented in the appendix (A2.2 and A2.3, respectively).

2.5 Molecular biomarkers

Total proteins (n=36), total glycogen and glucose (n=24) and total triglycerides (n=24) were quantified from frozen fish liver samples. Livers were homogenized in 500 µL MilliQ-grade water using an electric homogenizer (Homogenizer 150, Fisherbrand). Homogenates were then centrifuged at 4 ˚C for 10 min at 12,000 rpm and the supernatants were collected. 100 µl of each supernatant was diluted 1:2 in PBS 2x and used for total protein quantification. The remaining volume (400 µl) was boiled for 10 min and stored at -80 ˚C for glycogen and glucose quantification.

Quantification of total proteins was performed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) by measuring sample absorbance at 280 nm, and calculating protein concentration using the molar extinction coefficient of BSA (bovine serum albumin) 43,824 $cm^{-1}M^{-1}$ and a pathlength of 10 mm.

Glycogen was quantified through a colorimetric assay based on the enzymatic degradation of glycogen into glucose subunits, adapted from Huijing, (1970). Initially, 50 µl of each sample, previously diluted in MilliQ-grade water (1:5) were added to split microtubes. Subsequently, 50 µl of an enzyme mix (α-glucosidase (Sigma-Aldrich, USA) 50 $\mu g.ml^{-1}$ and α-amilase (Sigma-Aldrich, USA) 80 $\mu g.ml^{-1}$ in 0.1M of sodium acetate buffer pH 4.8) were added to each sample, mixed and incubated for one hour for enzymatic degradation of glycogen into glucose subunits. Afterwards, to detect glucose subunits, 100 µL of glucose oxidase mix (0.3 $mg.ml^{-1}$ glucose oxidase (Merck, Germany), 0.03 $mg.ml^{-1}$ horseradish peroxidase (Merck, Germany), 0.1 $mg.ml^{-1}$ O-dianisidine dihydrochloride (Sigma-Aldrich, USA) in Tris-phosphate-glycerol buffer) were added and incubated for one hour at 30 ˚C. In order to stop the reaction, 200 µL of 5 M HCl were added to the mixture and centrifugated for 5 min at 3,000 rpm. 150 µL duplicates were pipetted into microplate wells and the absorbance was measured at 540 nm in a Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific, USA). To quantify glucose subunits, an eight-point calibration curve (0-125 $\mu g.ml^{-1}$) was performed using glycogen standards from oyster (Sigma-Aldrich, USA). The final glycogen content (degraded in glucose units) was obtained by subtracting the glucose background, measured for each sample by replicating all the steps above excluding the initial step of enzymatic degradation of glycogen into glucose. Background glucose was also quantified using

an additional eight-point calibration curve (0-125 ug.ml^{-1}) with glucose standards from MilliporeSigma, (USA).

Total triglycerides were quantified following instructions in the Triglyceride Colorimetric Assay Kit (Cayman Chemical, USA). Briefly, liver tissue was homogenized in NP40 Substitute Assay Reagent from Cayman Chemical containing EDTA 1 mM as a chelating agent to inhibit protease activity, and were centrifuged for 10 min, 10,000 rpm at 4 ˚C. Supernatants were collected and diluted (1:5) using the NP40 Substitute Assay Reagent from Cayman Chemical prior to the assay. Duplicates of 10 µL of each sample were put into a 96-well microplate and 150 µL Enzyme Mixture solution (from Cayman Chemical, USA, containing lipoprotein lipase, glycerol kinase, glycerol phosphate oxidase and peroxidase) were added to each well to start the reaction. The microplate was afterwards incubated for 30 minutes at 37 ˚C and the absorbance measured at 530 nm using a Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific, USA). To quantify total triglycerides, an eight-point calibration curve (2000 µg/mL to 0 µg/mL) was calculated using the Triglyceride Standards (Cayman Chemical, USA).

RNA:DNA ratios were also calculated from the previously extracted nucleic acids of fish livers.
Biomarker results were normalized by liver weight and given in mg.g^{-1} liver.

2.6 Histological analysis

After 24 h – 48 h of fixation in Davidson's solution, whole fish were washed in a successive sequence with MilliQ-grade water (15 min × 4), and stored in 70% ethanol. The samples were then placed in histological cassettes in a tissue processor (Shandon Pathcentre Enclosed Tissue Processor, Thermo Electron Corporation) and dehydrated in vacuum in a successive series of ethanol (10 min with ethanol 70%, 30 min with ethanol 95%, 40 min with ethanol 100%), xylene washes (30 min) and finally embedded in liquid paraffin and left in an incubator at 65 ˚C until further use. Histological sections (5 µm thick) were obtained from paraffin blocks using a RM2245 rotary microtome, and slides were stained either with Hematoxylin and Eosin (H&E) or a tetrachrome histochemical (TC) technique (Costa, 2017). Briefly, both techniques involved previous deparaffination of slides and dehydration after staining (as described in Costa, 2018). Hematoxylin and Eosin technique combines an alum hematoxylin basic dye that stains acid structures (purple) and an acidic counterstain, Eosin (reddish). This method was performed by staining with Hematoxylin (2min), followed by washes in tap water (2 x 2min) and ethanol 70% (3min) and concluding with Eosin (1 min). The tetrachrome technique combined the use of several dyes and successive washes in MilliQ-grade water. In summary, Alcian blue was used (30 min) for staining blue acidic polysaccharides in cartilage matrices followed by two quick washes in MilliQ-grade water. Afterwards, the Periodic acid Schiff's staining (PAS) was used (Periodic acid – 10 min, Schiff's reagent – 15 min) to detect polysaccharides (i.e glycogen) and mucosubstances (i.e glycoprotein and glycolipids) adding two washes with MilliQ-grade water in-between both dyes (1 running wash then 2x2min). Weigert's iron hematoxylin (10 min) is then used for nuclear staining followed by a quick wash

in ferric chloride solution for removal of Weigert's iron hematoxylin's excess. To conclude the TC technique staining, a final wash in MilliQ-grade water was performed and picric acid was employed (30 sec) to stain yellow muscle fibers. The slides were afterwards mounted using DPX resinous media (BDH, Poole, UK). Tissue sections were analysed with a microscope equipped with a MC 190 HD camera, both from Leica Microsystems. Leica Application Suite was used to capture the images from the microscope. Further image processing and enhancement was done using ImageJ (Schneider et al., 2012) and GIMP (v2.10.32) (Montesanto, 2015).

Seven histopathological traits, divided into four reaction patterns (circulatory disturbances, inflammation, regressive alterations and parasites) were chosen for a qualitative, followed by a semi-quantitative analysis (calculation of histopathological index), as demonstrated in Costa (2017). Briefly, this approach is based on the product between the biological significance of each chosen trait (defined as weight) and its degree of dissemination (defined as score). The histopathological weights are based on proposals made by Bernet et al., (1999) and Costa et al., (2009) and the degree of dissemination ranged between 0 (absent) and 6 (diffuse). A series of blind reviews were conducted to assess the accuracy of the semi-quantitative analysis.

2.7 Fish condition

Body condition indices such as the Fulton's condition factor (Fulton's K) and hepatosomatic index (HSI = liver weight / total fish weight * 100) were calculated after weight and length measurements performed in the field. Fulton's body condition was calculated following the formula $K = 100M_t/L_t^3$, where M_t is the total wet mass (mg) and L_t is the total length (mm).

2.8 Statistics and bioinformatics

Shapiro-Wilk and Levene's (R package car v0.1-8) tests were used to assess the normal distribution and homoscedasticity of biomarker, histopathological index, and fish condition datasets (Fox & Weisberg, 2019). After validation of these procedures, parametric statistics ANOVA and post-hoc Tukey's tests were employed to test the main effects of season and location, as well as their interaction, on these datasets. In order to analyse the integration and interaction between multi-level end-points (molecular to whole body), the Integrated Biomarker Response was calculated according to Beliaeff & Burgeot, (2002). This tool provides a numeric standardized value that integrates all end-points studied, synthesizing all responses. Star plots were then used to display the score results.

A significance threshold of 0.05 was used for all analyses. The packages gplots (Warnes et al., 2022) and IBRtools (Resende & Pereira, 2022) were used for plotting (Star plot and Heatmap) and the package preprocessCore (Bolstad, 2021) was used for data normalization before plotting. All statistics were performed using the software R 4.2.0 (Ihaka & Gentleman, 1996).

3

RESULTS

3.1 Environmental data

Air temperature during fish collection varied from 18.0 ˚C to 20.8 ˚C in spring (May) and from 22.8 ˚C to 28.2 ˚C in summer (July) (in Ria de Aveiro and Tróia, respectively) at the hottest day hours. Water parameters measured in both locations at the sampling's days are summarized in Table 3.1. Mean seawater temperature in shallow waters during collection ranged from 19.0 ˚C to 27.0 ˚C in the different locations and seasons, with Tróia consistently showing higher temperatures when compared to Ria de Aveiro (on average, ~4.55 ˚C higher). Salinity displayed higher variation between seasons in Ria de Aveiro (27.6 ± 1.03 in spring vs. 34.0 ± 0.4 in summer), whereas in Tróia, values were relatively stable around 36-38). Oxygen concentrations overall ranged from 7.2 ppm to 8.3 ppm and were higher in summer, whereas pH varied around 0.1 units between seasons, being overall higher in Ria de Aveiro.

Table 3.1 Environmental data collected *in situ* at the sampling sites. Data (mean±SD temperature, salinity, dissolved oxygen concentration and pH) measured in shallow water at each sampling location (Ria de Aveiro and Tróia) and time (Spring season – May, and Summer season – July) during fish collection.

	Ria de Aveiro		***Tróia***	
	Spring	**Summer**	**Spring**	**Summer**
Temperature (˚C)	21.1 ± 0.8	19.0 ± 1.6	22.0 ± 0.9	27.2 ± 3.1
Salinity	27.6 ± 1.03	34.0 ± 0.4	38.0 ± 1.6	36.0 ± 1.8
[O_2] ppm	NA	7.6 ± 0.3	7.2 ± 0.9	8.3 ± 1.1
pH	8.2 ± 0.06	8.0 ± 0.04	7.9 ± 0.08	8.1 ± 0.2

The annual seawater temperature measured with field data loggers (Figure 3.1) to assess the thermal fluctuations to which fish are subjected to in their natural habitat showed variations among the two analysed zones: in Ria de Aveiro, maximum temperature was reached in May 2021 (23.9 ºC), the minimum in November-December 2021 (11.1 ºC) and the month with highest temperature amplitude was May 2021 (9ºC) (from 1 data logger). In Tróia the maximum temperature reached was in July 2022 (31.7 ºC), the minimum in January 2022 (7.5ºC) and the month with the highest temperature amplitude (max-min) was April 2021 (19.7 ºC) (averages from 3 data loggers). Nevertheless, the absolute maximum seawater temperature detected in Tróia (in 1 of the data loggers) was 37.6 ºC in May 2022. Overall, the mean temperature was slightly higher in summer months (June-September) than in spring

months (March-June), in both locations, however data showed that Tróia has an overall warmer regime with larger temperature fluctuations when compared to Ria de Aveiro.

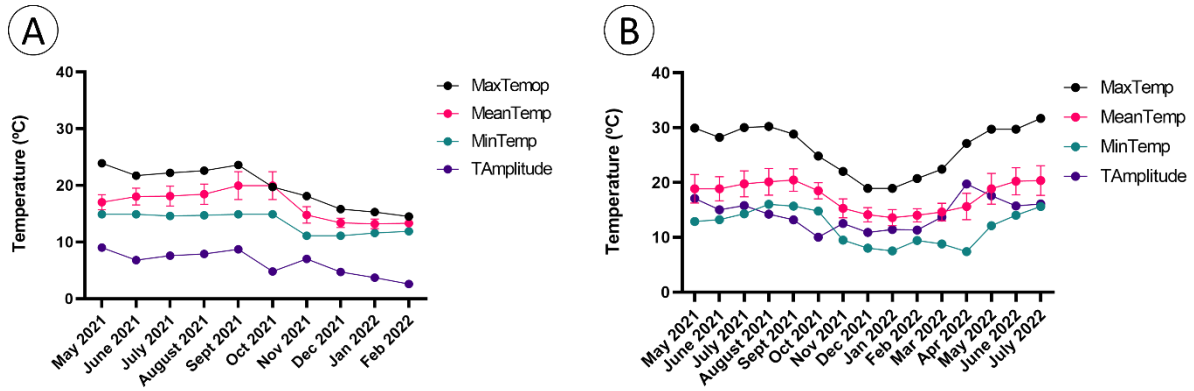

Figure 3.1 Monthly temperature profiles (maximum, mean±SD, minimum and thermal amplitude) of the sampling locations measured by field-deployed probes: A) Ria de Aveiro, B) Tróia.

3.2 RNA-seq

The assembled transcriptome of *Pomatoschistus microps* (liver) yielded a combined total of 340 455 contigs (Table 3.2). Of these, 129 196 were identified as potential open reading frames (ORFs, i.e., contigs with protein-encoding regions) of which 97 318 presented conclusive homology-matching against records in the Swiss-Prot database. Subsequently, the assembled transcriptome of *Pomatoschistus microps* yielded 21 differentially expressed genes (DEGs, 0.006% of the contigs) in spring vs. summer comparisons, both locations combined (Table 3.2). Of these, 31.8% were overexpressed in summer, while the vast majority representing 68.2% of the total DEGs was underexpressed in summer, when compared to spring. DEGs between seasons at each study site are presented in Figure 3.2, showing a total of 18 DEGs in Ria de Aveiro and 4 DEGs in Tróia (1 DEG is common to both locations).

Table 3.2 Selection of the transcripts of interest after de novo transcriptome assembly. Number of transcripts after each stage of analysis.

Analytical stage	Stage objective	Transcripts	Percentage* (%)
1	Transcriptome Assembly	340 455	100
2	Transcripts with Coding Regions	129 196	37.9
3	Functional Annotation	97 318	28.6
4	Differentially Expressed Genes	21	0.006
5	ORFs with Differential Expression	22	0.006

*Percentages were drawn against the total number of transcripts after transcriptome assembly (100%).

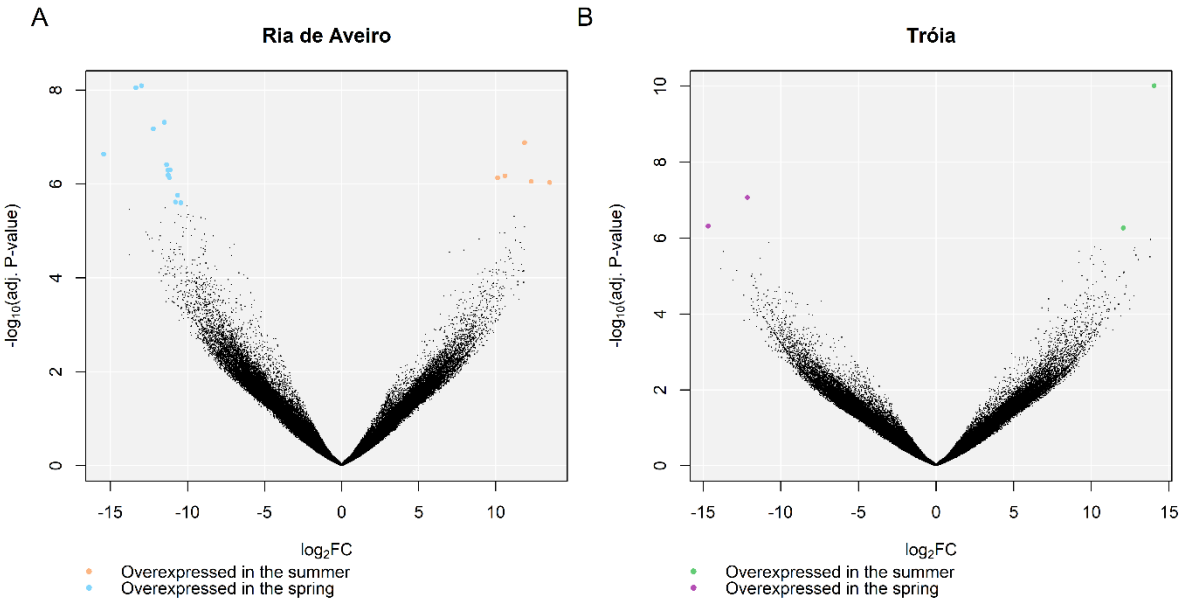

Figure 3.2 Volcano plots illustrating the differentially-expressed genes (DEGs). A) between Spring and Summer in Ria de Aveiro (13 overexpressed DEGs in spring and 5 overexpressed DEGs in summer, in blue and orange, respectively). B) between Spring and Summer in Tróia (2 overexpressed in spring and 2 overexpressed in summer, in purple and green, respectively). The black dots represent transcripts that were not differentially-expressed between seasons. Cut-off for differential expression set at $|\log_2 FC| > 1.5$ and FDR-adjusted $p < 0.05$.

From these DEGs, a total of 22 open readings frames (ORFs) were predicted based on functional annotation (see Table 3.3, 7 DEGs had no available annotation, 8 DEGs had 1 ORF, 4 DEGs had 2 ORFs each and the remaining 2 DEGs had 3 ORFs each).

Table 3.3 List of the 22 ORFs differentially expressed between seasons at each sampling location. Regulation refers to expression observed in summer when compared to spring. The FDR-adjusted p-value (< 0.05 for significance) associated with each log_2FC value (cut-off > 1.5) are also represented.

Ria de Aveiro										
Gene name	**Expression**	**Log_2FC**	**FDR p-value**	**Coded protein**	**Aminoacid nr.**	**Uniprot Acession**	**%ID**	**e-value**	**Generic function/role**	**Organism**
HMGCR	overexpressed	13.494	9.25E-07	3-hydroxy-3-methylglutaryl-coenzyme A reductase	888	A7Z064	64.516	0.00E+00	Cellular cholesterol homeostasis	*Bos taurus*
SIK2	underexpressed	-13.348	8.89E-09	Serine/threonine-protein kinase SIK2	798	Q9IA88	66.184	0.00E+00	Modulates efficiency of insulin signal transduction, inhibits CREB activity	*Gallus gallus*
Fah	underexpressed	-12.224	6.68E-08	Fumarylacetoacetase	419	P25093	71.802	0.00E+00	L-phenylalanine degradation	*Rattus norvegicus*
cyp2k1	underexpressed	-11.500	4.85E-08	Cytochrome P450 2K1	504	Q92090	61.934	1.00E-152	Catalytic activity on xenobiotics, steroids, fatty acids, vitamins and prostaglandins	*Oncorhynchus mykiss*
rnps1-b	underexpressed	-11.500	4.85E-08	RNA-binding protein with serine-rich domain 1-B	283	Q3KPW1	65.502	7.31E-59	Enhances the formation of the ATP-dependent A complex of the spliceosome, participate in mRNA 3'-end cleavage, mediates increase of mRNA abundance and translational efficiency	*Xenopus laevis*
hif1α	underexpressed	-11.353	3.86E-07	Hypoxia-inducible factor 1-alpha	766	Q98SW2	65.941	0.00E+00	Master transcriptional regulator of the adaptive response to hypoxia	*Oncorhynchus mykiss*
hif1α	underexpressed	-11.266	5.11E-07	Hypoxia-inducible factor 1-alpha	766	Q98SW2	66.453	0.00E+00		*Oncorhynchus mykiss*
LPL	underexpressed	-11.184	7.32E-07	Lipoprotein lipase	490	P11602	53.846	4.16E-15	Lipid clearance from the blood stream, lipid utilization and storage	*Gallus gallus*
LPL	underexpressed	-11.184	7.32E-07	Lipoprotein lipase	475	P06858	68.687	1.10E-153		*Homo sapiens*
Nfat5	underexpressed	-11.124	4.98E-07	Nuclear factor of activated T-cells 5	1534	Q9WV30	39.674	1.02E-21	Transcriptional regulation of osmoprotective and inflammatory genes, Regulates hypertonicity-induced cellular accumulation of osmolytes	*Mus musculus*
NFAT5	underexpressed	-11.124	4.98E-07	Nuclear factor of activated T-cells 5	1531	O94916	80.323	0.00E+00	Transcriptional regulation of osmoprotective and inflammatory genes, Mediates the transcriptional response to hypertonicity	*Homo sapiens*
NFAT5	underexpressed	-11.124	4.98E-07	Nuclear factor of activated T-cells 5	1531	O94916	49.18	7.67E-06		*Homo sapiens*

TNNI 2	underexpressed	-10.778	2.42E-06	Troponin I, fast skeletal muscle	183	P68247	63.006	3.47E-76	Confers calcium-sensitivity to striated muscle actomyosin ATPase activity	*Coturnix japonica*
C5	underexpressed	-10.438	2.51E-06	Complement C5	1681	P08650	33.299	0.00E+00		*Rattus norvegicus*
C5	underexpressed	-10.438	2.51E-06	Complement C5	1681	P08650	38.235	8.99E-53	Initiates the spontaneous assembly of the late complement components, C5-C9, into the membrane attack complex	*Rattus norvegicus*
C5	underexpressed	-10.438	2.51E-06	Complement C5 (Hemolytic complement)	1680	P06684	38.462	3.85E-51		*Mus musculus*
LYL1	underexpressed	-10.645	1.73E-06	Protein lyl-1	280	P12980	57.862	3.08E-50	Transcription regulation	*Homo sapiens*
Gima p9	underexpressed	-10.645	1.73E-06	GTPase IMAP family member 9	291	G3X987	30.516	1.39E-29	GTP metabolism	*Mus musculus*

Tróia

Gene name	**Expression**	**Log2FC**	**FDR p-value**	**Coded protein**	**Aminoacid nr.**	**Uniprot Acession**	**%ID**	***e*-value**	**Generic Function/Role UniProt**	**Organism**
TRIM 39	Overexpressed	14.043	9.76E-11	E3 ubiquitin-protein ligase TRIM39	518	Q9HCM9	31.373	6.58E-23	Facilitates apoptosis, Regulates the G1/S transition of the cell cycle and DNA damage-induced G2 arrest	*Homo sapiens*
ZP1	Overexpressed	12.053	5.42E-07	Zona pellucida sperm-binding protein 1	627	I6M4H4	38.362	6.67E-47	Induction of the acrosome reaction and prevents post-fertilization polyspermy	*Oryctolagus cuniculus*
PALM3	Underexpressed	-14.673	4.83E-07	Paralemmin-3	673	A6NDB9	52.239	2.34E-12	ATP-binding protein, which may act as an adapter in the Toll-like receptor (TLR) signalling	*Homo sapiens*
psbp 2	Underexpressed	-14.673	4.83E-07	Saxitoxin and tetrodotoxin-binding protein 2	391	Q90WJ9	26.455	5.22E-06	Toxin accumulation and/or excretion	*Takifugu pardalis*
Fah	Underexpressed	-12.146	8.50E-08	Fumarylacetoacetase	419	P25093	71.802	0.00E+00	L-phenylalanine degradation	*Rattus norvegicus*

These results show that most ORFs were underexpressed during seasonal warming and that there was a higher number of differentially expressed ORFs in Ria de Aveiro's fish population in summer (vs. spring) when compared to Tróia's population. Moreover, whereas in fish from Ria de Aveiro, there was only one overexpressed gene in summer when compared to spring (HMGCR, related with cellular cholesterol homeostasis), in Tróia there were two overexpressed genes (TRIM39, which facilitates apoptosis and ZP1, related with oocyte structural integrity). On the contrary, fish from Ria de Aveiro displayed 17 underexpressed genes in summer (vs. spring) (top 3 genes with higher fold-change were SIK2, Fah and cyp2k1, which have roles in insulin signal transduction, amino-acid degradation and catalytic activity on multiple substrates, respectively) and fish from Tróia showed only 3 underexpressed genes (PALM3, psbp2 and Fah, with roles in Toll-like receptor signalling, toxin metabolism and aminoacid degradation, respectively). Additionally, seasonal warming affected fish from different locations in a distinct manner (via different pathways) as only one of the predicted ORF's with differential expression occurred in both locations simultaneously (underexpressed in both cases).

There was a distinct a separation of the samples (shown by the heatmap in Figure 3.3) between seasons and locations into three clusters (horizontal dendrogram): the grey cluster contains the samples from Ria de Aveiro during summer; the orange cluster contains the samples from Tróia during summer, and lastly, the red cluster contains all samples from the Spring season (of both locations). Moreover, there is a clear grouping of 16 genes in a bigger cluster (purple, vertical dendrogram) related to biological processes such as immune response mechanisms (e.g., C5 – Complement 5), energy metabolism (e.g., LPL – lipoprotein lipase, and cyp2k1 – cytochrome P450 2K1) and regulation of transcription (e.g., hif1α – hypoxia-inducible factor 1-alpha, NFAT5 – nuclear factor of activated T-cells 5, and LYL1 – protein lyl-1). Additionally, all genes related to these biological processes are underexpressed in the Summer of Ria de Aveiro (grey cluster) when compared to the other smaller gene clusters which are mostly overexpressed.

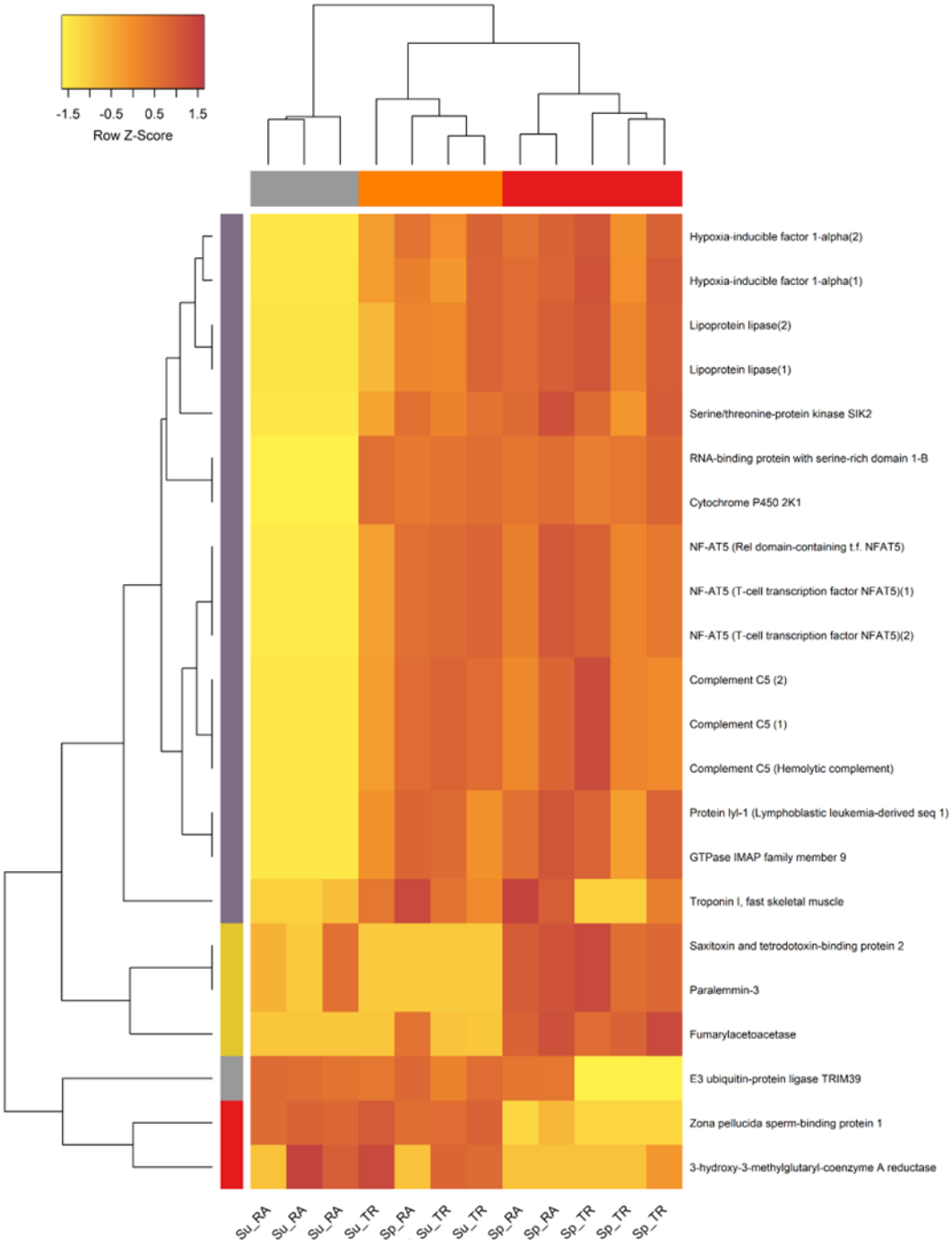

Figure 3.3 Heatmap illustrating relative gene expression of the differentially-expressed genes between Spring vs Summer in both locations. Cut-off for differential expression was set at $|\log_2 FC| > 1.5$ and FDR-adjusted $p < 0.05$. Rows represent different genes (note: in similar gene names, a number was used to separate different gene IDs) and columns represent different samples (note: in sample codes, Sp = Spring, Su =summer, RA = Ria de Aveiro and TR = Tróia). The horizontal dendrogram shows the association between replicate female fish liver samples, whereas the vertical dendrogram shows the association between protein-coding genes. Overexpressed genes are represented in red while underexpressed genes are represented in yellow. The metric and function of the cluster analysis is Euclidian distances and complete linkage, respectively.

Gene ontology (GO) of the differentially expressed genes (for detailed GO Terms associated to each gene see Table A.1 in Appendix) showed that they were overall associated with 5 main types of biological processes namely apoptosis, protein turnover (i.e., ubiquitination processes), immune

response, energy metabolism and regulation of transcription. Furthermore, molecular functions included DNA- and RNA- binding (e.g. hif1α, NFA5 and rnps1-b) and multiple types of catalytic activity (i.e., transferase such as SIK2, hydrolase such as C5, oxidoreductase such as cyp2k1), with gene expression occurring in diverse cellular components, mainly in the nucleus (e.g. hif1α, NFA5), endoplasmic reticulum (e.g. cyp2k1, HMGCR), plasma membrane (e.g. C5, LPL), extracellular space (e.g., C5, LPL) and cytosol (e.g. NFAT5), among others. Despite the similar percentages of ORFs that most genes showed we can see that for, instance, biological processes related with the biosynthesis of lipophilic molecules such as cholesterol, steroids and isoprenoids and regulation of protein metabolic processes (summer in Ria de Aveiro, Figure 3.4A) were overexpressed in the season of the cooler regime, whereas in the warmer season and regime, overexpressed genes were mainly related with apoptosis, protein stabilization and protein ubiquitination (summer in Tróia, Figure 3.5A). On the contrary, underexpressed genes in the warmer season and cooler regime were related with transcription regulation and inflammatory responses (summer in Ria de Aveiro, Figure 3.4B), whereas underexpressed genes in the warmer season and regime were mainly related to lipid metabolism and aminoacid catabolism (summer in Tróia, Figure 3.5B). Moreover, the metabolic processes identified as over or underexpressed during seasonal change differed between study locations (i.e., with different climatic regimes).

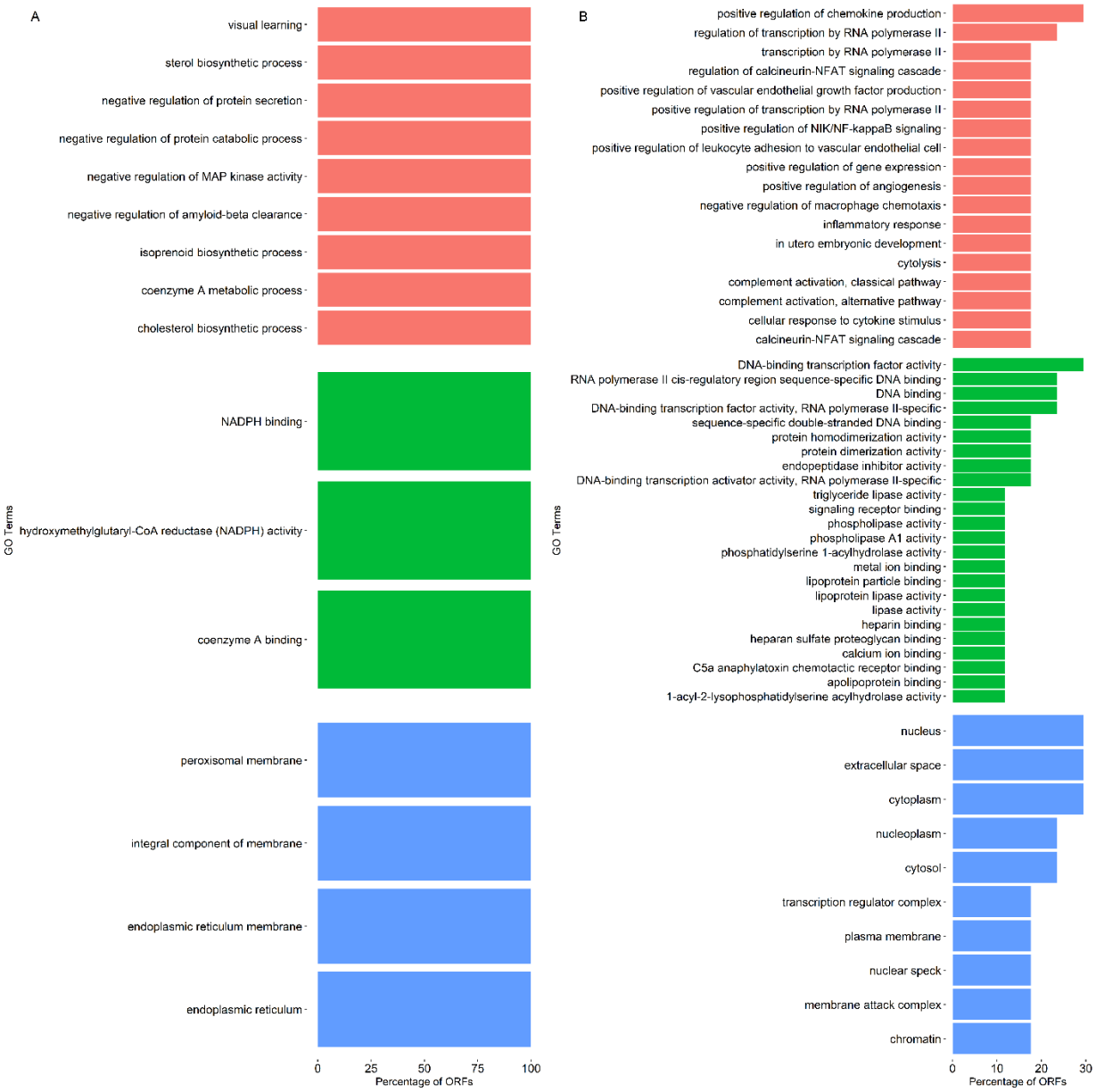

Figure 3.4 Top GO Terms of the differentially-expressed genes from Ria de Aveiro. Horizontal bars show the percentage of the respective ORFs that were: A) upregulated in Summer*;* B) downregulated in Summer. Top GO terms upregulated in Summer from Ria de Aveiro had a percentage of ORFs of 100% and Top GO terms downregulated in Summer from Ria de Aveiro had a percentage of ORFs of 29,4%, 23.5% and 17.6%. Note that the same DEG can associate with multiple GO terms.

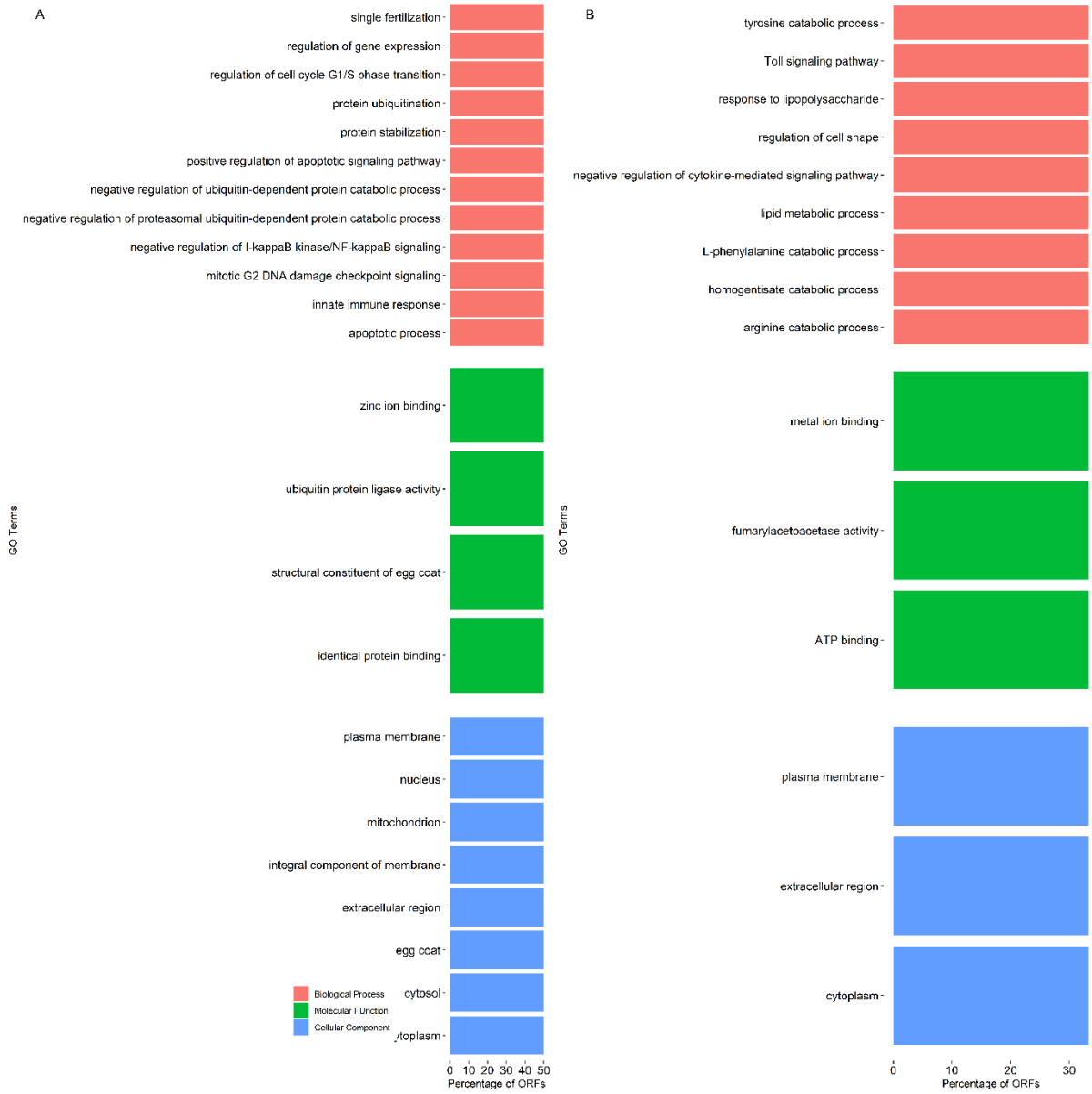

Figure 3.5 Top GO Terms of the differentially-expressed genes from Tróia. Horizontal bars show the percentage of the respective ORFs that were: A) upregulated in Summer; B) downregulated in Summer. Top GO terms upregulated in Summer from Tróia had a percentage of ORFs of 100% and Top GO terms downregulated in Summer from Tróia had a percentage of ORFs of 100%. Note that the same DEG can associate with multiple GO terms.

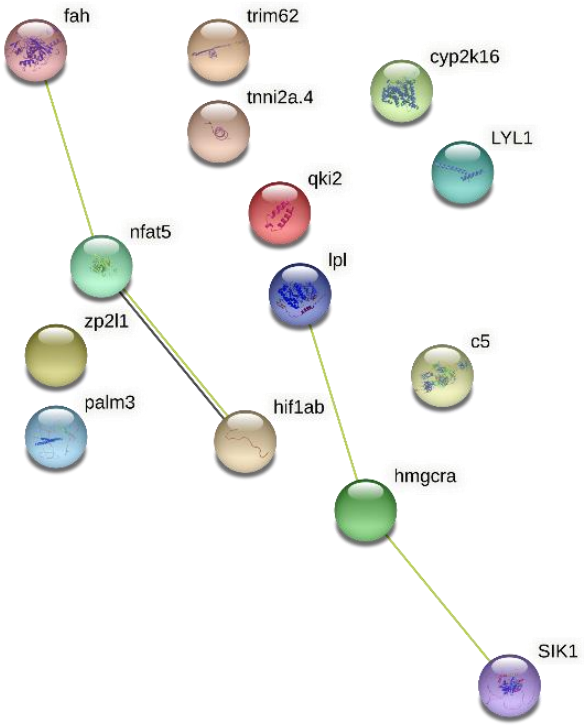

Figure 3.6 Protein-protein interaction (PPI) network for the subset of differentially-expressed genes. The proteins are depicted by colourful circles. Connections between these proteins (either directly or through intermediary proteins) are depicted by the lines. The confidence threshold was set at 0.150 (less stringent, due to reduced number of DEGs and poorly annotated target species).

Protein-protein association network (PPI) with homology matching genes against the organism *Danio rerio*, showed the formation of two groups of interrelated genes (either directly, or through intermediary proteins), jointly contributing to shared functions, namely regulation of gene expression (fah, nfat5 and hif1αb, respectively) and energy metabolism (lpl, hmgcra and SIK1) (Figure 3.6).

3.3 Molecular biomarkers

The factorial ANOVA results (Table 3.4) testing the main and interactive effects of season and location in biomarker levels showed the existence of a significant effect of the factor Season in Glycogen, Glucose and Tprotein, and the factor Location in the Glycogen, Glucose, Tprotein. Moreover, the interaction of both factors showed a significant effect in Glucose and Tprotein.

Table 3.4 ANOVA results of all biomarkers analysed (Glycogen, Glucose, Tprotein – Total protein, Triglycerides and RNA:DNA ratio). Statistical significance was considered at p-value < 0.05. Significant results are presented in bold.

		F	MS	*p*-value
Glycogen	Season	**9.975**	**504.7**	**0.005**
	Location	**4.464**	**225.9**	**0.047**
	Season:Location	0.443	22.4	0.513

Glucose	Season	**24.03**	**15.228**	**<0.001**
	Location	**21.66**	**13.722**	**<0.001**
	Season:Location	**20.77**	**13.163**	**<0.001**
Tprotein	Season	**5.779**	**8624**	**0.022**
	Location	**5.892**	**8793**	**0.021**
	Season:Location	**7.712**	**11509**	**0.009**
Triglycerides	Season	0.485	3.701	0.494
	Location	0.064	0.487	0.803
	Season:Location	1.836	14.007	0.191
RNA:DNA ratio	Season	0.804	22.589	0.381
	Location	0.311	8.729	0.584
	Season:Location	0.26	7.299	0.616

Post-hoc Tukey's tests for significant interactions between season and location are presented in Figure 3.7 for selected comparisons of interest. Results showed significant differences for glucose between 'summer Tróia' and the remaining groups (Figure 3.7a), as well as significant differences for Tprotein between seasons in Tróia and the warmer season of both locations (i.e., summer of Tróia vs summer of Ria de Aveiro) (Figure 3.7b). Tukey's tests for the remaining biomarkers are provided in the Appendix (see Figure A.2).

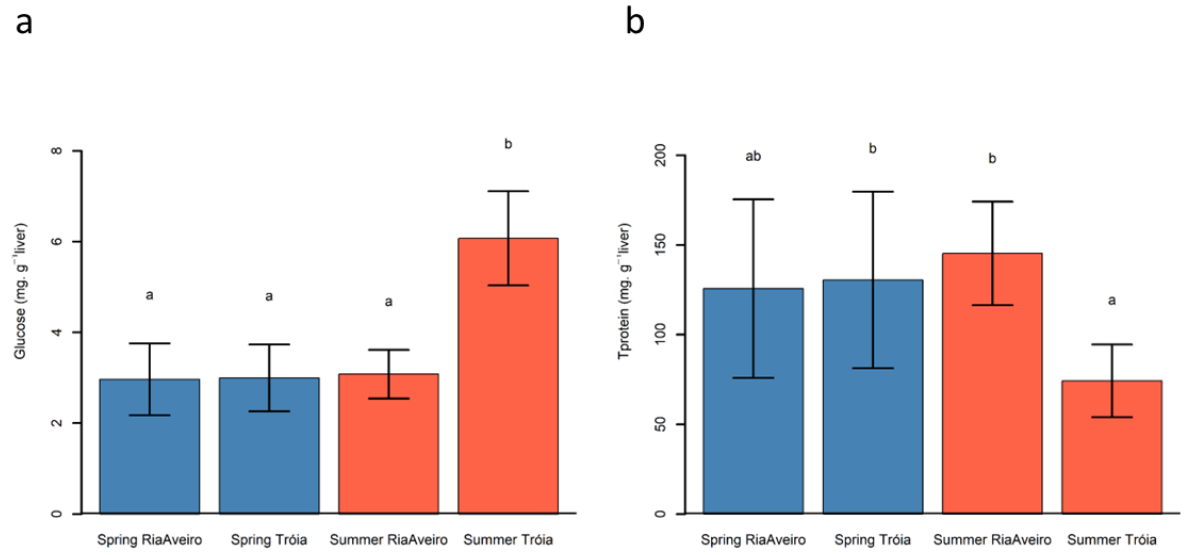

Figure 3.7 Post-hoc Tukey tests for significant interactions of season with location for: a) Glucose; b) Tprotein.

3.4 Histopathology

Fish collected in both seasons and locations showed visible structural alterations in vital organs such as kidney, liver and muscle (Table 3.5 and Figure 3.8). Overall, the most common histopathological traits found were related with circulatory disturbances (e.g., hyperaemia – swollen blood vessels due to increased pre-mortem blood pressure/flow, and haemorrhage), inflammation (e.g., identified by melanomacrophage aggregates and immune cells infiltration), regressive alterations (e.g., muscle atrophy) and presence of parasites (mainly metazoan and protozoan). The frequency of these histopathological alterations were distinct in fish liver, especially between the different locations (Figure 3.8A-B). Fish from Ria de Aveiro displayed, in general, higher diversity of alterations than fish from Tróia (e.g., in the prevalence of parasites found as well as the presence of circulatory disturbances). Kidneys (trunk) showed a similar histopathological pattern between fish from either season and location. In general, this was the most affected organ, revealing high incidence of parasites (mostly myxozoa-like), hyperaemia, haemorrhage and melanomacrophage aggregates (Figure 3.8C-D). Besides the occurrence of helminth-like parasites (Figure 3.8F), muscle also displayed presence of Mesomycetozoea parasites (most likely *Ichthyosporea*) in both seasons and latitudes (Figure 3.8E). Results of the qualitative analyses performed for both seasons and latitudes targeting the three organs are summarized in the Table 3.5. Overall, fish from Ria de Aveiro showed more histopathological alterations related with circulatory disturbances (i.e., hyperaemia and haemorrhage) when compared to Tróia. Both locations had a similar behaviour in the remaining histopathological traits in the three vital organs analysed. The number of parasites was higher in the spring when compared to the summer in Tróia. Additionally, it was observed muscular atrophy in the spring season. Ria de Aveiro showed a similar behaviour in structural alterations between seasons.

Table 3.5 Qualitative analysis of histopathological alterations observed in fish collected from the sampling seasons (Spring and Summer) and from the cooler climate regime (Ria de Aveiro) and warmer climate regime (Tróia).

Ria de Aveiro					
	Reaction pattern	**Histopathological alteration**	**Target organs**		
			Liver	Kidney	Skeletal muscle
Spring (n=5)	Circulatory disturbances	Haemorrhage	0	++	0
		Hyperaemia	0	++	0
	Inflammation	Melanomacrophage aggregates	0	++	0
		Immune cells infiltration	-	0	-
	Regressive alterations	Muscle atrophy	0	0	0
	Parasites	Metazoan	++	++	±
		Protozoan	0	0	-
Summer (n=5)	**Reaction pattern**	**Histopathological alteration**	**Target organs**		
			Liver	Kidney	Skeletal muscle
	Circulatory	Haemorrhage	0	++	0

	disturbances	Hyperaemia	±	++	0
	Inflammation	Melanomacrophage aggregates	0	++	0
		Immune cells infiltration	-	0	0
	Regressive alterations	Muscle atrophy	0	0	0
	Parasites	Metazoan	++	++	±
		Protozoan	0	0	-
Tróia					
	Reaction pattern	**Histopathological alteration**	**Target organs**		
			Liver	Kidney	Skeletal muscle
Spring (n=5)	Circulatory disturbances	Haemorrhage	0	±	0
		Hyperaemia	0	±	0
	Inflammation	Melanomacrophage aggregates	0	++	0
		Immune cells infiltration	++	0	-
	Regressive alterations	Muscle atrophy	0	0	-
	Parasites	Metazoan	++	++	±
		Protozoan	0	0	-
	Reaction pattern	**Histopathological alteration**	**Target organs**		
			Liver	Kidney	Skeletal muscle
Summer (n=5)	Circulatory disturbances	Haemorrhage	0	-	0
		Hyperaemia	0	±	0
	Inflammation	Melanomacrophage aggregates	0	±	0
		Immune cells infiltration	-	0	-
	Regressive alterations	Muscle atrophy	0	0	0
	Parasites	Metazoan	±	±	-
		Protozoan	0	0	-

0 not found, - rare, ± occasional, + frequent, ++ diffuse.

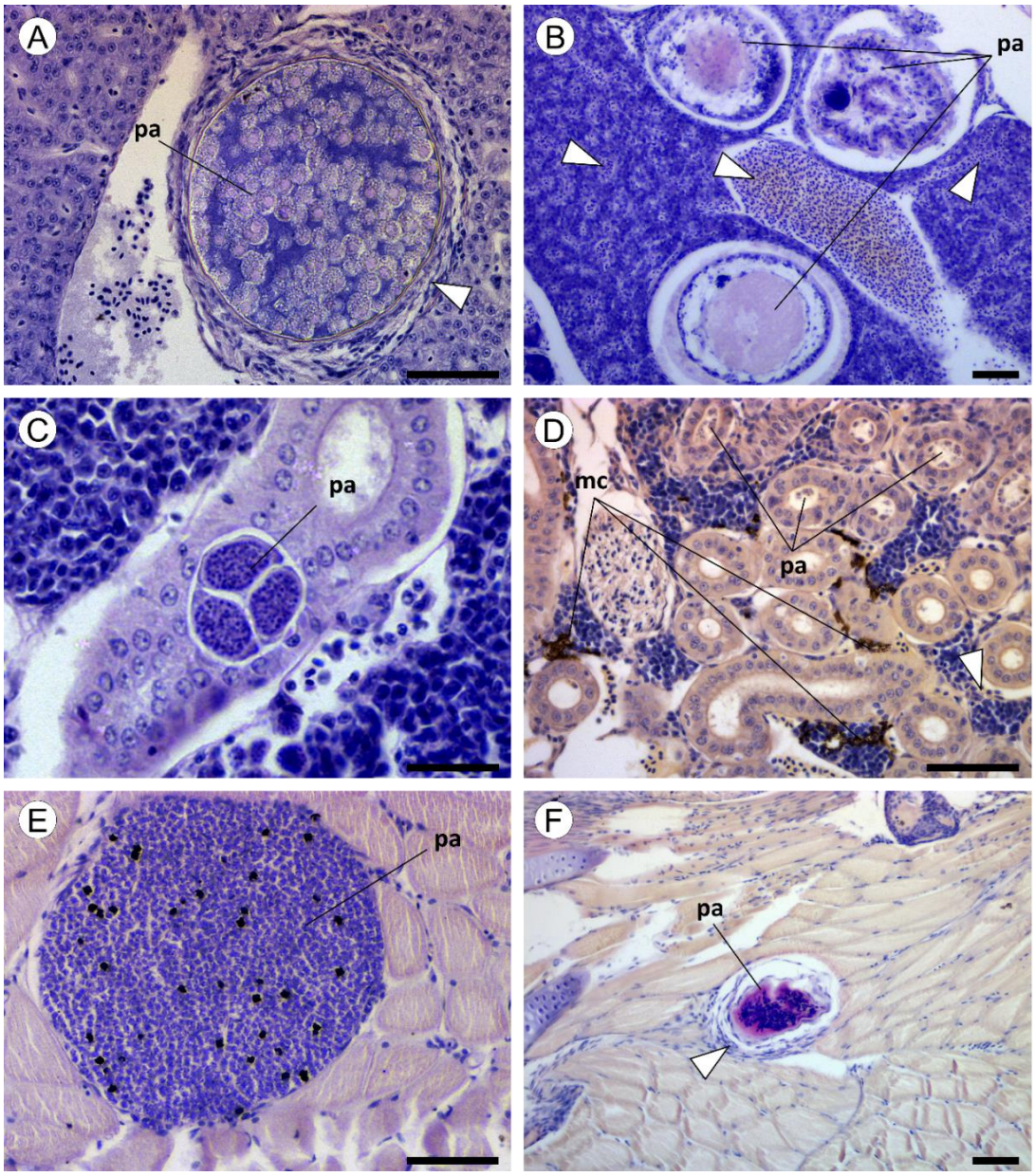

Figure 3.8 Representative histopathological traits found in fish collected from the sampling seasons (Spring and Summer) from the cooler regime (Ria de Aveiro) and the warmer regime (Tróia). A) Liver of fish collected from Spring of Tróia containing a metazoan parasite (*pa*) with immune cells infiltration (*arrowhead*); B) Liver of fish collected from Spring of Ria de Aveiro containing metazoan parasites (*pa*) and with swollen blood capillaries (thus indicating pre-mortem condition), suggesting hyperaemia (*arrowhead*); C) Kidney of fish collected from Spring of Tróia containing intralumial myxozoa-like parasites (pa); D) Kidney of fish collected from Summer of Tróia showing signs of hyperaemia (*arrowhead*), melanomacrophage aggregates (*mc*) and the presence of intralumial myxozoa-like parasites (*pa*); E) Skeletal muscle of fish collected from Spring of Tróia containing a mass of protozoan parasites, potentially as sporogonic stage (*pa*); F) Skeletal muscle of fish collected from Summer of Tróia showing the presence of a metazoan parasite (*pa*). *Scale bar* 50 µm.

Histological sections of fish gonads collected from spring of both locations presented either mature gonads (Tróia, majority of vitellogenic follicules) or gonads still in maturation stage (Ria de Aveiro, Figure 3.9A, with some pre-vitellogenic and some vitellogenic folilcules). Fish from the warmer season (summer) of Ria de Aveiro presented mature gonads showing typically vitellogenic follicles whereas, in

Tróia in the warmer season (summer) fish presented immature gonads (Figure 3.9B, majority of pre-vitellogenic follicules).

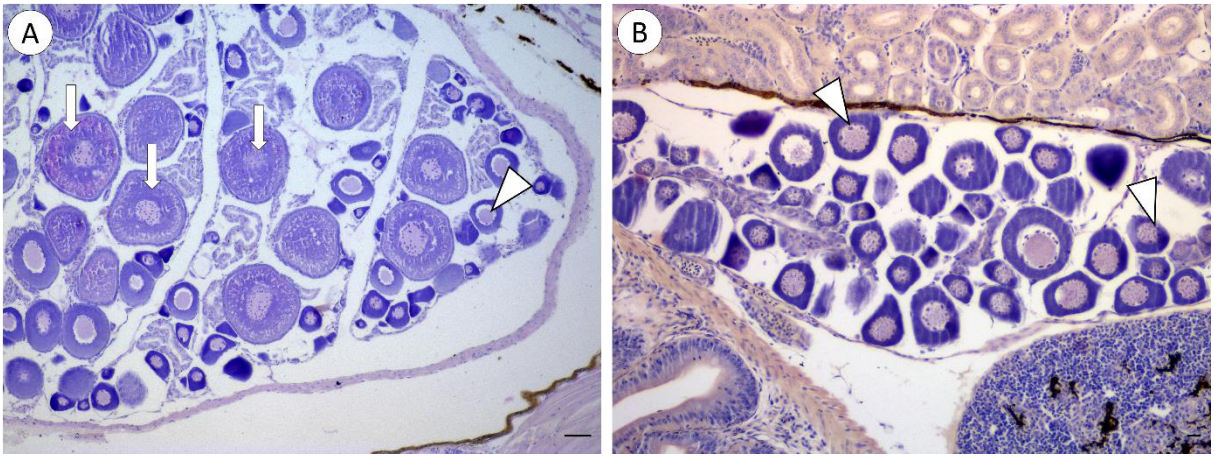

Figure 3.9 Histological sections of female fish gonads. A) Female gonad (stained with H&E) collected during Spring of Ria de Aveiro, showing pre-vitellogenic follicles (*arrowheads*) and vitellogenic follicles (*arrows*). B) Female gonad (stained with H&E) collected during Summer of Tróia, showing mostly pre-vitellogenic follicles (*arrowheads*). *Scale bar* 25 µm.

Tissue compositional differences (i.e., in the deposition of energy reserves) were also observed in fish livers between locations, especially in summer collected specimens. Histochemical analyses showed that while fish from Tróia displayed high glycogen contents, fish from Ria de Aveiro did not, detected by reactivity with PAS-compounds. Figure 3.10A presents hepatocytes that reacted poorly with PAS-positive compounds, while Figure 3.10B shows hepatocytes that present high amounts of glycogen (positive reaction for PAS-compounds, stained bright magenta/pink).

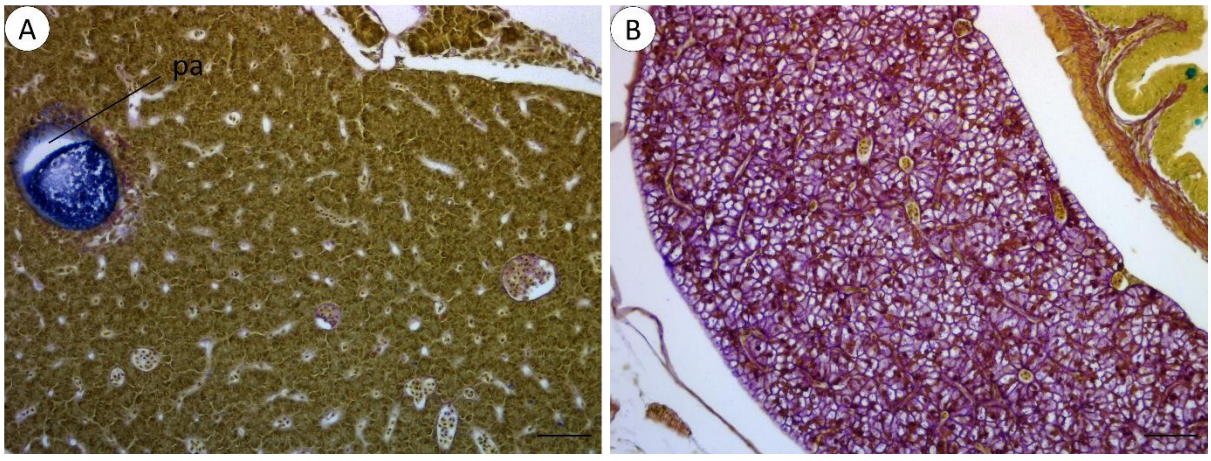

Figure 3.10 Liver sections of *P. microps* (TC staining). A) Liver of fish collected from Spring of Tróia illustrating low content of PAS-reactive compounds and the presence of a metazoan parasite (*pa*); B) Fish liver collected from Summer of Tróia illustrating high content of PAS-reactive compounds (stained bright magenta/pink). *Scale bar* 50 µm.

Table 3.6 Variables affecting the histopathological index (HI) in fish from Spring vs Summer and Ria de Aveiro vs Tróia.

	F	MS	*p*-Value
Season	1.151	0.013	0.296
Location	**21.554**	**0.236**	**<0.001**
Season:Location	**9.098**	**0.099**	**0.007**

Boldface characters indicate variable with significant effect on HI (ANOVA, p < 0.05).

Results from the ANOVA testing the main and interactive effects of season and location on histopathological index showed significantly different results for location (F=21.554, MS=0.236, p-value<0.001) and the interaction of season with location (F=9.098, MS=0.099, p-value=0.007) (Table 3.6).

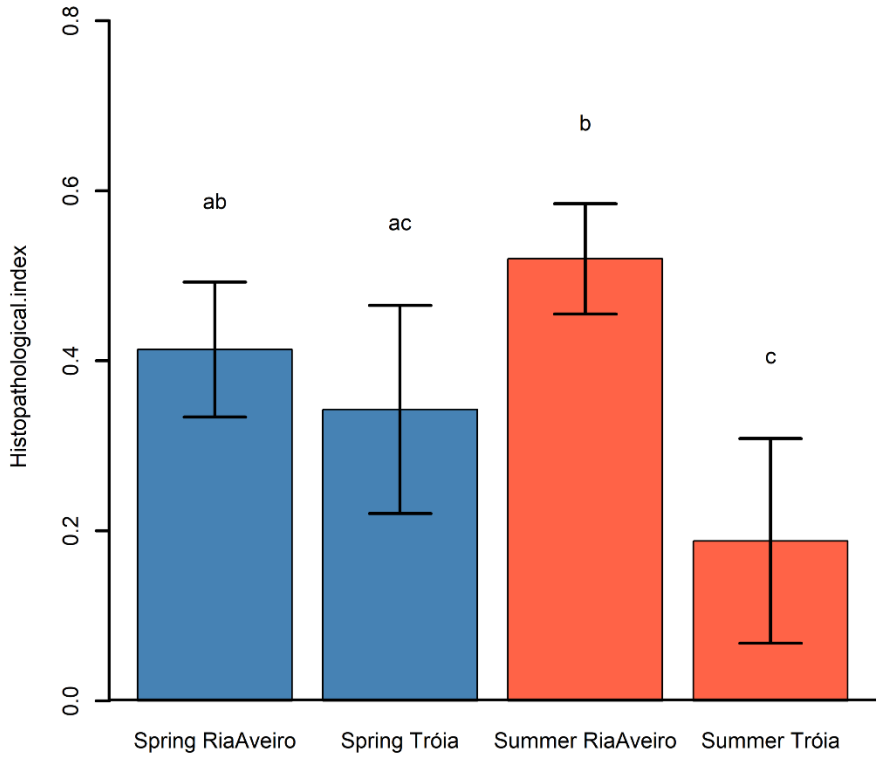

Figure 3.11 Results of post-hoc Tukey tests significant effects of interaction of season with location on histopathological index values. Mean±SD (Spring Ria de Aveiro – 0.413 ± 0.079; Spring Tróia – 0.343 ± 0.122; Summer Ria de Aveiro – 0.520 ± 0.065; Summer Tróia – 0.188 ± 0.120) were used for plotting.

Histopathological indices were higher (36.2%) in the summer season of Ria de Aveiro when compared with Tróia, showing significant differences between the locations (Figure 3.11). Besides increased mean values in different seasons in Ria de Aveiro and Tróia, no significant differences were detected (e.g., spring of Tróia vs summer Tróia and spring Ria de Aveiro vs summer of Ria de Aveiro).

3.5 Fish condition

ANOVA results showed significant differences only for the effects of factor interactions (Season:Location, Table 3.7) in Fulton's K body condition. Post-hoc Tukey tests showed that during summer, fish from Ria de Aveiro displayed higher body condition than fish from Tróia (Figure 3.12). HSI showed no significant differences and is presented in Figure A.2 in the Appendix.

Table 3.7 ANOVA results of the Hepatosomatic index and Fulton's K. Statistical significance was considered at p-value < 0.05. Significant results are presented in bold.

Hepatosomatic index	Season	3.577	7.162	0.064
	Location	0.849	1.699	0.361
	Season:Location	0.029	0.057	0.866
Fulton's K	Season	0.927	0.018	0.340
	Location	3.275	0.065	0.076
	Season:Location	**4.819**	**0.096**	**0.032**

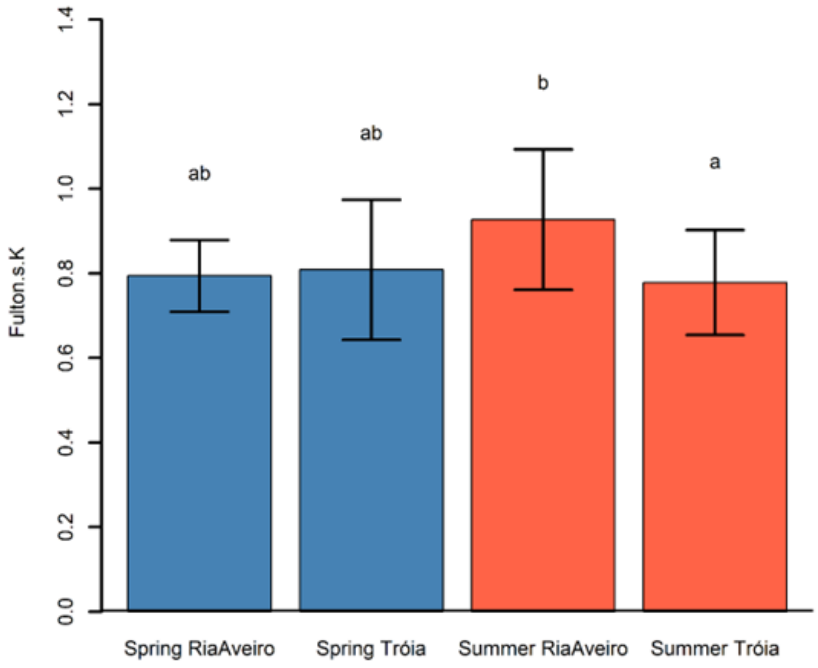

Figure 3.12 Results of post-hoc Tukey tests for the effects of interaction of season with location on Fulton's K. Mean±SD (Spring Ria de Aveiro – 0.794 ± 0.084; Spring Tróia – 0.808 ± 0.166; Summer Ria de Aveiro – 0.927 ± 0.166; Summer Tróia – 0.794 ± 0.085) were used for plotting.

3.6 IBR INDEX

Overall, IBR values were higher in the warmer season (Summer) (8.718 and 10.415 in Ria de Aveiro and Tróia, respectively) when compared to the cooler season (Spring) of each study location (0.000 and 4.191 in Ria de Aveiro and Tróia, respectively). The main variables contributing to high IBR in summer (including both locations) were glucose, glycogen, triglycerides, Fulton's K and HSI. The highest IBR value was found for Summer Tróia when compared to the remaining groups. Star plot (Figure 3.13) showed that, in this specific group, higher overall scores for carbohydrate and lipid energy reserves (Glycogen, Glucose and Triglycerides) were observed when compared with all the other conditions. In contrast, the histopathological index and Tprotein were the lowest (i.e., Summer Tróia). In the second group with highest IBR (Summer Ria de Aveiro), the highest scores were found for Fulton's K, HSI and HI, whereas the lower score was found for RNA:DNA ratios.

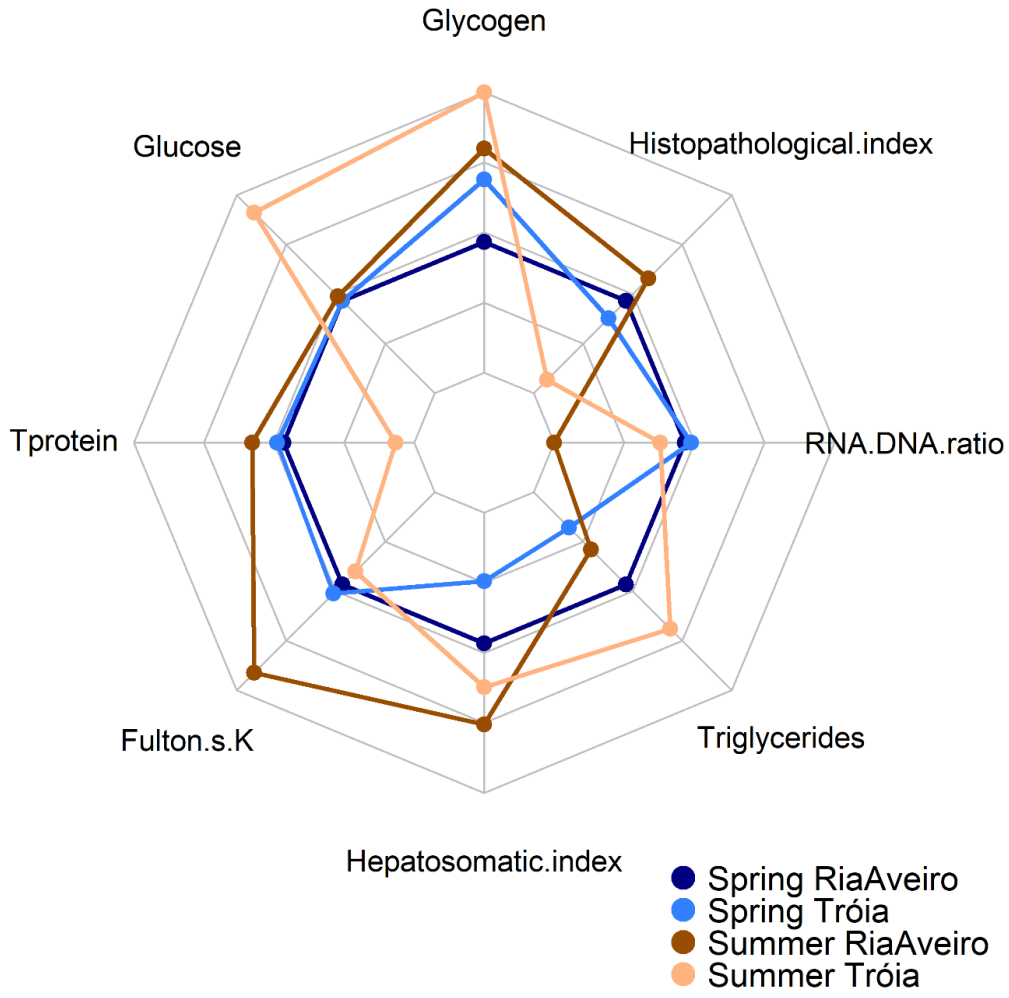

Figure 3.13 Star plot with mean scores of each biomarker. IBR index values for each group were as follows: 'Spring Ria de Aveiro' = 0.000, 'Spring Tróia' = 4.191, 'Summer Ria de Aveiro' = 8.718, 'Summer Tróia' = 10.415. Note that for star plot construction and IBR index calculations, all phenotypic variables were used: energy biomarkers, RNA:DNA ratios, HSI, body condition Fulton's K, as well as the histopathological index.

4

DISCUSSION

Coastal fish populations, especially those inhabiting shallow waters are currently experiencing environmental changes induced by climate change, such as shifts to warmer regimes, greater seasonal thermal variability and climate extremes. The current work aimed to understand how a species of marine coastal fish, the common goby *Pomatoschistus microps* undergoes physiological acclimatization to cope with such environmental changes and infer on the species adaptation potential to future stressful conditions. To do so, we investigated the molecular mechanisms (i.e., gene expression) and their plasticity, as well as consequent phenotypic variation conferring different populations across a latitudinal thermal gradient (north vs. centre of the Portuguese coast) the ability to withstand seasonal warming (spring vs. summer seasons).

4.1 Maximum temperatures and thermal fluctuations are higher in shallow water habitats at lower latitude

Overall, our environmental data showed that shallow waters in both locations displayed mean annual temperatures of ~ 17 ºC, slightly lower than those measured by satellite for Portuguese coastal waters (~18ºC -19 ºC, Data collected from SeaTemperature.org (https://www.seatemperature.org/), accessed on 30 September 2022). However, maximum temperatures recorded in field during the summer season in shallow waters ranged from 22 ºC to 31 ºC, therefore reaching values of +8 ºC when compared to the maximum of 21-23 ºC observed in subtidal waters during the same annual period (Data collected from SeaTemperature.org (https://www.seatemperature.org/), accessed on 30 September 2022). This is an expected result, given the low thermal inertia observed in shallow waters along the coastline, coupled with the reduced water renewal during low tides in estuaries and coastal lagoons, with consequent intense temperature fluctuations (Lopes et al., 2019; Vinagre et al., 2021). Large spatial-temporal temperature gradients are therefore a common feature of Mediterranean climates, as also previously observed by Cardoso et al., (2019). Comparing the two sampling sites, Tróia is overall warmer than Ria de Aveiro throughout the year, as the latter is located at higher latitude having greater Atlantic influence, whereas the former has greater Mediterranean influence (Baptista et al., 2018). Both sampling seasons (Spring – May and Summer – July) in Tróia showed higher maximum water temperature, mean water temperature and temperature amplitude, typical of a Köppen-Geiger classification of Csa). In contrast, Ria de Aveiro is characterized by a more temperate climate with dry milder summers (Köppen-Geiger classification of Csb) and thus, exhibits cooler water temperatures. In fact, the absolute maximum seawater temperature registered in Tróia with our field probes was 37.6 ºC, while the maximum temperature registered in Ria de Aveiro was 23.9 ºC during low tide, in May 2022 and 2021, respectively.

This shows how extreme temperatures can be for marine fauna inhabiting in shallow waters in estuaries and coastal lagoons, especially at lower latitudes. These high temperatures recorded in May also corroborate the projections of extended summers (lasting from the beginning of May to the end of September) in RCP8.5 scenario for Portugal (see (Cardoso et al., 2019)), with maximum temperatures exceeding the historical 90th percentile during more than 50% of summer duration. Additionally, in the summer months the thermal amplitude of Tróia was two times higher than Ria de Aveiro, underlining the critical differences that this species undergoes in the two distinct locations, differing by ~ 2° in latitude (40° 37' 43.85'' N in Ria de Aveiro vs. 38° 29' 06.9'' N in Tróia). Moreover, seawater parameters measured in the field sites during sampling in low tides showed higher temperature, salinity, and O_2 concentrations in Tróia (but lower pH) when compared to Ria de Aveiro. Within Tróia, the similar high salinity values observed between seasons can be explained by a general lack of rain during 2021 in the peninsula and therefore reduced freshwater input from the Sado river into the estuary. The observed increase in O_2 concentrations in the summer (despite the increase in water temperature) can be explained by higher macroalgae photosynthesis during this season, as photoperiod is longer and cloud cover is minimal, meaning that more oxygen is being produced and released into the seawater (Cornwall et al., 2013). Within Ria de Aveiro, a decrease in water temperature was observed from May to July 2021, most likely due to the cooler summer wind from northerly directions which usually occurs between June and August (with some interannual variation), in which case warm water temperatures may only occur later in September (Lopes et al., 2019; Sousa et al., 2020). This cooler wind is more prone to impact higher latitude zones in the coastal area of Portugal, favouring a phenomenon called *upwelling* which results in the replacement of the warmer surface water by the sea bottom cooler water (Alvarez et al., 2013). Besides surface water temperature, salinity is also impacted by this phenomenon. Since saltwater tends to stay deeper to due to its higher density, salinity values increased from Spring to Summer in Ria de Aveiro (from 27.6 ± 1.03 to 34.0 ± 0.4) (Vaz et al., 2005). The variation of pH in 0.1 units between seasons for both locations likely results from a combination of variation in local temperature (main factor driving seasonal pH changes in lower latitudes), dissolved inorganic carbon (DIC) and total alkalinity (main factors driving pH changes at higher latitudes), as well as net community production and vertical mixing (Hagens & Middelburg, 2016). For example, if productivity is high and water mixing is reduced in summer, this would lead to reduced surface water DIC and pCO_2 and consequent higher pH (Hagens & Middelburg, 2016), as observed in Tróia during Summer season. Also, the registered ΔpH in both locations (±0.01 pH units) is in line with observed annual variations in other locations where temperature variability is in the range of ~9 °C (e.g., North Mediterranean Sea (Marcellin Yao et al., 2016). These differences in environmental parameters between both sampling seasons and locations were shown to affect *P. microps* through i) the induction of molecular response mechanisms, namely through changes in gene transcription, ii) energy reserves (namely glucose, glycogen and total proteins), as well as iii) Fulton's K condition index and iv) histopathological index of fish populations inhabiting in these fluctuating habitats.

4.2 Common gobies downregulate gene expression during summer as a mechanism for saving energy and maintaining body condition

Typically, molecular alterations associated with thermal stress tend to be tissue specific in fish (de Nadal et al., 2011; Madeira et al., 2014). The liver is a crucial organ in physiological homeostasis maintenance due to its central role in many metabolic activities, from bile production, amino-acid regulation, glucose processing, vitamin storage, detoxification and resistance to infections (Heymann & Tacke, 2016; Hinton et al., 2017). Changes in this vital organ therefore may provide an insight not only into effects but also into responses to environmental stress (Chien & Hwang, 2001; Skjæraasen et al., 2010; Tao et al., 2018). Previous studies showed that gene expression levels are usually higher in the liver than in other tissues in response to temperature extremes, making it an excellent model organ to study thermally induced mechanisms in stressful environments (Das et al., 2005; Song et al., 2015; Y. Zhu et al., 2013), such as intertidal areas. The present work revealed the differential expression of 21 genes between spring vs summer comparisons. This can be considered a relatively low number of detected DEGs, as previous studies have shown that fish usually display hundreds to a few thousands of DEGs when exposed to temperature increase (see B. Li et al., 2019; Y. Li et al., 2017). Nonetheless, given that the degree of repeatability in environmental fluctuation dictates its predictability (implying past precedence in the occurrence of stressful events) (Hoffmann & Bridle, 2022), regular seasonal cyclic changes may have elicited an early response in fish that could not be entirely detected in our analyses. For instance, during spring, temperature and photoperiod begin to increase concomitantly (Huber & Bengtson, 1999), acting as a cue for the organism to physiologically prepare for summer. This occurs through the induction of feedforward response mechanisms (see Bernhardt et al., 2020), which have evolved to rely on regular environmental variation for long time-periods. These molecular mechanisms (activated through neuronal and hormonal pathways) lead to rapid changes in organisms' phenotypes to prepare them for a later stage, reducing time lags between environmental conditions and internal states and avoiding performance declines once the summer arrives (and with it multiple thermal challenges) (Beaman et al., 2016; Bernhardt et al., 2020; Verd et al., 2019). Previous field studies with fish, such as Feidantsis et al., (2013) found that in liver of *Sparus aurata* inhabiting shallow waters (0-2 m depth), the highest changes in concentration levels of proteins involved in cellular stress responses such as mitogen-activated protein kinases (MAPKs) and extracellular-signal-regulated kinases (ERKs) actually occurred in April and May, respectively, and afterwards, either remained stable until summer (in the former) or decreased in summer (in the latter). Moreso, expression levels of heat shock proteins in most organs of this fish species peaked in spring, with no further induction detected later than May (Feidantsis et al., 2013). This may explain why fish sampled in the summer displayed a low number of DEGs when compared to spring, as it is possible that most gene expression changes for heat acclimatization occur earlier in the year, during the period that fish come out of the winter syndrome.

Overall, *P. microps* presented more underexpressed genes (68.2% of total DEGs) than overexpressed genes (31.8% of total DEGs) in the warmer season when compared to the cooler season. These results

suggest that this eurythermal species, especially at higher latitudes responds to the warmer season by downregulating genes, likely resulting in more energy savings, since the upregulating of more genes usually leads to higher metabolism (López-Maury et al., 2008). In this particular case however, downregulation of cellular metabolism likely enables fish to balance their dynamic energy budgets (Nisbet et al., 2012), avoiding increasing their oxygen consumption and metabolic rates, as an acclimatization mechanism to warmer waters. Energy savings suggested by differences observed in gene transcription were corroborated by biomarker analyses, which confirmed that fish had a better nutritional status in the warmer season, with higher accumulation of energy reserves such as glycogen, glucose and total protein, that can be later used in processes such as growth or reproduction. The ability of fish to save energy under periods of environmental stress was further evidenced in Ria de Aveiro's summer population by the significant increase measured in Fulton's K condition index. In fact, seasonal cycles of reserve deposition and utilization in ectothermic animals are crucial to alleviate temporal mismatches of energy supply and demand (Larson, 1991). It is known that for many fish species in the Atlantic, winter is normally the food-limiting season due to lower primary productivity (Speers-Roesch et al., 2018), whereas in summer, higher temperatures promote increased appetite and feeding rates (Guderley & Leroy, 2001) and food availability is higher. Previous studies with teleost fish also corroborate our findings, as for instance, (C. Yang et al., 2022) observed a downregulation of genes in the freshwater fish *Micropterus salmoides* exposed to heat stress. This response is not exclusive of vertebrates, as studies with invertebrate models such as *Drosophila melanogaster* came to similar results (e.g., downregulation of genes in the GO catalytic activity, hydrolase activity and peptidase activity,(Sørensen et al., 2005), saving energy and suggesting that heat stress responses are fairly conserved among animals. An increasing number of studies has also documented the importance of seasonal warming in reserve deposition in fish, for instance, (MacDonald et al., 2018) found that for the species *Ammodytes marinus*, the accumulation of energy reserves during the warm feeding season is crucial for these animals to acquire the threshold size necessary for the onset of the wintering period.

4.3 Downregulation of immune response, energy metabolism and transcription pathways during thermal challenges may compromise tissue health

The top five underexpressed genes with higher fold change in Ria de Aveiro were related with different metabolic activities (SIK2, Fah, cyp2k1, rnps1-b and hif1α, which have roles in melanogenesis/insulin signal transduction, catabolic processes, metabolic processes, mRNA processing and glucose homeostasis, respectively). The downregulation of SIK2 (serine/threonine-protein kinase 2) is an interesting finding, as this gene has a relevant role in repressing eumelanogenesis in vertebrates (Horike et al., 2010), regulating the synthesis of melanin. Melanin has an important effect on the surface colouring and daily activities of fish (WANG et al., 2020), and the underexpression of SIK2 during warming may contradict the current thermal melanism hypothesis, which proposes that climate change will generally lead to lighter coloured animals, given the role of colouration in thermoregulation in ectothermic animals (Delhey et al., 2020). Alternatively, the downregulation of this gene may be related

to fish sexual maturation, as the reproductive season in Ria de Aveiro's population usually occurs during summer and is typically a period of fish life-cycle in which body colouring patterns are more conspicuous as a means to attract mates (Arruda et al., 1993). SIK2 also plays a role in insulin regulation and autophagosome processing as well as in maintaining the cellular protein pool homeostasis (Yanget al., 2013) and its downregulation in the summer may be related to cellular stress response pathways, since SIK2 stability is known to rely on Hsp90, and is negatively regulated by proteasome-mediated turnover, but this link to the CSR needs further clarification (Yang et al., 2013). The downregulation of Fah (fumarylacetoacetase) gene in this population may suggest a disadvantage, as lower levels of this hydrolase may result in higher accumulation of fumarylacetoacetate, which is hepatotoxic and, in excessive amounts, leads to cell cycle arrest, potentially compromising hepatocyte regeneration (Paulk et al., 2012; Wang, 2019). The differential expression of cyp2k1 (cytochrome P450 monooxygenase) also corroborates our previous findings, as this gene, belonging to the cytochrome P450 (CYP) superfamily, is responsible for the elimination of a variety of endogenous and exogenous noxious compounds, such as toxic metabolites and xenobiotics, fatty acids and steroids (Cok et al., 1998; Mininni et al., 2014). It has been shown that thermal stress regulates the response of this superfamily of enzymes (Cok et al., 1998; Sarasquete & Segner, 2000), suggesting that the underexpression of cyp2k1 indicates that detoxification and elimination of some compounds, and therefore the metabolic rate, were reduced in response to thermal stress. On the other hand, the underexpression of hif1α (hypoxia-inducible factor 1-alpha) in this population was not entirely expected, given that the activation of this gene is thought to be crucial for heat acclimatization in both invertebrates and vertebrates, as it targets other genes important to maintain homeostasis under chronic stressful situations (including warming-induced hypoxia), such as heme oxygenase-1 (HO1), erythropoietin (Epo), and vascular endothelial growth factor (Maloyan et al., 2005). Nevertheless, this can be explained by the fact that in the locations where the gobies were collected, oxygen levels in seawater are known to increase in summer as algae photosynthesis increases, so seasonal warming was not directly associated with seawater oxygen deprivation. This, coupled to reduction of fish metabolic rate (as suggested by the maintenance of total protein contents and increase in body condition during warming in this population), was likely crucial in preventing deleterious tissue hypoxia that can occur due to excessive oxygen demand. Yet, we cannot disregard our results from histological assessment that point towards increased liver hyperaemia in summer in fish from Ria de Aveiro. The occurrence of hyperaemia may, to a certain extent suggest a higher partial pressure of CO_2 in blood which can act as a vasodilator (Yang et al., 2017) and hence explain the increase in blood flow to this tissue. Albeit this could merely reflect functional hyperaemia as a regulatory response to a warmer environment (e.g. see (Liu et al., 2017)), reactive hyperaemia cannot be excluded, since the downregulation of both Fah and cyp2k1 suggest that there may be metabolic and/or toxic waste accumulation in liver tissue (Zhu et al., 2006), which is known to induce vasodilation in an attempt that increased blood flow will wash out the waste from tissues. To a lesser extent, there was also downregulation of C5 (complement 5) and NFAT5 (nuclear factor of activated T-cells 5) genes, the first being an essential component of the defensive complement system in all vertebrates (Johansen et al., 2019), and the second activating T-cells/macrophages (Lee et al., 2019; Lorgen et al., 2017), both having important roles in stress and disease, so their underexpression

indicates reduced immune function. This result was also confirmed by the increase in histopathological index observed in fish from Ria de Aveiro during summer. Overall, our results point towards a physiological investment of fish from Ria de Aveiro in saving energy, but this may occur at the expense of other important biological processes, such as detoxification, liver renewal/function and disease resistance. However, these results may not be completely illustrative of the impact of seasonal warming but rather reflect acclimatization to continuous temperature fluctuations, as there may also be temporary periods of water cooling in summer in Ria de Aveiro, due to northerly winds inducing coastal upwelling, as discussed earlier.

4.4 Overexpression of ubiquitin pathways and parasitisation suggests heat stress during summer in fish populations from Mediterranean hot dry climate regions

In fish from Tróia's population, the highest overexpressed gene, TRIM39 (E3 ubiquitin protein ligase TRIM39) plays important roles in protein ubiquitination (regulating the turnover of proteins), apoptosis induction and immune function (for detailed characterization of TRIM proteins in fish, see e.g. Langevin et al., 2019 and Zhang et al., 2012) whereas the underexpressed genes with higher fold-changes, PALM3 (paralemmin-3) and psbp2 (saxitoxin/tetrodotoxin binding protein 2), were related with signalling pathways (e.g., Toll signalling pathway, important in eliciting innate immune responses) and toxin accumulation and/or excretion, in the warmer season. Previous studies from liver transcriptome in response to heat shock stress have also identified DEGs that typically code to biological functions such as protein stabilization (e.g., protein folding), immune response and energy metabolism in, for instance, the rainbow trout *Oncorhynchus mykiss* (Y. Li et al., 2017) or in the crimson spotted rainbowfish *Melanotaenia duboulayi* (Smith et al., 2013). Therefore, the overexpression of genes related with apoptosis and protein ubiquitination suggests that fish from the warmer regime (Tróia) might be more susceptible to protein damage when compared with the cooler regime (Ria de Aveiro), as they have endured higher maximum temperatures. Although histological assessments did not show increased frequency of apoptotic cells in tissues from fish collected in summer in Tróia, increased signs of inflammation were detected, again supporting a decrease in resistance to disease, similar to Ria de Aveiro's population. In the case of Tróia's population, higher overall water temperatures occur throughout the year, which may induce or inhibit different components of the immune system in certain periods (as observed by overexpression of TRIM39 but underexpression of PALM3 in summer), regulating the articulation of responses at the levels of the innate and adaptive immune systems. Increased temperature is also usually associated to a higher number of pathogens and disease outbreaks in wild populations of many marine species (see for a review, Lõhmus & Björklund, 2015; Macnab & Barber, 2012), including *P. microps* (see Cereja et al., 2018), which could explain a decrease in disease resistance. In spite of this, histopathology evidenced that *P. microps* can be infected with both metazoan and protozoan parasites in multiple seasons, although pathogen virulence may be

enhanced under warming conditions, as shown in other studies (Harvell et al., 2002; Lv et al., 2006; Stewart et al., 2018). Notwithstanding, Bowden, (2008) also showed that fluctuations in environmental parameters other than water temperature, such as salinity, oxygen and pH can also lead to changes in organism immune function, which can be enhanced or reduced depending on fish tolerance and acclimatization ability to these environmental stressors.

4.5 Fish populations from warmer and highly fluctuating regimes rely on gene frontloading as a physiological strategy to cope with thermal stress

It should be noted that the different number of DEGs in response to seasonal change between Ria de Aveiro and Tróia points to different physiological acclimatization strategies employed by the two populations to cope with environmental stress. In Ria de Aveiro, there was a total of 18 DEGs, whereas in Tróia, there was only a total of 4 DEGs. The lower number of DEGs observed in Tróia suggests that this population, being adapted to a climatic regime in which high temperatures are frequent, likely resorts to the frontloading of genes, a process by which long-term changes occur in the level of constitutive gene expression (Barshis et al., 2013), better preparing the organism for frequently encountered stressful conditions (Collins et al., 2021). For instance, in other marine organisms such as corals, which are frequently exposed to El Niño events and heatwaves, some genes have a higher basal expression (i.e., frontloading), allowing the corals to be more readily prepared for increases in sea surface temperature and promoting thermotolerance (Brener-Raffalli et al., 2019; Vidal-Dupiol et al., 2022), and even cross-tolerance between environmental stressors (Collins et al., 2021). The higher number of DEGs observed in fish from Ria de Aveiro with a cooler environment suggest, on the other hand, that these fish encounter thermal stress events less regularly and therefore use an alternative strategy – the induction or inhibition of gene expression only when necessary, when facing environmental stress. Both these physiological acclimatization strategies are used by a multitude of marine species, and previous studies have shown that not only populations can employ them differently, but under particular conditions, even one same population can use both strategies depending on tidal height and microhabitat thermal differences, as shown for crustaceans from intertidal rocky reefs (D. Madeira et al.,). In addition, the fact that there was only one common DEG observed in both locations also shows that populations from different latitudes are employing diverse response pathways to deal with environmental stress. This means that there may be local adaptation to specific stressors or specific magnitude of change of those stressors, resulting in considerable inter-population molecular and phenotype variation.

It is not surprising that fish from Tróia, which should endure higher temperatures throughout the whole year might be more acclimatized to thermal stress than the ones from cooler, northern latitudes. To cope with warming seasons and events, the two populations must ultimately possess different adaptations to survive (e.g., such as different nutrition and energy sources, different enzyme flexibility, presence or absence of certain enzyme isoforms, different mitochondrial density, different concentration levels of

intracellular stabilizers, as their baseline physiology, see (Angilletta Jr., 2009; Angilletta et al., 2003). For instance, biomarker analyses showed that there was a significant disproportionate increase of glucose in fish livers in summer of Tróia population, when compared to the population from summer of Ria de Aveiro. This result suggests that during periods of high thermal stress, glucose tends to be preferably accumulated and seems crucial to answer metabolic demands, functioning as an energy provision source for fish inhabiting in central western coast of Portugal with warmer climatic regime. Feidantsis et al., 2021 observed a similar response in the gilthead seabream *Sparus aurata*, which during thermal stress accumulates plasma glucose as a provision of energy. As glucose increased, total proteins decreased in fish livers from summer Tróia, however in populations from Ria de Aveiro, total protein contents in fish livers were maintained throughout seasons, which would be expected from a carnivorous fish that utilizes proteins and aminoacids as its major source of energy for aerobic metabolism (see Falco et al., 2020). These results further underline how the same species copes with thermal stress differently depending on the thermal regime in which they live and have evolved in. Differences in the deposition and utilization of energy reserves may also suggest that dietary habits of populations at distinct latitudes likely vary or that populations show asynchronous development along the Portuguese coast, prioritizing or inhibiting different metabolic pathways according to life-cycle and/or maturation stages and health status. Fulton's K body condition index might corroborate the previous hypotheses of dietary and/or developmental stage differences, since variation in temperature, coupled to variation in the diet of fish in different latitudes can result in changes in their body size and weight over time, and change fish maturation speed. Particularly, our observations from histochemical and histological analysis of fish livers and gonads suggest that specimens collected during summer in Tróia were still juveniles undergoing a growth phase (as evidenced by high concentration of liver glycogen reserves, detected as PAS-positive compounds), whereas specimens collected from the same season in Ria de Aveiro were already undergoing sexual maturation, as evidenced by lower concentration of glycogen reserves in liver, coupled to the presence of developed oocytes and spermatocytes in female and male gonads, respectively. The ecological implications of these observations become relevant in the light of species thermal windows, given that in ectothermic species thermal windows are wider in juveniles and non-reproducing adults, and narrower in eggs, larvae and spawning adults (see Dahlke et al., 2020; Pörtner & Knust, 2007; Pörtner et al., 2017). This means that while fish populations in Tróia during summer season are essentially composed of younger, more thermally tolerant cohorts during a period in which the occurrence of acute thermal stress events is highly likely to occur (e.g., higher frequency of tropical nights, marine heatwaves, etc), increasing specimens' survival chance; the opposite occurs in Ria de Aveiro, where during the warmer season, the population is mostly composed of reproducing adults, which is considered a thermal bottleneck stage in the life-cycle, during which organism vulnerability to stressful events is increased. In the long-run, this may compromise population renewal and self-sustainability in Ria de Aveiro, unless the population undergoes an adaptive phenological change by altering the timing of the onset of reproduction, either anticipating or delaying it to avoid periods of potential heat stress.

5

CONCLUSION AND FUTURE PERSPECTIVES

Overall, the present work demonstrated that the mechanisms of thermal acclimatization and adaptation of *P. microps* from areas with different thermal regimes are, at least in part, modulated by changes in gene expression networks that likely involve a few genes that have a pivotal role in the control of metabolic processes and energy balance.

Altogether, the main take-home messages from this thesis are the following:
(i) Large spatial-temporal temperature fluctuations are a common feature of shallow waters in Mediterranean climates, especially at lower latitudes where climatic regimes are generally warmer;

(ii) As inhabitants of these shallow water coastal ecosystems, common gobies cope with the potential deleterious effects of temperature fluctuations and latitudinal thermal gradients through population-specific molecular mechanisms, physiological strategies and phenotypic adaptations;

(iii) As a species, *P. microps* showed the capacity to acclimate to seasonal periods of thermal stress through energy-saving mechanisms by down-regulating cellular metabolism, enabling fish to maintain balanced energy budgets, although at the expense of other biological processes such as liver renewal/function (populations from Ria de Aveiro) as well as detoxification and disease resistance (populations from both Ria de Aveiro and Tróia);

(iv) Fish populations in Ria de Aveiro deal with seasonal warming mainly through the induction of gene expression changes, whereas fish populations in Tróia, which encounter more frequent thermal challenges, rely essentially on frontloading of genes, being however sensitive to protein damage caused by high maximum temperatures;

(v) *P. microps* populations utilize energy reserves differently in summer indicating either dietary differences associated to the specific food-webs of each location or asynchronous development and different life-cycle stages between populations in each season;

(vi) Fish from Tróia populations live close to their thermal limits, as their critical thermal maximum is 32 ºC - 33 ºC (determined in a previous study by Cereja et al. 2018), and maximum habitat temperatures

during the summer season in Tróia have shown to surpass these values during low tides, indicating that this population may have very low or negative thermal safety margins, which may impact its ability to cope and survive;

(vii) Populations in Ria de Aveiro live far from their thermal limits but are particularly sensitive in summer as they reproduce during this season, and spawning adults of ectothermic species are known to have reduced thermal windows, therefore being vulnerable to any potential thermal extreme events that occur during this period.

Overall, this study improved our understanding of climate change impacts on a key marine fish, concluding that despite *P. microps* having specific physiological mechanisms to cope with temperature stress, molecular and phenotypic plasticity may not always mean that population tolerance and resilience will be re-defined by acclimatization ability, especially in naturally fluctuating environments, where multi-stressors may occur (e.g., salinity, pH, hypoxia, disease outbreaks, etc). This is especially relevant, as this estuarine species plays an important role on ecosystem processes and biological-environmental interactions - and the potential local extinction of some populations of an intermediary predator can disrupt food webs (e.g., such as estuarine ones in Tróia). Lastly, we conclude that if we extrapolate these results to other marine species that may respond similarly, there may be significant implications of these findings for coastal biodiversity management and inland aquaculture production. Critical knowledge of the inherent physiological variability that underlies inter-population acclimatization ability and survival odds in face of global change stressors seems a crucial aspect to consider when improving local and regional approaches to marine conservation, fisheries management and food production.

As future perspectives, It would be relevant to develop further studies focused on i) multi-omics comparative and integrated analyses (e.g., adding epigenomics, proteomics and metabolomics to transcriptomics analysis) as a true Systems-Biology approach to study fish adaptation mechanisms at multiple biological organization levels to build predictive models of the magnitude of climate change impacts in species physiology; ii) extend the present field study with wild fish from these same populations to compare responses during a regular summer vs. summer with heatwave(s); iii) multiple life-stage assessments to understand the different acclimatization abilities and physiological strategies employed by different developmental stages (from egg to adult) of this species; and iv) experimental biology approaches, where the most vulnerable populations could be selected for further testing, namely extreme climatic events, such as single or sequential heatwaves of different magnitudes and duration. The combination of these topics will certainly provide relevant advances in climate change biology science.

6

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A

APPENDIX

A.1 Figures and Tables

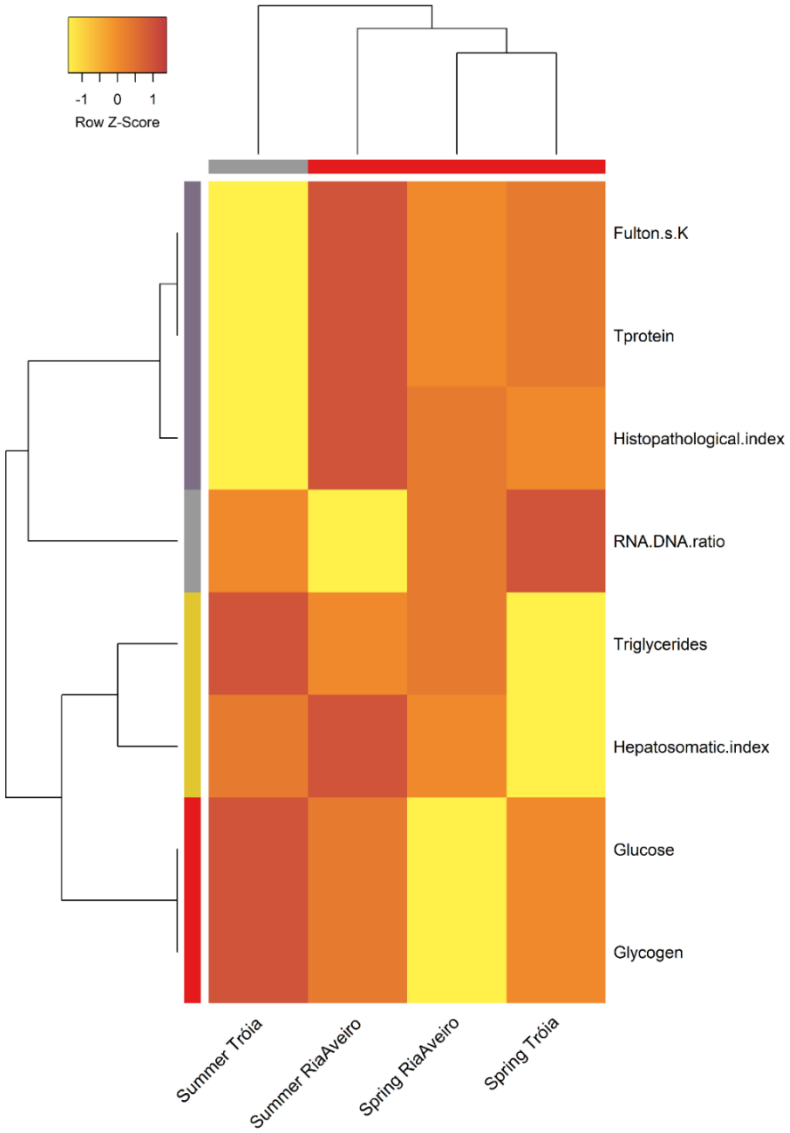

Figure A.1 Heatmap illustrating the differences in mean values of the biomarkers in each study condition. The metric and function of the cluster analysis is Euclidian distances and complete linkage, respectively.

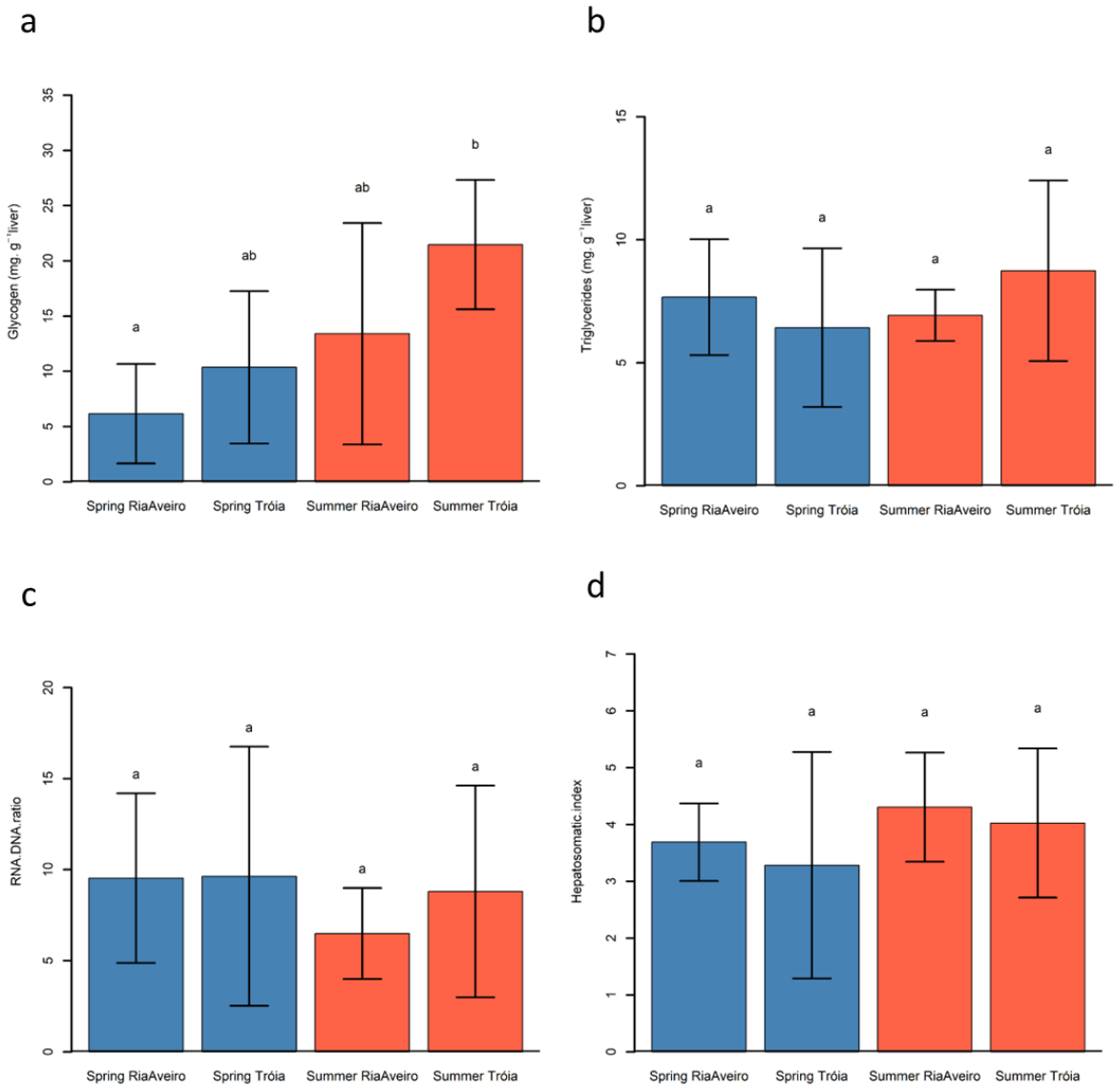

Figure A.2 Results of post-hoc Tukey tests for the effects of interaction of season with location of the remaining biomarkers: a) Glycogen; b) Triglycerides; c) RNA:DNA ratio; d) Hepatosomatic index (HIS).

Table A.1 List of GO Terms associated with each gene.

Gene name	Organism	Protein	GO BiologicalProcess
NFAT5	*Homo sapiens*	Nuclear factor of activated T-cells 5, NF-AT5 (T-cell transcription factor NFAT5) (Tonicity-responsive enhancer-binding protein, TonE-binding protein, TonEBP)	[GO:0033173];[GO:0071474];[GO:0071345];[GO:0010628]; [GO:1904996];[GO:1901224];GO:0045944];[GO:0070884]; [GO:0006357]; [GO:0007165];[GO:0006366]
hif1a	*Oncorhynchus mykiss*	Hypoxia-inducible factor 1-alpha, HIF-1-alpha, HIF1-alpha	[GO:0001678]
SIK2	*Gallus gallus*	Serine/threonine-protein kinase SIK2, EC 2.7.11.1 (Qin-induced kinase) (Salt-inducible kinase 2, SIK-2) (Serine/threonine-protein kinase SNF1-like kinase 2)	[GO:0035556];[GO:0000226];[GO:0006468];[GO:0046626]
HMGCR	*Bos taurus*	3-hydroxy-3-methylglutaryl-coenzyme A reductase, HMG-CoA reductase, EC 1.1.1.34	[GO:0006695];[GO:0015936];[GO:0008299];[GO:1900222]; [GO:0043407];[GO:0042177];[GO:0050709];[GO:0016126]; [GO:0008542]
LPL	*Homo sapiens*	Lipoprotein lipase, LPL, EC 3.1.1.34 (Phospholipase A1, EC 3.1.1.32)	[GO:0071398];[GO:0031670];[GO:0042632];[GO:0034371]; [GO:0006633];[GO:0006631];[GO:0016042];[GO:0055096]; [GO:1900077];[GO:0006644];[GO:2000343];[GO:0032722]; [GO:0010886];[GO:0045600];[GO:0050729];[GO:0032731]; [GO:0032755];[GO:0010884];[GO:0010744];[GO:0010890]; [GO:0032760];[GO:0009617];[GO:0009409];[GO:0009749]; [GO:0009410];[GO:0019432];[GO:0019433];[GO:0070328]; [GO:0006641];[GO:0034372]
cyp2k1	*Oncorhynchus mykiss*	Cytochrome P450 2K1, EC 1.14.14.1 (CYPIIK1) (Cytochrome P450 LMC2)	[GO:0046222];[GO:0048252];[GO:0002933]
TNNI2	*Coturnix japonica*	Troponin I, fast skeletal muscle (Troponin I, fast-twitch isoform)	NA
rnps1-b	*Xenopus laevis*	RNA-binding protein with serine-rich domain 1-B	[GO:0006397]; [GO:0008380]
C5	*Rattus norvegicus*	Complement C5 [Cleaved into: Complement C5 beta chain; Complement C5 alpha chain; C5a anaphylatoxin; Complement C5 alpha' chain]	[GO:0006874];[GO:0006935];[GO:0006957];[GO:0006958]; [GO:0097273];[GO:0019835];[GO:0032835];[GO:0042593]; [GO:0001701];[GO:0006954];[GO:0090594];GO:0001822]; [GO:0002523];GO:0033602];[GO:0010760];[GO:0010700];

			[GO:0001780];[GO:0045766];[GO:0032722];[GO:0050921]; [GO:0007204]; [GO:0010575]
C5	*Mus musculus*	Complement C5 (Hemolytic complement) [Cleaved into: Complement C5 beta chain; Complement C5 alpha chain; C5a anaphylatoxin; Complement C5 alpha' chain]	[GO:0006956];[GO:0006957];[GO:0006958];[GO:0019835]; [GO:0001701];[GO:0006954];[GO:0010760];[GO:0045766]; [GO:0032722];[GO:0050778];[GO:0010575]
LYL1	*Homo sapiens*	Protein lyl-1 (Class A basic helix-loop-helix protein 18, bHLHa18) (Lymphoblastic leukemia-derived sequence 1)	[GO:0030183];[GO:0001955];[GO:0060216];[GO:0045893]; [GO:0006357];[GO:0006355]
Gimap9	*Mus musculus*	GTPase IMAP family member 9 (Immune-associated nucleotide-binding protein 7, IAN-7) (Immunity-associated nucleotide-binding protein 7)	[GO:0046039]
Nfat5	*Mus musculus*	Nuclear factor of activated T-cells 5, NF-AT5 (Rel domain-containing transcription factor NFAT5) (T-cell transcription factor NFAT5)	[GO:0033173];[GO:0071345];[GO:0010628];[GO:1904996]; [GO:1901224];[GO:0045944];[GO:0070884];[GO:0006357]; [GO:0006970];[GO:0006366]; [GO:0006351]
LPL	*Gallus gallus*	Lipoprotein lipase, LPL, EC 3.1.1.34 (Phospholipase A1, EC 3.1.1.32)	[GO:0042632];[GO:0034371];[GO:0006633];[GO:0006631]; [GO:0016042];[GO:0055096];[GO:0032722];[GO:0010886]; [GO:0045600];[GO:0010890];[GO:0019433];[GO:0070328]; [GO:0034372]
Fah	*Rattus norvegicus*	Fumarylacetoacetase, FAA, EC 3.7.1.2 (Beta-diketonase) (Fumarylacetoacetate hydrolase)	[GO:0006527];[GO:1902000];[GO:0006559];[GO:0006629]; [GO:0006572]
ZP1	*Oryctolagus cuniculus*	Zona pellucida sperm-binding protein 1 (Zona pellucida glycoprotein 1, Zp-1) [Cleaved into: Processed zona pellucida sperm-binding protein 1]	[GO:0007338]
TRIM39	*Homo sapiens*	E3 ubiquitin-protein ligase TRIM39, EC 2.3.2.27 (RING finger protein 23) (RING-type E3 ubiquitin transferase TRIM39) (Testis-abundant finger protein) (Tripartite motif-containing protein 39)	[GO:0006915];[GO:0045087];[GO:0007095];[GO:0043124]; [GO:0032435];[GO:2000059];[GO:2001235];[GO:0050821]; [GO:0016567];[GO:1902806]; [GO:0010468]
PALM3	*Homo sapiens*	Paralemmin-3	[GO:0001960];[GO:0008360];[GO:0032496];[GO:0008063]
psbp2	*Takifugu pardalis*	Saxitoxin and tetrodotoxin-binding protein 2	NA

A.2 R Scripts

A.2.1 R Script for Energy Biomarkers, Condition Indices and Statistics

```
##Packages

library(car)
library(multcompView)
library(gplots)
library(RColorBrewer)
library(IBRtools)
library(preprocessCore)

setwd("\\[PATH]\\Reservas Energéticas\\R studio energy_reserves")
FishData_set<-read.csv("fishData.csv",sep=",",header=TRUE, row.names=1)
FishData_set$SubsetData<-paste(FishData_set$Season,FishData_set$Location)
FishData_set<-FishData_set[,c(1,2,12,3:11)]

for(i in 1:4){
  FishData_set[,i]<-as.factor(FishData_set[,i])
}

#####Determining normality and homoscedasticity of data#####

##Normality (Sharpiro-Wilks' test)

  nFactors<-nlevels(FishData_set[,3])
  Factors<-levels(FishData_set[,3])

  for(i in 5:ncol(FishData_set))
  {
    for(j in 1:nFactors)
    {
print(paste(sep="","Variable:",colnames(FishData_set)[i],";","Factor:",Factors[j]))
print(shapiro.test(FishData_set[FishData_set[,3]==Factors[j],i]))
    }
  }

##Homoscedasticity (Levene's test)

  for(i in 5:ncol(FishData_set))
  {
  print(paste(sep=" ","Variable:",colnames(FishData_set)[i]))
  print(leveneTest(FishData_set[,i]~as.factor(FishData_set[,3]),center=median))
```

```
  }

####################Parametric tests####################

###ANOVA and Tukey's test###

  letAux<-matrix(data=NA,nrow=4,ncol=(ncol(FishData_set)-
5+1),dimnames=list(c("SpringAveiro",
"MidSummerTroia","SpringTroia","MidSummerAveiro"),colnames(FishData_set)[5:ncol(Fis
hData_set)]))
  for(i in 5:ncol(FishData_set)){
     AnovaHI<-aov(data=FishData_set,FishData_set[,i]~Season*Location)
     print(colnames(FishData_set)[i])
     print(summary(AnovaHI))
     TukeyHI<-TukeyHSD(AnovaHI)
     print(TukeyHI)
     Tukey_values<-as.matrix(as.numeric(TukeyHI$'Season:Location'[,4]))
     rownames(Tukey_values)<-gsub(" ","",rownames(TukeyHI$'Season:Location'))
     let<-multcompLetters(Tukey_values[,1])
     letAux[,i-4]<-let$Letters
     print(let)
  }

## Means and StDs of data##

  Means<-
aggregate(FishData_set[,5:ncol(FishData_set)],list(FishData_set[,3]),mean,na.rm=TRU
E)
  SDs<-
aggregate(FishData_set[,5:ncol(FishData_set)],list(FishData_set[,3]),sd,na.rm=TRUE)
  colnames(Means)[1]<-c("SubsetData")
  colnames(SDs)[1]<-c("SubsetData")

  Means<-Means[c(3,4,1,2),]
  Means[,1]<-c("Spring  RiaAveiro",  "Spring  Tróia",  "Summer  RiaAveiro",  "Summer
Tróia")

  SDs<-SDs[c(3,4,1,2),]
  SDs[,1]<-c("Spring RiaAveiro", "Spring Tróia","Summer RiaAveiro", "Summer Tróia")

  ColUnits<-colnames(Means)
  ColUnits[c(2,3,4,7)]<-c(expression(paste("Glycogen  (mg."  ~"g"^-1,  "liver)"))  ,
expression(paste("Glucose  (mg."~"g"^-1,  "liver)")),    expression(paste("Tprotein
(mg."~"g"^-1, "liver)")), expression(paste("Triglycerides (mg." ~"g"^-1, "liver)")))

##Graphs of biomarkers##

  letAux<-letAux[c(1,3,4,2),]
```

```
  for(i in 2:ncol(Means)){
    if(
      i==2||i==6
    ) {
      windows()
      par(mfrow=c(2,2)
      )
    }

    SDu<-Means[,i]+SDs[,i]
    SDl<-Means[,i]-SDs[,i]
    MeanPlot<-barplot2(

      Means[,i],
      names.arg=Means[,1],
      xlab=expression(italic("")),
      ylab=ColUnits[i],
      ylim=c(0,zapsmall(max(SDu)+max(SDu)*0.38,2)),
      lwd=2,
      plot.ci=TRUE,
      ci.u=SDu,
      ci.l=SDl,
      ci.lty="solid",
      ci.lwd="2",
      ci.width=0.4,
      cex.lab=1,
      cex.axis=1,
      col=c("steelblue","steelblue","tomato","tomato"),
      cex.names = 1
    )
    abline(h=0,lwd=3)

    text(
      MeanPlot[,1],
      (Means[,i]+SDs[,i])+max(SDl)*0.21,
      letAux[,i-1],
      cex=1
    )
    mtext(side=3,letters[i-1],at=-0.5,line=2,cex=2)
    if(
      i==5||i==9
    )
      dev.print(tiff, paste("Tukey_Statistics", i, ".tiff", sep=""), height=30, width
= 30, units ="cm", res = 600)

  }
```

```
####Heatmap####

##Preparing data for plotting##

    Means_normalized<-
normalize.quantiles(as.matrix(Means[,2:ncol(Means)]),copy=FALSE)
    rownames(Means_normalized)<-Means[,1]
    clustFunction <- function(x) hclust(x, method="complete")
    distFunction <- function(x) dist(x, method="euclidean")
    cCol<-colorRampPalette(brewer.pal(9, "Set1"))
    colFit<-clustFunction(distFunction(Means_normalized))
    cClusters<-cutree(colFit,h=23)
    cHeight<-length(unique(as.vector(cClusters)));
    colHeight = cCol(cHeight)
      cRow<-colorRampPalette(brewer.pal(9, "Set1"))
      rowFit<-clustFunction(distFunction(t(Means_normalized)))
      rClusters<-cutree(rowFit,h=10)
      rHeight<-length(unique(as.vector(rClusters)));
      rowHeight = cRow(rHeight)
      plotColGenes<-rowHeight[rClusters]

##Plotting Heatmap##
    heatColour<-colorRampPalette(c("#fff04a", "#f28e2b", "#c23e40"))(n = 100)
    windows()
    heatmap.2(as.matrix(t(Means_normalized)),
              hclust=clustFunction,
              distfun=distFunction,
              ColSideColors=colHeight[cClusters],
              RowSideColors=plotColGenes,
              density.info="none",
              col=heatColour,
              trace="none",
              scale="row",
              cexCol = 1.4,
              lhei = c(1.5, 9, 0.04),
              lwid = c(1.5, 5.7, 0.5),
              offsetRow = 0,
              offsetCol = 0,
              srtCol = 45,
              key.title = NA,
              margins = c(9, 20)
              )
    dev.print(tiff, "HeatMap.tiff", height=25, width = 20, units ="cm", res = 600)
```

```
####IBR Starplot####

##Starplot Graph##

  starplot<-ibrv2_bdi(Means)
  starplot_index<-ibrv2_index(Means)

  windows()
  par(xpd = TRUE, mar = c(5, 2.5, 4, 2) + 0.1)
  colorvector<- c(rgb(0,0,0.5), rgb(0.2,0.5,1), rgb(0.6,0.3,0), rgb(1,0.7,0.5))

  ibr_chart(starplot, legend = F, pcol = colorvector, pfcol = NULL, axistype = 0)
  legend(x=0.5, y=-1.15, legend = Means[,1], bty = "n", pch=20 , col=colorvector ,
text.col = "black", cex=0.9, pt.cex=2)
  dev.print(tiff, "Starplot.tiff", height=15, width = 15, units ="cm", res = 600)

  write.table(starplot, "Starplot.csv", sep = ",", col.names=NA)
  write.table(starplot_index, "Starplot_index.csv", sep = ",", col.names=NA)
```

A.2.2 R Script for Differential-expression analysis

```
##Packages##

library(tximport)
library(limma)
library(edgeR)
library(seqinr)
library(rhdf5)
library(UniprotR)
library(ggplot2)
library(cowplot)
library(RColorBrewer)
library(gridGraphics)
library(gplots)

setwd("\\[PATH]\\ExtremeOceans_RNA-Seq\\DIffgenes")

#####Differentially-expressed analysis#####
sampleName <- c(
  "F14_Sp_TR",
  "F15_Su_TR",
  "F16_Su_RA",
  "F18_Sp_TR",
  "F19_Sp_RA",
  "F2_Su_TR",
  "F21_Sp_RA",
  "F21_Sp_TR",
  "F27_Sp_RA",
  "F30_Su_TR",
  "F38_Su_RA",
  "F4_Su_RA"
)
Location <- c(
  "Tróia",
  "Tróia",
  "Ria de Aveiro",
  "Tróia",
  "Ria de Aveiro",
  "Tróia",
  "Ria de Aveiro",
  "Tróia",
  "Ria de Aveiro",
  "Tróia",
  "Ria de Aveiro",
  "Ria de Aveiro"
)
Season <- c(
  "Spring",
```

```
  "Summer",
  "Summer",
  "Spring",
  "Spring",
  "Summer",
  "Spring",
  "Spring",
  "Spring",
  "Summer",
  "Summer",
  "Summer"
)
Level <- c(
  "SpTR",
  "SuTR",
  "SuRA",
  "SpTR",
  "SpRA",
  "SuTR",
  "SpRA",
  "SpTR",
  "SpRA",
  "SuTR",
  "SuRA",
  "SuRA"
)


Kallistofolder <- "\\[PATH]\\ExtremeOceans_RNA-Seq\\Kallisto"
Trinityfile <-
  "\\[PATH]\\ExtremeOceans_RNA-Seq\\Trinity\\Trinity.Trinity.fasta.gz"


##Table for experimental design
dataMatrix <- as.data.frame(cbind(sampleName,
                                  Location,
                                  Season,
                                  Level))
write.table(dataMatrix,
            "DEG\\dataMatrix.csv",
            sep = ";",
            col.names = NA)

##Data table
##Importing the results

samples <- dir(Kallistofolder)
file <-
  c(paste(sep = "", Kallistofolder, "\\", samples, "\\", "abundance.h5"))
```

```
names(file) = samples
print(names(file))
Tsv <-
  tximport(file,
           type = "kallisto",
           txOut = TRUE,
           countsFromAbundance = "lengthScaledTPM")
print(head(Tsv$counts))
print(dim(Tsv$counts))
print(colnames(Tsv$counts))
Fa <- read.fasta(Trinityfile, as.string = TRUE)
print(head(Fa))
TxID <- getName(Fa)
TxSEQ <- unlist(getSequence(Fa, as.string = TRUE))
TxSEQ <- as.data.frame(cbind(TxID, TxSEQ))
print(head(TxSEQ))
colnames(TxSEQ)[1] <- "ID"
print(nrow(TxSEQ))
Counts <- Tsv$counts
Counts <- cbind(as.data.frame(row.names(Counts)), Counts)
colnames(Counts)[1] <- "ID"
Full <- merge(Counts, TxSEQ, by = "ID")
colnames(Full) <- c("ID", sampleName, "sequence")
write.table(Full, "DEG\\Full.csv", sep = ";", col.names = NA)


##Statistics

##Create a DGEList object from a table of counts

Data <-
  DGEList(counts = Full[, 2:13],
          group = Level,
          genes = Full[, 1])

##Calculate the normalization factors

Data <- calcNormFactors(Data)

##Create the generalized linear models for comparing the expression levels from the
two seasons for each location

Design <- model.matrix( ~ 0 + Level, data = Data$samples)
colnames(Design) <- levels(Data$samples$group)
Data <- estimateDisp(Data, Design)
fit <- glmFit(Data, Design)
```

```
##Estimate the relative expression (log2FC positive - Overexpressed in summer compared
to spring; Negative - underexpressed in summer compared to spring)

SuTRvSpTR <- makeContrasts(contrasts = "SuTR-SpTR", levels = Design)
SuRAvSpRA <- makeContrasts(contrasts = "SuRA-SpRA", levels = Design)
print(SuTRvSpTR)
print(SuRAvSpRA)
lrtSuTR <- glmLRT(fit, contrast = SuTRvSpTR)
lrtSuRA <- glmLRT(fit, contrast = SuRAvSpRA)
print(topTags(lrtSuTR))
print(topTags(lrtSuRA))
Results <- cbind(Full$ID, lrtSuTR$table, lrtSuRA$table)
colnames(Results) <-
  c(
    "ID",
    "log2FC_SuTR",
    "log2CPM_SuTR",
    "LR_SuTR",
    "FDRp_SuTR",
    "log2FC_SuRA",
    "log2CPM_SuRA",
    "LR_SuRA",
    "FDRp_SuRA"
  )
save(Full, Results, file = "DEG\\FullResults.RData")
save(Data, file = "DEG\\Data.RData")
load("DEG\\FullResults.RData")


##decideTest identifies the differentially-expressed genes according to the cut-offs
(log2FC and FDR)
##0 means no differential expression
##1 means overexpressed
##-1 means underexpressed

expressionTable <-
  decideTests(
    Results[, grepl("FDRp", colnames(Results))],
    coefficients = Results[, grepl("log2FC", colnames(Results))],
    lfc = 1.5,
    adjust.method = "fdr"
  )
colnames(expressionTable) <- c("SuTR", "SuRA")
head(expressionTable)
nrow(expressionTable)
DEG <- cbind(Results, expressionTable)
degTable <-
  DEG[which(abs(DEG["SuTR"]) == 1 | abs(DEG["SuRA"]) == 1), ]
```

```
print(head(degTable))
nrow(degTable)
write.table(degTable,
            "DEG\\degTable.csv",
            sep = ";",
            col.names = NA)
write.table(DEG, "DEG\\DEG.csv", sep = ";", col.names = NA)
save(lrtSuTR, lrtSuRA, file = "DEG\\lrt.RData")
save(degTable, file = "DEG\\degTable.RData")
save(DEG, file = "DEG\\DEG.RData")

##Annotation

write.fasta(
  sequences = as.list(Full$sequence),
  names = Full$ID,
  file.out = "DEG\\Sequences.fasta"
)

##Smear plot

smearPlot <-
  function(degTable,
           lrt,
           colour,
           legend,
           title,
           Condition,
           j) {
    Colours <- matrix(data = NA, nrow = nrow(degTable))
    for (i in 1:nrow(degTable)) {
      if (degTable[i, paste("log2FC_", Condition, sep = "")] > 0)
        Colours[i, 1] <- colour[1, 1]
      else
        Colours[i, 1] <- colour[2, 1]
    }
    plotSmear(
      lrt,
      de.tags = rownames(degTable),
      cex = 0.5,
      deCol = Colours,
      xlab = log2CPM,
      ylab = log2FC,
      main = title,
      panel.first = {
        points(0,
               0,
               pch = 16,
```

```
              cex = 1e6,
              col = "grey95")
        grid(col = "white", lty = 1)
      }
    )
    legend(
      "bottomleft",
      inset = c(-0.06, -0.3),
      xpd = TRUE,
      pch = 20,
      bty = "n",
      col = c(colour[1, ], colour[2, ]),
      legend = c(legend[1, ], legend[2, ])
    )
    mtext(
      side = 3,
      LETTERS[j],
      at = -6.8 - (j - 1) * 0.4,
      line = 2,
      cex = 1.5
    )
  }

##Plotting Smear plot

load("DEG\\lrt.RData")
windows()
par(mfrow = c(1, 2), mar = c(6, 4.5, 3.5, 2))
for (i in 1:length(Condition)) {
  if (i == 1) {
    lrt <- lrtSuTR
    degTableaux <- subset(degTable, abs(degTable[, "SuTR"]) == 1)
  }
  else{
    lrt <- lrtSuRA
    degTableaux <- subset(degTable, abs(degTable[, "SuRA"]) == 1)
  }

  smearPlot(degTableaux, lrt, colour[Condition[i]], legend[Condition[i]], title[i],
Condition[i], i)

}
dev.print(
  tiff,
  "DEG\\PlotSmear.tif",
  height = 15,
  width = 30,
  units = 'cm',
```

```
  res = 600
)

##Volcano plot

window()
signif <- -log10(DEG[, paste("FDRp_", "SuTR", sep = "")])
plot(DEG[, paste("log2FC_", "SuTR", sep = "")],
     signif,
     pch = ".",
     xlab = "log2FC",
     ylab = "-log10FDR")

load(\\[PATH]\\DEG\\DEG.RData")
print(colnames(DEG))

log2FC <- expression(paste("log"[2], "FC"))
log2CPM <- expression(paste("Average log"[2], "CPM"))
log10FDR <- expression(paste("-log"[10], "(adj. P-value)"))
colour <- data.frame(SuTR = c("#fcb683", "#83d6fc"),
                     SuRA = c("#f7add0", "#adf7ef"))
legend <-
  data.frame(
    SuTR = c("Overexpressed in the summer", "Overexpressed in the spring"),
    SuRA = c("Overexpressed in the summer", "Overexpressed in the spring")
  )
title <- c("Troia" , "Ria de Aveiro")
Condition <- c("SuTR", "SuRA")

volcanoPlot <- function(DEG, colour, legend, title, Condition, i) {
  signif <- -log10(DEG[, paste("FDRp_", Condition, sep = "")])
  plot(
    DEG[, paste("log2FC_", Condition, sep = "")],
    signif,
    pch = ".",
    xlab = log2FC,
    ylab = log10FDR,
    main = title,
    panel.first = {
      points(0,
             0,
             pch = 16,
             cex = 1e6,
             col = "grey95")
      grid(col = "white", lty = 1)
    }
  )
  points(DEG[which(DEG[Condition] == 1), paste("log2FC_", Condition, sep = "")],
```

```
        -log10(DEG[which(DEG[Condition] == 1), paste("FDRp_", Condition, sep =
"")]),
        pch = 20,
        cex = 0.8,
        col = colour[1, ])
  points(DEG[which(DEG[Condition] == -1), paste("log2FC_", Condition, sep = "")],
        -log10(DEG[which(DEG[Condition] == -1), paste("FDRp_", Condition, sep =
"")]),
        pch = 20,
        cex = 0.8,
        col = colour[2, ])
  legend(
    "bottomleft",
    inset = c(-0.06, -0.3),
    xpd = TRUE,
    pch = 20,
    bty = "n",
    col = c(colour[1, ], colour[2, ]),
    legend = c(legend[1, ], legend[2, ])
  )
  mtext(
    side = 3,
    LETTERS[i],
    at = -19.6 + (-i + 1) * 3,
    line = 2,
    cex = 1.5
  )
}


##Plotting Volcano plot

windows()
par(mfrow = c(1, 2), mar = c(6, 4, 3.5, 2))
for (i in 1:length(Condition)) {
  volcanoPlot(DEG,     colour[Condition[i]],     legend[Condition[i]],     title[i],
Condition[i], i)
}
dev.print(
  tiff,
  "DEG\\VolcanoPlot.tif",
  height = 15,
  width = 30,
  units = 'cm',
  res = 600
)
```

```
##Reading results from Blast

blastfile <-
  " \\[PATH]\\Blastp\\ExtremeOceans_Trim_Blastp_SwissProt.csv"
blastp <- read.csv(blastfile, sep = ";", header = F)
blastpID <-
  unlist(sapply(blastp[, 1], function(x)
    unlist(strsplit(x, ".", fixed = TRUE))[1]))
blastp <- cbind(blastpID, blastp)

##Merge the results from BLAST with the Differential Expression Analysis

degAnnotAll <-
  merge(degTable, blastp, by.x = "ID", by.y = "blastpID")


degAnnotAll[, (ncol(degAnnotAll) + 1)] <-
  unlist(sapply(degAnnotAll[, 13], function(x)
    unlist(strsplit(x, "|", fixed = TRUE))[2]))
degAnnot <- degAnnotAll[, c(12, 2, 3, 5, 6, 7, 9, 10, 11, 27, 14, 22)]
save(degAnnotAll, degAnnot, file = "Annotated\\degAnnotAll.RData")

##Downloading information from UniProt

Accession <- unique(degAnnot[, 10])
NameTaxa <- GetNamesTaxa(Accession)
SubcellLocation <- GetSubcellular_location(Accession)
GOTerms <- GetProteinGOInfo(Accession)
AccessionInfo <- merge(NameTaxa, SubcellLocation, by = "row.names")
AccessionInfo <-
  merge(AccessionInfo, GOTerms, by.x = "Row.names", by.y = "row.names")
save(NameTaxa,     SubcellLocation,     GOTerms,     AccessionInfo,     file     =
"Annotated\\AccessionInfo.RData")
Uniprot <-
  merge(
    degAnnot,
    AccessionInfo,
    by.x = 10,
    by.y = "Row.names",
    sort = FALSE
  )
Uniprot_final <- Uniprot[, c(2:10, 1, 11, 12, 14, 16, 20, 22, 32:34)]
colnames(Uniprot_final) <-
  c(
    "TrinityID",
    colnames(Uniprot_final)[2:9],
    "Accession",
    "Blastp_%ID",
```

```
    "Blastp_Evalue",
    "Entry name",
    "Gene name",
    "Organism",
    "Protein",
    "GO_BiologicalProcess",
    "GO_MolecularFUnction",
    "GO_CellularComponent"
  )
Uniprot_final <-
  Uniprot_final[order(
    Uniprot_final$Blastp_Evalue,
    -Uniprot_final$`Blastp_%ID`,
    -Uniprot_final[, "log2FC_SuTR"],
    Uniprot_final[, "FDRp_SuTR"],
    -Uniprot_final[, "log2FC_SuRA"],
    Uniprot_final[, "FDRp_SuRA"]
  ), ]
Uniprot_final_SuTR <-
  subset(Uniprot_final, abs(Uniprot_final$SuTR) == 1)
Uniprot_final_SuRA <-
  subset(Uniprot_final, abs(Uniprot_final$SuRA) == 1)

save(Uniprot, Uniprot_final, file = "Annotated\\Uniprot_final_BeforeDeletion.RData")
save(Uniprot_final_SuRA,            Uniprot_final_SuTR,            file            =
"Annotated\\Uniprot_final_SuTR_SuRA.RData")

write.table(
  Uniprot_final_SuRA[, c(1, 5, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19)],
  "Annotated\\Uniprot_final_SuRA.csv",
  sep = ",",
  col.names = NA
)
write.table(
  Uniprot_final_SuTR[, c(1, 2, 4, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19)],
  "Annotated\\Uniprot_final_SuTR.csv",
  sep = ",",
  col.names = NA
)


##Function for GO terms

GOTerm <- function(GOCategory, Uniprotfinal, Location, Sign) {
  GOTerms <- c()
  NACount <- 0
  for (i in 1:nrow(Uniprotfinal)) {
    GOAux <-
      sapply(Uniprotfinal[i, GOCategory], function(x)
```

```
        unlist(strsplit(x, "; ", fixed = TRUE)))
    if (!is.na(GOAux[1]))
      GOTerms <- rbind(GOTerms, GOAux)
    else
      NACount <- NACount + 1
  }
  GOTerms <- as.data.frame(GOTerms)
  colnames(GOTerms) <- "GOTerm"
  GOUnique <- unique(GOTerms)
  GO <-
    sapply(GOUnique[, 1], function(x)
      length(GOTerms[which(GOTerms[, 1] %in% x), ]))
  GO <- GO[order(-GO)]
  GO <- data.frame(GO, GO / nrow(Uniprotfinal) * 100)
  colnames(GO) <- c("NumberORFs", "Percentage")
  save(
    GOTerms,
    GOUnique,
    NACount,
    GO,
    file = paste(
      sep = "",
      "GO terms\\" ,
      GOCategory,
      "_",
      Location,
      "_",
      Sign,
      ".RData"
    )
  )
  write.table(
    GO,
    paste(sep = "",  "GO terms\\" , GOCategory, "_", Location, "_", Sign, ".csv"),
    sep = ";",
    col.names = NA
  )
  return(GO)
}

##Tables with GO terms

col <- c(8, 9)
sign <- c("over", "under")
cat <-
  c("GO_BiologicalProcess",
    "GO_MolecularFUnction",
    "GO_CellularComponent")
loc <- c("SuTR", "SuRA")
```

```
for (i in 1:length(loc)) {
  if (i == 1)
    Uniprotfinal <- Uniprot_final_SuTR
  else
    Uniprotfinal <- Uniprot_final_SuRA
  for (j in 1:length(sign)) {
    if (j == 1)
      Uniprotfinalaux <-
        subset(Uniprotfinal, Uniprotfinal[, col[i]] == 1)
    else
      Uniprotfinalaux <-
        subset(Uniprotfinal, Uniprotfinal[, col[i]] == -1)
    for (k in 1:length(cat))
    {
      GOTerm(cat[k], Uniprotfinalaux, loc[i], sign[j])


    }
  }
}

##Plot GO terms

GOGraph <-
  function(Sign,
           Location,
           GOCategory,
           legendposition) {
    for (i in 1:length(GOCategory)) {
      print(paste(
        sep = "",
        "GO terms\\" ,
        GOCategory,
        "_",
        Location,
        "_",
        Sign,
        ".RData"
      ))
      load(paste(
        sep = "",
        "GO terms\\" ,
        GOCategory[i],
        "_",
        Location,
        "_",
        Sign,
```

```
      ".RData"
    ))

    GOGraphAux <- GOGraphData(GO, GOCategory[i])
    if (i == 1)
      GOGraph <- GOGraphAux
    else
      GOGraph <- rbind(GOGraph, GOGraphAux)
  }

  ggplot(GOGraph, aes(reorder(GOTerm, Numbers), Numbers, fill = group)) +
    geom_col() +
    facet_wrap( ~ group, nrow = 3, scales = "free_y") +
    coord_flip() +
    xlab("GO Terms") +
    ylab("Percentage of ORFs") +
    theme(
      panel.grid = element_blank(),
      panel.background = element_blank(),
      strip.background = element_blank(),
      strip.text = element_blank(),
      axis.text = element_text(colour = "black"),
      legend.position = legendposition,
      legend.title = element_blank(),
      legend.direction = "vertical"
    )
}


##Preparing the data for plotting the Top10 GO Terms

GOGraphData <- function(GO, GOCategory) {
  if (nrow(GO) > 9)
    GOsame <- which(GO$Percentage == GO$Percentage[10])
  else
    GOsame <- nrow(GO)

  GOTop10 <- GO[1:GOsame[length(GOsame)], ]
  GOGraph <-
    data.frame(
      GOTerm = rownames(GO[1:GOsame[length(GOsame)], ]),
      Numbers = GOTop10$Percentage[1:GOsame[length(GOsame)]],
      group = GOCategory
    )
  GOGraph$GOTerm <-
    sapply(GOGraph$GOTerm, function(x)
      unlist(strsplit(x, " [", fixed = TRUE))[1])
  GOGraph$group <-
```

```
    sapply(GOGraph$group, function(x)
      unlist(strsplit(x, "GO_", fixed = TRUE))[2])
  GOGraph$group <-
    factor(gsub("(.)([[:upper:]])", "\\1 \\2", GOGraph$group))
  return(GOGraph)
}

##Top "10" GO Terms

windows()
for (i in 1:length(Condition)) {
  for (j in 1:length(sign)) {
    if (i == 1 && j == 1)
      legendposition <- c(-1, 0.05)
    else
      legendposition <- "none"
    assign(
      paste(Condition[i], sign[j], sep = ""),
      GOGraph(sign[j], Condition[i], cat, legendposition)
    )
  }
}
plot_grid(
  SuRAover,
  SuRAunder,
  SuTRover,
  SuTRunder,
  labels = "AUTO",
  label_fontface = "plain",
  label_size = 12,
  vjust = 2.5,
  hjust = -3.5
)
dev.print(
  tiff,
  "GO terms\\GOTerms.tif",
  height = 40,
  width = 40,
  units = 'cm',
  res = 600
)

##Heatmap

load("DEG\\Data.RData")
cpms <- cpm(Data, normalized.lib.sizes = TRUE, log = T)
Trinity_ID <- as.matrix(Data$genes)
rownames(cpms) <- Trinity_ID
```

```
ID <-
  unlist(sapply(Uniprot_final[, 1], function(x)
    unlist(strsplit(x, ".", fixed = TRUE))[1]))
blastpaux <- cbind(ID, Uniprot_final)
heatNorm <-
  merge(blastpaux, cpms, by.x = "ID", by.y = "row.names")
heatNorm$ProteinNames <-
  c(
    "Zona pellucida sperm-binding protein 1",
    "Lipoprotein lipase(1)",
    "Lipoprotein lipase(2)",
    "NF-AT5 (T-cell transcription factor NFAT5)(1)",
    "NF-AT5 (Rel domain-containing t.f. NFAT5)",
    "NF-AT5 (T-cell transcription factor NFAT5)(2)",
    "Troponin I, fast skeletal muscle",
    "3-hydroxy-3-methylglutaryl-coenzyme A reductase",
    "GTPase IMAP family member 9",
    "Protein lyl-1 (Lymphoblastic leukemia-derived seq 1)",
    "Serine/threonine-protein kinase SIK2",
    "Cytochrome P450 2K1",
    "RNA-binding protein with serine-rich domain 1-B",
    "Hypoxia-inducible factor 1-alpha(1)",
    "Hypoxia-inducible factor 1-alpha(2)",
    "Paralemmin-3",
    "Saxitoxin and tetrodotoxin-binding protein 2",
    "E3 ubiquitin-protein ligase TRIM39",
    "Fumarylacetoacetase",
    "Complement C5 (1)",
    "Complement C5 (2)",
    "Complement C5 (Hemolytic complement)"
  )
heatData <- as.matrix(heatNorm[, 21:32])
rownames(heatData) <- heatNorm[, 33]
clustFunction <- function(x)
  hclust(x, method = "complete")
distFunction <- function(x)
  dist(x, method = "euclidean")
cCol <- colorRampPalette(brewer.pal(9, "Set1"))
colFit <- clustFunction(distFunction(t(heatData)))

colCutoff <- 20

cClusters <- cutree(colFit, h = colCutoff)
cHeight <- length(unique(as.vector(cClusters)))

colHeight = cCol(cHeight)

cRow <- colorRampPalette(brewer.pal(9, "Set1"))
rowFit <- clustFunction(distFunction(heatData))
```

```
rowCutoff <- 21
rClusters <- cutree(rowFit, h = rowCutoff)
rHeight <- length(unique(as.vector(rClusters)))

rowHeight = cRow(rHeight)
plotColGenes <- rowHeight[rClusters]

#Plotting Heatmap

heatColour <-
  colorRampPalette(c("#fff04a", "#f28e2b", "#c23e40"))(n = 100)
windows()
heatmap.2(
  heatData,
  hclust = clustFunction,
  distfun = distFunction,
  ColSideColors = colHeight[cClusters],
  RowSideColors = plotColGenes,
  density.info = "none",
  col = heatColour,
  trace = "none",
  scale = "row",
  cexCol = 1.1,
  cexRow = 0.92,
  lhei = c(0.8, 5),
  lwid = c(1.5, 5.5, 0.5),
  offsetRow = 0,
  offsetCol = 0,
  srtCol = 45,
  key.title = NA,
  margins = c(7, 27),
  labRow = rownames(heatData)
)
dev.print(
  tiff,
  "DEG\\Heatmap.tif",
  height = 30,
  width = 25,
  units = 'cm',
  res = 600
)



##How many ORFs there were in the DEGs

Fa <-
  read.fasta(
```

```
    "\\[PATH]\\TransDecoder\\Trinity.Trinity.fasta.transdecoder.pep",
    as.string = TRUE,
    seqtype = "AA"
  )
Tri_ID <- getName(Fa)
Tri_ID <- as.data.frame(Tri_ID)
Tri_ID$col <-
  unlist(sapply(Tri_ID[, 1], function(x)
    unlist(strsplit(x, ".", fixed = TRUE))[1])) /
  Tri_IDmerged <- merge(degTable, Tri_ID, by.x = 1, by.y = 2)


##Not annotated

Lost_ORFs <- setdiff(Tri_IDmerged[, 12], degAnnotAll[, 12])

##Annotated

Common_ORFs <- merge(Tri_IDmerged, degAnnotAll, by = 12)

##Multiple ORFs

Duped_ORFs <- Common_ORFs[duplicated(Common_ORFs[, 2]), ]
```

A.2.3 Command line - server

```
##FastQC

/[PATH]/FastQC/fastqc          /[PATH]/F14.Sp_TR-N3182_R1.fastq.gz          --outdir
//[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F14.Sp_TR-N3182_R2.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F15.Su_TR-N3186_R1.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F15.Su_TR-N3186_R2.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F16.Su_RA-N3192_R1.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F16.Su_RA-N3192_R2.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F18.Sp_TR-N3183_R1.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F18.Sp_TR-N3183_R2.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F19.Sp_RA-N3188_R1.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F19.Sp_RA-N3188_R2.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F2.Su_TR-N3185_R1.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F2.Su_TR-N3185_R2.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F21.Sp_RA-N3189_R1.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F21.Sp_RA-N3189_R2.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F21.Sp_TR-N3184_R1.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F21.Sp_TR-N3184_R2.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F27.Sp_RA-N3190_R1.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F27.Sp_RA-N3190_R2.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F30.Su_TR-N3187_R1.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F30.Su_TR-N3187_R2.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F38.Su_RA-N3193_R1.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F38.Su_RA-N3193_R2.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
```

```
/[PATH]//FastQC/fastqc          /[PATH]/F4.Su_RA-N3191_R1.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
/[PATH]//FastQC/fastqc          /[PATH]/F4.Su_RA-N3191_R2.fastq.gz          --outdir
/[PATH]/Statistics/FastQC
```

##Trimming

```
trim_galore --paired --illumina --phred33 --output_dir /[PATH]/Trimming --length 20
--stringency 1 -e 0.1 --gzip /[PATH]/F14.Sp_TR-N3182_R1.fastq.gz /[PATH]/F14.Sp_TR-
N3182_R2.fastq.gz
trim_galore --paired --illumina --phred33 --output_dir /[PATH]/Trimming --length 20
--stringency 1 -e 0.1 --gzip /[PATH]/F15.Su_TR-N3186_R1.fastq.gz /[PATH]/F15.Su_TR-
N3186_R2.fastq.gz
trim_galore --paired --illumina --phred33 --output_dir /[PATH]/Trimming --length 20
--stringency 1 -e 0.1 --gzip /[PATH]/F16.Su_RA-N3192_R1.fastq.gz /[PATH]/F16.Su_RA-
N3192_R2.fastq.gz
trim_galore --paired --illumina --phred33 --output_dir /[PATH]/Trimming --length 20
--stringency 1 -e 0.1 --gzip /[PATH]/F18.Sp_TR-N3183_R1.fastq.gz /[PATH]/F18.Sp_TR-
N3183_R2.fastq.gz
trim_galore --paired --illumina --phred33 --output_dir /[PATH]/Trimming --length 20
--stringency 1 -e 0.1 --gzip /[PATH]/F19.Sp_RA-N3188_R1.fastq.gz /[PATH]/F19.Sp_RA-
N3188_R2.fastq.gz
trim_galore --paired --illumina --phred33 --output_dir /[PATH]/Trimming --length 20
--stringency 1 -e 0.1 --gzip /[PATH]/F2.Su_TR-N3185_R1.fastq.gz /[PATH]/F2.Su_TR-
N3185_R2.fastq.gz
trim_galore --paired --illumina --phred33 --output_dir /[PATH]/Trimming --length 20
--stringency 1 -e 0.1 --gzip /[PATH]/F21.Sp_RA-N3189_R1.fastq.gz /[PATH]/F21.Sp_RA-
N3189_R2.fastq.gz
trim_galore --paired --illumina --phred33 --output_dir /[PATH]/Trimming --length 20
--stringency 1 -e 0.1 --gzip /[PATH]/F21.Sp_TR-N3184_R1.fastq.gz /[PATH]/F21.Sp_TR-
N3184_R2.fastq.gz
trim_galore --paired --illumina --phred33 --output_dir /[PATH]/Trimming --length 20
--stringency 1 -e 0.1 --gzip /[PATH]/F27.Sp_RA-N3190_R1.fastq.gz /[PATH]/F27.Sp_RA-
N3190_R2.fastq.gz
trim_galore --paired --illumina --phred33 --output_dir /[PATH]/Trimming --length 20
--stringency 1 -e 0.1 --gzip /[PATH]/F30.Su_TR-N3187_R1.fastq.gz /[PATH]/F30.Su_TR-
N3187_R2.fastq.gz
trim_galore --paired --illumina --phred33 --output_dir /[PATH]/Trimming --length 20
--stringency 1 -e 0.1 --gzip /[PATH]/F38.Su_RA-N3193_R1.fastq.gz /[PATH]/F38.Su_RA-
N3193_R2.fastq.gz
trim_galore --paired --illumina --phred33 --output_dir /[PATH]/Trimming --length 20
--stringency 1 -e 0.1 --gzip /[PATH]/F4.Su_RA-N3191_R1.fastq.gz /[PATH]/F4.Su_RA-
N3191_R2.fastq.gz
```

##Trinity

```
Trinity        --seqType        fq        --left        /[PATH]/Trimming/F14.Sp_TR-
N3182_R1_val_1.fq.gz,/[PATH]/Trimming/F15.Su_TR-
N3186_R1_val_1.fq.gz,/[PATH]/Trimming/F16.Su_RA-
```

```
N3192_R1_val_1.fq.gz,/[PATH]/Trimming/F18.Sp_TR-
N3183_R1_val_1.fq.gz,/[PATH]/Trimming/F19.Sp_RA-
N3188_R1_val_1.fq.gz,/[PATH]/Trimming/F2.Su_TR-
N3185_R1_val_1.fq.gz,/[PATH]/Trimming/F21.Sp_RA-
N3189_R1_val_1.fq.gz,/[PATH]/Trimming/F21.Sp_TR-
N3184_R1_val_1.fq.gz,/[PATH]/Trimming/F27.Sp_RA-
N3190_R1_val_1.fq.gz,/[PATH]/Trimming/F30.Su_TR-
N3187_R1_val_1.fq.gz,/[PATH]/Trimming/F38.Su_RA-
N3193_R1_val_1.fq.gz,/[PATH]/Trimming/F4.Su_RA-N3191_R1_val_1.fq.gz          --right
/[PATH]/Trimming/F14.Sp_TR-N3182_R2_val_2.fq.gz,/[PATH]/Trimming/F15.Su_TR-
N3186_R2_val_2.fq.gz,/[PATH]/Trimming/F16.Su_RA-
N3192_R2_val_2.fq.gz,/[PATH]/Trimming/F18.Sp_TR-
N3183_R2_val_2.fq.gz,/[PATH]/Trimming/F19.Sp_RA-
N3188_R2_val_2.fq.gz,/[PATH]/Trimming/F2.Su_TR-
N3185_R2_val_2.fq.gz,/[PATH]/Trimming/F21.Sp_RA-
N3189_R2_val_2.fq.gz,/[PATH]/Trimming/F21.Sp_TR-
N3184_R2_val_2.fq.gz,/[PATH]/Trimming/F27.Sp_RA-
N3190_R2_val_2.fq.gz,/[PATH]/Trimming/F30.Su_TR-
N3187_R2_val_2.fq.gz,/[PATH]/Trimming/F38.Su_RA-
N3193_R2_val_2.fq.gz,/[PATH]/Trimming/F4.Su_RA-N3191_R2_val_2.fq.gz     --max_memory
100G --CPU 8 --no_salmon --full_cleanup --output /[PATH]/Trinity

##Nx Statistics

/[PATH]//Trinityrnaseq-v2.6.6/util/TrinityStats.pl /[PATH]/Trinity.Trinity.fasta

##ExN50 Statistics

module load kallisto-0.43.0

/[PATH]//Trinityrnaseq-v2.6.6/util/align_and_estimate_abundance.pl     --transcripts
/[PATH]/Trinity.Trinity.fasta   --seqType   fq   --left   /[PATH]/Trimming/F14.Sp_TR-
N3182_R1_val_1.fq.gz   --right   /[PATH]/Trimming/F14.Sp_TR-N3182_R2_val_2.fq.gz   --
est_method    kallisto    --output_dir    /[PATH]/Statistics/Ex90N50/F14.Sp_TR    --
prep_reference
/[PATH]//Trinityrnaseq-v2.6.6/util/align_and_estimate_abundance.pl     --transcripts
/[PATH]/Trinity.Trinity.fasta   --seqType   fq   --left   /[PATH]/Trimming/F15.Su_TR-
N3186_R1_val_1.fq.gz   --right   /[PATH]/Trimming/F15.Su_TR-N3186_R2_val_2.fq.gz   --
est_method    kallisto    --output_dir    /[PATH]/Statistics/Ex90N50/F15.Su_TR    --
prep_reference
/[PATH]//Trinityrnaseq-v2.6.6/util/align_and_estimate_abundance.pl     --transcripts
/[PATH]/Trinity.Trinity.fasta   --seqType   fq   --left   /[PATH]/Trimming/F16.Su_RA-
N3192_R1_val_1.fq.gz   --right   /[PATH]/Trimming/F16.Su_RA-N3192_R2_val_2.fq.gz   --
est_method    kallisto    --output_dir    /[PATH]/Statistics/Ex90N50/F16.Su_RA    --
prep_reference
/[PATH]//Trinityrnaseq-v2.6.6/util/align_and_estimate_abundance.pl     --transcripts
/[PATH]/Trinity.Trinity.fasta   --seqType   fq   --left   /[PATH]/Trimming/F18.Sp_TR-
N3183_R1_val_1.fq.gz   --right   /[PATH]/Trimming/F18.Sp_TR-N3183_R2_val_2.fq.gz   --
```

```
est_method kallisto --output_dir /[PATH]/Statistics/Ex90N50/F18.Sp_TR --
prep_reference
/[PATH]//Trinityrnaseq-v2.6.6/util/align_and_estimate_abundance.pl --transcripts
/[PATH]/Trinity.Trinity.fasta --seqType fq --left /[PATH]/Trimming/F19.Sp_RA-
N3188_R1_val_1.fq.gz --right /[PATH]/Trimming/F19.Sp_RA-N3188_R2_val_2.fq.gz --
est_method kallisto --output_dir /[PATH]/Statistics/Ex90N50/F19.Sp_RA --
prep_reference
/[PATH]//Trinityrnaseq-v2.6.6/util/align_and_estimate_abundance.pl --transcripts
/[PATH]/Trinity.Trinity.fasta --seqType fq --left /[PATH]/Trimming/F2.Su_TR-
N3185_R1_val_1.fq.gz --right /[PATH]/Trimming/F2.Su_TR-N3185_R2_val_2.fq.gz --
est_method kallisto --output_dir /[PATH]/Statistics/Ex90N50/F2.Su_TR --
prep_reference
/[PATH]//Trinityrnaseq-v2.6.6/util/align_and_estimate_abundance.pl --transcripts
/[PATH]/Trinity.Trinity.fasta --seqType fq --left /[PATH]/Trimming/F21.Sp_RA-
N3189_R1_val_1.fq.gz --right /[PATH]/Trimming/F21.Sp_RA-N3189_R2_val_2.fq.gz --
est_method kallisto --output_dir /[PATH]/Statistics/Ex90N50/F21.Sp_RA --
prep_reference
/[PATH]//Trinityrnaseq-v2.6.6/util/align_and_estimate_abundance.pl --transcripts
/[PATH]/Trinity.Trinity.fasta --seqType fq --left /[PATH]/Trimming/F21.Sp_TR-
N3184_R1_val_1.fq.gz --right /[PATH]/Trimming/F21.Sp_TR-N3184_R2_val_2.fq.gz --
est_method kallisto --output_dir /[PATH]/Statistics/Ex90N50/F21.Sp_TR --
prep_reference
/[PATH]//Trinityrnaseq-v2.6.6/util/align_and_estimate_abundance.pl --transcripts
/[PATH]/Trinity.Trinity.fasta --seqType fq --left /[PATH]/Trimming/F27.Sp_RA-
N3190_R1_val_1.fq.gz --right /[PATH]/Trimming/F27.Sp_RA-N3190_R2_val_2.fq.gz --
est_method kallisto --output_dir /[PATH]/Statistics/Ex90N50/F27.Sp_RA --
prep_reference
/[PATH]//Trinityrnaseq-v2.6.6/util/align_and_estimate_abundance.pl --transcripts
/[PATH]/Trinity.Trinity.fasta --seqType fq --left /[PATH]/Trimming/F30.Su_TR-
N3187_R1_val_1.fq.gz --right /[PATH]/Trimming/F30.Su_TR-N3187_R2_val_2.fq.gz --
est_method kallisto --output_dir /[PATH]/Statistics/Ex90N50/F30.Su_TR --
prep_reference
/[PATH]//Trinityrnaseq-v2.6.6/util/align_and_estimate_abundance.pl --transcripts
/[PATH]/Trinity.Trinity.fasta --seqType fq --left /[PATH]/Trimming/F38.Su_RA-
N3193_R1_val_1.fq.gz --right /[PATH]/Trimming/F38.Su_RA-N3193_R2_val_2.fq.gz --
est_method kallisto --output_dir /[PATH]/Statistics/Ex90N50/F38.Su_RA --
prep_reference
/[PATH]//Trinityrnaseq-v2.6.6/util/align_and_estimate_abundance.pl --transcripts
/[PATH]/Trinity.Trinity.fasta --seqType fq --left /[PATH]/Trimming/F4.Su_RA-
N3191_R1_val_1.fq.gz --right /[PATH]/Trimming/F4.Su_RA-N3191_R2_val_2.fq.gz --
est_method kallisto --output_dir /[PATH]/Statistics/Ex90N50/F4.Su_RA --
prep_reference
/[PATH]//Trinityrnaseq-v2.6.6/util/abundance_estimates_to_matrix.pl --est_method
kallisto --gene_trans_map none --out_prefix kallisto --name_sample_by_basedir
/[PATH]/Statistics/Ex90N50/F14.Sp_TR/abundance.tsv
/[PATH]/Statistics/Ex90N50/F15.Su_TR/abundance.tsv
/[PATH]/Statistics/Ex90N50/F16.Su_RA/abundance.tsv
/[PATH]/Statistics/Ex90N50/F18.Sp_TR/abundance.tsv
/[PATH]/Statistics/Ex90N50/F19.Sp_RA/abundance.tsv
```

```
/[PATH]/Statistics/Ex90N50/F2.Su_TR/abundance.tsv
/[PATH]/Statistics/Ex90N50/F21.Sp_RA/abundance.tsv
/[PATH]/Statistics/Ex90N50/F21.Sp_TR/abundance.tsv
/[PATH]/Statistics/Ex90N50/F27.Sp_RA/abundance.tsv
/[PATH]/Statistics/Ex90N50/F30.Su_TR/abundance.tsv
/[PATH]/Statistics/Ex90N50/F38.Su_RA/abundance.tsv
/[PATH]/Statistics/Ex90N50/F4.Su_RA/abundance.tsv


echo "Kallisto done"

/[PATH]//Trinityrnaseq-v2.6.6/util/misc/contig_ExN50_statistic.pl
kallisto.isoform.TMM.EXPR.matrix /[PATH]/Trinity.Trinity.fasta > ExN50.stats
/[PATH]//Trinityrnaseq-v2.6.6/util/misc/plot_ExN50_statistic.Rscript ExN50.stats

##TransDecoder

/[PATH]//TransDecoder/TransDecoder.LongOrfs -t /[PATH]/Trinity.Trinity.fasta
/[PATH]//TransDecoder/TransDecoder.Predict -t /[PATH]/Trinity.Trinity.fasta

##Blastp

/[PATH]//ncbi-blast-2.13.0+/bin/makeblastdb                                    -in
/[PATH]//databases/SwissProtRelease202202/uniprot_reviewed.fasta               -out
/[PATH]//databases/SwissProtRelease202202/uniprot_reviewed -dbtype prot
/[PATH]//ncbi-blast-2.13.0+/bin/blastp                                      -query
/[PATH]/TransDecoder_Output/Trinity.Trinity.fasta.transdecoder.pep             -db
/[PATH]//databases/SwissProtRelease202202/uniprot_reviewed  -max_target_seqs  1  -
max_hsps 1 -outfmt '10 delim=; qseqid sseqid pident length mismatch gapopen qstart
qend sstart send evalue bitscore sacc qcovs stitle' -evalue 1e-5 -num_threads 20 >
/[PATH]/Blastp/ExtremeOceans_Trim_Blastp_SwissProt.csv



##Pfam

/[PATH]//hmmer/bin/hmmpress /[PATH]//databases/pfam/Pfam-A.hmm

/[PATH]//hmmer/bin/hmmscan --cpu 20 --seed 1 --domtblout ExtremeOceans_pfam.domtblout
--tblout        ExtremeOceans_pfam.tblout        /[PATH]//databases/pfam/Pfam-A.hmm
/[PATH]/TransDecoder_Output/Trinity.Trinity.fasta.transdecoder.pep
```

A.2.3 R Script for Histopathological analysis

```
##Weighted histopathological condition indices##

##Functions
hIndice<-function(histoTable, histoWeights, histoRP, nRP){
 nTraits<-length(histoWeights)
 messageText<-paste0(
  "Estimating global histopathological condition indice (Ih)\n",
  "(",  nTraits, " traits )"
 )
 printMessage(messageText)
 maxScore<-0
 tableScore<-NULL
 traitNames<-colnames(histoTable)
 for(i in 1:nTraits){
  assign(
   as.character(colnames(histoTable)[i]),
   histoTable[,i]*histoWeights[i]
  )
  maxScore<-maxScore+6*histoWeights[i]
  tableScore<-as.data.frame(
   cbind(
    tableScore, get(as.character(colnames(histoTable)[i]))
   )
  )
 }
 hIndice<-(apply(tableScore, 1, sum))/maxScore
 tableScore<-cbind(tableScore, hIndice)
 colnames(tableScore)<-c(paste0("Ij(", traitNames, ")"), "Ih")
 printMessage("Done.")
  if(nRP > 1){
  rpNames<-levels(histoRP)
  nCasesInitial<-summary(histoRP)[1]
  messageText<-paste0(
   "Estimating partial indice for reaction pattern: ",
   rpNames[1], "\n",
   "(",  nCasesInitial, " traits)"
  )
  printMessage(messageText)
  maxScore<-6*sum(histoWeights[1:nCasesInitial])
  rpScore<-(apply(tableScore[,1:nCasesInitial], 1, sum))/maxScore
  tableScore<-cbind(tableScore, rpScore)
  colnames(tableScore)[ncol(tableScore)]<-paste0("Ih(", rpNames[1], ")")
  printMessage("Done.")
  for(i in 2:nRP){
   messageText<-paste0(
    "Estimating partial indice for reaction pattern: ",
    rpNames[i], "\n",
```

```
    "(", summary(histoRP)[i], " traits)"
   )
   printMessage(messageText)
   nCases<-summary(histoRP)[i]
   startPos<-nCasesInitial+1
   stopPos<-nCasesInitial+nCases
   maxScore<-6*sum(histoWeights[startPos:stopPos])
   if(nCases>1){
    rpScore<-(apply(tableScore[,startPos:stopPos], 1, sum))/maxScore
   } else {
   rpScore<-tableScore[,startPos]/maxScore
   }

   nCasesInitial<-stopPos
   tableScore<-cbind(tableScore, rpScore)
   colnames(tableScore)[ncol(tableScore)]<-paste0("Ih(", rpNames[i], ")")
   printMessage("Done.")
  }
 }
 return(tableScore)
}

loadData<-function(fileName){
 printMessage(
  paste(sep = " ",
   "Reading data from file:",
   paste(sep="/", getwd(), fileName)
  )
 )
 tryCatch(
  {
   histoData<-read.table(
    fileName,
    sep=",",
    header=FALSE
   )
   return(histoData)
   printMessage("Data read successfully...")
  },
  warning = function(w){
   printMessage("Problem found reading file, check data...")
  },
  error = function(e){
   printMessage("Error reading file. File/folder not found or corrupt.")
  }
 )
}

saveResults<-function(finalTable, fileName){
```

```
 printMessage(
  paste(sep = " ",
   "Writing data to file:",
   paste(sep="/", getwd(), fileName)
  )
 )
 tryCatch(
  {
   write.table(
    finalTable,
    fileName,
    sep=";",
    col.names=NA
   )
   printMessage("Results saved successfully...")
  },
  warning = function(w){
   printMessage("Problem found writing file, check data...")
  },
  error = function(e){
   printMessage("Error writing to file. Folder not found or file open elsewhere.")
  }
 )
}

printMessage<-function(messageText){
 cat(messageText, "\n")
}

#Data input
fileName<-"HistoData.csv"
histoData<-loadData(fileName)
histoRP<-as.factor(
 histoData[1,2:ncol(histoData)]
)
nRP<-nlevels(histoRP)
histoTraits<-histoData[2,2:ncol(histoData)]
histoWeights<-as.numeric(histoData[3,2:ncol(histoData)])
histoTable<-(histoData[4:nrow(histoData), 2:ncol(histoData)])
histoTable<-matrix(
 unlist(
  lapply(histoTable, as.numeric)
 ),
 nrow(histoTable)
)
colnames(histoTable)<-histoTraits
rownames(histoTable)<-histoData[4:nrow(histoData),1]
printMessage(
 paste(
```

```
  sep=" ",
  "Number of cases:", nrow(histoTable), "|",
  "Total number of traits:", ncol(histoTable), "|",
  "Reaction Patterns:", nRP
 )
)

#Hindice estimation
finalTable<-hIndice(histoTable, histoWeights, histoRP, nRP)
finalTable<-cbind(
 finalTable[, -c((ncol(finalTable)-nRP):ncol(finalTable))],
 finalTable[, c((ncol(finalTable)-nRP+1):ncol(finalTable), ncol(finalTable)-nRP)]
)

##Result output
zapsmall(finalTable, 3)
View(finalTable)
summary(finalTable)
saveResults(finalTable, "HistoResults.csv")
printMessage("Analysis complete.")

##END
```

A.3 List of thesis outputs

Participation in workshops:

● **Nunes M** (2021). Epigenomics Analysis Workshop (advanced training course), 13th-15th September 2021, Canadian Bioinformatics Workshops, online.

Oral communications in international congresses:

● **Nunes, M.** (presenter); Moutinho Cabral, I.; Gonçalves, C.; Fernandes, J.F.; Missionário, M.; Travesso, M.; Grosso, A.R.; Madeira, D.; Costa, P.M.; Madeira, C. (2022) Goby fish populations in intertidal environments: gene networks and epigenetic regulators modulating energy metabolism in response to seasonal warming and local climate regimes. ICYMARE - International Conference for Young Marine Researchers, 13th-16th September, Bremerhaven, Germany.

Poster presentations in international congresses:

● **Nunes, M** (presenter).; Missionário, M.; Guerreiro, B.; Gonçalves, C.; Travesso, M.; Fernandes, J.F.; Madeira, D.; Mendonça, V.; Vinagre, C.; Costa, P.M.; Madeira, C. (2022) Tissue level effects of season warming and thermal gradient in common gobies: structural and biochemical changes assessed by histopathological screening. ICYMARE - International Conference for Young Marine Researchers, 13-16th September, Bremerhaven, Germany.

● Missionário, M.; António, C.; Rodrigues, A.M.; Madeira, D.; **Nunes, M.;** Fernandes, J.F.; Travesso, M.; Madeira, D.; Mendonça, V. Vinagre, C.; Costa, P.M.; Madeira, C (presenter). (2022). Plasticity in fluctuating environments: metabolic networks modulated by season and latitude in an intertidal fish. EMBO - EMBL Symposium: Plasticity across scales - from molecules to phenotypes, 26th-29th October, Heidelberg, Germany.

● **Nunes, M.** (presenter); Moutinho Cabral, Inês; Gonçalves, C.; Missionário, M.; Fernandes, J.F.; Travesso, M.; Madeira, Diana; Costa, Pedro M.; Madeira, Carolina. (2022). Transcriptome profiling of the common goby *Pomatoschistus microps* exposed to seasonal warming reveals gene downregulation as an energy-saving mechanism to cope with thermal stress". 1st Egas Moniz One Health Symposium - Teaming up for a better Public Health, 3th November, Caparica, Portugal.

Oral communications in student and lab meetings:

● **Nunes, M.** (presenter); Costa, P.M.; Madeira, C. (2021). Gene networks and epigenetic regulators modulating heat adaptation in sand gobies: influence of seasonal environment and climate extremes in thermal tolerance. SeaTox Lab Meetings, 30 de setembro, Faculdade de Ciências e Tecnologia da Universidade NOVA de Lisboa, Caparica, Portugal.

● **Nunes, M.** (presenter); Costa, P.M.; Madeira, C. (2022). Gene networks and epigenetic regulators modulating heat adaptation in sand gobies: influence of seasonal environment and climate extremes in thermal tolerance. Jornadas Intercalares dos Mestrados (JIM) do DQ e do DCV, 10 e 11 fevereiro. Faculdade de Ciências e Tecnologia da Universidade NOVA de Lisboa, Caparica, Portugal.