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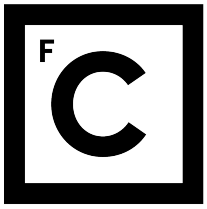

Ciências
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Responses of congeneric freshwater fish, *Squalius carolitertii* and *Squalius torgalensis*, to future climate change

A molecular and physiological approach.

Doutoramento em Biologia
Especialidade em Biologia evolutiva

Tiago Filipe Salgueiro de Jesus

Tese orientada por:
Professora Doutora Maria Manuela Gomes Coelho Noronha Trancoso
Professora Doutora Vera Maria Fonseca de Almeida e Val

Documento especialmente elaborado para a obtenção do grau de doutor

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Júri

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Nota prévia

A presente tese apresenta resultados de trabalhos já publicados ou em preparação para publicação (capítulos 2 e 3), de acordo com o previsto no nº 2 do artigo 25º do regulamento de Estudos Pós-graduados da Universidade de Lisboa, publicado no Diário de República II série nº 57 de 23 de Março de 2015. Tendo os trabalhos sido realizados em colaboração, o candidato esclarece que participou integralmente na conceção dos trabalhos, obtenção dos dados, análise e discussão dos resultados, bem como na redação dos manuscritos.

Lisboa, Maio de 2017

Tiago Filipe Salgueiro de Jesus

Abstract

Climate changes are exposing freshwater fish to higher water temperatures and acidification. Once studies evaluating freshwater fish responses to these challenges are scarce, the main objective of this thesis is to comprehend how two Iberian freshwater fish species cope with future climate change. *Squalius carolitertii* and *Squalius torgalensis*, which are endemic of two distinct regions of the Iberian Peninsula, live in different environmental conditions. Herein, their thermal stress responses were firstly accessed by the expression of two genes involved in the heat shock response (HSR) (*hsp70* and *hsc70*). Afterwards, we conducted a transcriptome-wide study of fish exposed to acute thermal stress. Results suggest that *S. torgalensis* handled with stressing thermal conditions differently than *S. carolitertii*. While *S. torgalensis* redirects resources from cell division and growth processes to the HSR, the induction of genes involved in the HSR was lower in *S. carolitertii*, which presented no re-adjustment of other energy consumption mechanisms. The long-term responses on gene expression and physiology of these two species to future warming (plus 3 °C) and acidification (ΔpH=-0.4) were evaluated herein, alongside with protein modeling of fourteen target genes. Findings suggest that *S. torgalensis* is better suited to cope with the projected climate change conditions, once it presents fewer changes in gene expression and in the physiological markers involved in the HSR and energy metabolism than *S. carolitertii*. Also, the HSP90 and GBP1 proteins of *S. torgalensis* have higher thermostability, suggesting that they function in a wider range of temperatures. Instead, *S. carolitertii* presents many changes in gene expression, including in genes involved in the thermal stress response as well as in energy metabolism, and a decrease in the aerobic metabolism coupled with an increase in the anaerobic metabolism. Remarkably, the projected climatic conditions elicit severe changes in the circadian (*cry1aa* and *per1a*) and immune (*gpb1*) related genes, as well as an increase in HSP70 protein content, which may hinder the survival

of both species. This work provide the first assessment of the ability of Iberian freshwater fish to deal with future climate change and shall be considered for conservation actions, particularly for the critically endangered *S. torgalensis*.

Keywords: acidification, climate change, freshwater fish, gene expression, protein modeling, thermal stress, warming

Resumo

As alterações climáticas estão a criar novos desafios, tanto em sistemas antropogénicos, como também nos ecossistemas naturais. Embora, no passado, tenham existido períodos em que o clima da Terra sofreu alterações profundas, nunca, como agora, essas alterações tinham sido tão fortemente influenciadas pelas actividades de uma só espécie. As actividades antropogénicas têm promovido um aumento da concentração atmosférica de CO_2 e de gases com efeito estufa, o que produz efeitos à escala global, dos quais o aquecimento global da temperatura do ar é o mais evidente. O Painel Intergovernamental sobre Alterações Climáticas (IPCC) prevê um aquecimento global da temperatura do ar entre os 0.3 °C e os 4.8 °C e um aumento das emissões de CO_2 entre 140 a 1910 giga toneladas de carbono até ao final deste século, com consequentes efeitos nefastos nos ecossistemas aquáticos. Embora grande parte da investigação feita até hoje se tenha concentrado nos efeitos das alterações climáticas em ecossistemas marinhos, os ecossistemas de água doce também estão sujeitos às mesmas pressões, que podem levar ao aquecimento e acidificação das águas. Até à data, existem poucos estudos sobre os efeitos das alterações climáticas em peixes de água doce. Contudo, alguns estudos publicados abordam os efeitos de alguns factores ambientais relacionados com as alterações climáticas em diversas espécies, incluindo algumas espécies de peixes de água doce.

O género *Squalius* é um grupo de peixes de água doce pertencente à família Cyprinidae e encontra-se representado na Península Ibérica por um grande número de endemismos. No território português existem 4 espécies de *Squalius* e um complexo alopoliploide de origem híbrida, todos endémicas da Península Ibérica. As 4 primeiras espécies de origem não híbrida encontram-se distribuídas em alopatria pelas bacias de Portugal e, por isso, sujeitas a diferentes pressões ambientais. *S. carolitertii* é uma espécie que vive na região norte de Portugal (a norte do rio Tejo), onde as condições ambientais apresentam menores variações de temperatura, comparativamente com outras regiões no

Sul da Península Ibérica. Por sua vez, *S. torgalensis* habita na bacia do rio Mira, que possui uma marcada alternância entre períodos de cheia e de seca. Durante a estação seca, os indivíduos desta espécie podem ficar sujeitos a condições bastante severas, nomeadamente da temperatura da água e do seu teor de oxigénio. Neste contexto, o principal objectivo desta tese é compreender de que forma estas duas espécies (*S. carolitertii* e *S. torgalensis*), vivendo em condições tão distintas, irão lidar com as alterações climáticas projectadas para o final deste século.

Para tal, estudaram-se os efeitos que as alterações de temperatura terão em ambas as espécies através de experiências de choque térmico, nas quais se expuseram peixes de ambas as espécies a alterações de temperaturas por um curto período de tempo. Através de uma abordagem de genes candidatos, na qual se analisaram as diferenças de expressão dos genes *hsp70* e *hsc70* ao aumento de temperatura, foi verificado que *S. carolitertii* e *S. torgalensis* apresentavam diferentes respostas. *S. carolitertii* não apresentou diferenças de expressão significativas, para ambos os genes, com o aumento de temperatura e alguns indivíduos não conseguiram sobreviver a 35 °C. Por sua vez, *S. torgalensis* induziu significativamente a expressão dos dois genes, quando exposto a 35 °C, tendo sobrevivido a todas as condições de teste. Dadas as diferentes respostas das duas espécies às condições de choque térmico, realizou-se, de seguida, uma análise comparativa do transcriptoma de ambas as espécies a duas temperaturas diferentes (18 °C e 30 °C). Contudo, ao passo que no primeiro desenho experimental a temperatura foi aumentada 1 °C por dia, nesta segunda experiência de choque térmico a temperatura foi aumentada 1 °C por hora. Nesta segunda experiência de choque térmico, observaram-se incrementos de expressão em genes envolvidos no *folding* de proteínas (por exemplo, *hsp70*, *hsp90* e *hsp40*) em ambas as espécies, contudo mais elevados para *S. torgalensis*. Para além disso, *S. carolitertii* apresentou um maior número de genes com aumento de expressão, maioritariamente enriquecidos em funções de regulação da transcrição. Por sua vez, *S. torgalensis* apresentou um maior número de genes, enriquecidos em funções de crescimento e divisão celular, com expressão significativamente diminuída. Estes resultados sugerem que nestas condições *S. carolitertii* tenta regular o seu metabolismo através do

aumento da expressão de genes envolvidos na regulação da expressão génica (factores de transcrição). Por outro lado, *S. torgalensis* apresenta uma estratégia diferente, na qual redireciona recursos do crescimento geral das células para os mecanismos de resposta ao *stress*. Deste último estudo foi também possível obter um painel de genes potencialmente úteis para o estudo de alterações de temperatura, particularmente para as espécies de ciprinídeos Ibéricos.

Posteriormente, com o objectivo de estudar os efeitos que o aquecimento e acidificação da água, provocados pelas alterações climáticas, tinham na expressão génica de *S. carolitertii* e *S. torgalensis*, indivíduos de ambas as espécies foram expostos, durante um mês, a um aumento de temperatura de 3 °C e a um ΔpH=-0.4 em relação à actual média de Verão destes parâmetros nos seus *habitats* naturais. Nestas condições experimentais a expressão de catorze genes (escolhidos com base no estudo comparativo dos transcriptomas), relacionados com o *folding* de proteínas, o metabolismo energético, o ritmo circadiano e a resposta imunitária, foi quantificada e comparada com a expressão desses mesmos genes na condição controlo. Relativamente aos genes envolvidos no *folding* de proteínas, *S. carolitertii* foi a espécie que apresentou mais alterações de expressão com diferenças significativas em 4 genes (*hsp90aa1*, *hsc70*, *fkbp4* e *stip1*). *S. torgalensis* apenas apresentou diferenças significativas na expressão do gene *stip1*. Demonstrou, ainda, uma maior capacidade do que *S. carolitertii* para produção de energia em hipercapnia, através de um aumento de expressão do gene *cs* e manutenção dos níveis de expressão do gene *ldha*. Estes resultados sugerem que *S. torgalensis* tem uma maior tolerância térmica, o que lhe permitiu aclimatar às condições ambientais simuladas experimentalmente, ou estas condições ambientais podem não ter sido suficientemente severas para que esta espécie apresentasse uma resposta de stress, na medida em que poderá já estar adaptada a condições semelhantes no seu habitat natural. Por outro lado, *S. carolitertii* apresenta diferenças significativas na expressão de 12 dos 14 genes estudados e uma resposta típica de stress, que poderá inviabilizar o futuro desta espécie a longo prazo. Não obstante, as alterações de expressão observadas nos genes envolvidos no ritmo circadiano (*cry1aa* e *per1a*) e resposta imunitária (*gbp1*) podem comprometer a persistência de ambas as espécies.

Para além da expressão génica, foi ainda averiguada a existência de diferenças estruturais entre as proteínas resultantes da tradução destes catorze genes, utilizando modelação de proteínas *in silico*. Cinco das proteínas modeladas apresentaram diferenças em parâmetros físico-químicos ou estruturais entre as duas espécies. Foram observadas diferenças estruturais nas proteínas de *folding* HSC70 e FKBP52, bem como nas proteínas HIF1α e GPB1, que embora estejam localizados em zonas de *coil* podem ter funções relevantes para a estabilidade das mesmas. Foi, também, encontrada uma maior estabilidade térmica para as proteínas HSP90 e GPB1 para a espécie *S. torgalensis*, o que poderá constituir uma vantagem em ambientes com temperaturas mais elevadas. Estas alterações estruturais e funcionais nestas proteínas poderão ter impacto na expressão génica, na medida em que *S. torgalensis* poderá ter proteínas mais eficientes, o que faz com que não necessite de aumentar expressão dos genes que as codificam.

Foram, ainda, efectuadas análises fisiológicas, com marcadores de stress (térmico e oxidativo) e de metabolismo energético, nos indivíduos experimentalmente expostos ao aumento de temperatura e diminuição de pH, durante um mês. Os resultados demonstram que o aquecimento (em normocapnia) promoveu um aumento da actividade do enzima lactato desidrogenase (LDH) em *S. carolitertii* e uma diminuição em *S. torgalensis*. Por sua vez, a actividade do enzima citrato sintase (CS) sofreu uma diminuição significativa em hipercapnia para *S. carolitertii*, enquanto *S. torgalensis* não apresentou diferenças significativas. No que refere à actividade de enzimas de *stress* oxidativo, não foram observadas diferenças relevantes para ambas as espécies. Contudo, *S. carolitertii* e *S. torgalensis* apresentaram um aumento da quantidade de proteínas de choque térmico (HSP70), no cenário sinergístico, e em hipercapnia, respetivamente. Estes resultados sugerem que *S. carolitertii* poderá ser mais vulnerável do que *S. torgalensis* às alterações climáticas simuladas neste estudo, dado que apresentou reduzida capacidade de produção energética (diminuição da actividade da CS) e um maior aumento da quantidade de HSP70.

Embora *S. torgalensis* pareça melhor adaptado para lidar com futuras alterações climáticas, esta é também uma espécie que, possivelmente, se encontra mais perto do seu limite de tolerância térmica, especialmente durante a estação seca. Para além disso, *S. torgalensis* vive num ambiente mais instável e cujos efeitos das alterações climáticas podem ser ainda mais severos. Neste sentido, as medidas de conservação para esta espécie criticamente ameaçada, deverão contemplar a manutenção dos seus refúgios estivais.

Palavras Chave: acidificação, alterações climáticas, aquecimento, expressão génica, modelação de proteínas, peixes de água doce, *stress* térmico

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Surge a dor,
surge a vontade
de fazer com amor
e habilidade.

A vontade aperta
o desespero aumenta
até que cedemos
e abrimos as comportas do nosso ser ao mundo.

Começa a fluir,
a cair, a cair, a cair
por vezes batendo
outras mergulhando tão fundo.

Tão fundo e tão leve,
com a doçura de quem te teve.
Te teve e não te voltará a ter mais,
até que uma nova vaga bata no cais.

Eu, 1 de Julho de 2012

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List of Abbreviations

AR5 - Fifth Assessment Report of the Intergovernmental Panel on Climate Change.
cDNA - Complementary DNA.
contigs - Set of overlapping DNA fragments that together represent a consensus region of DNA.
CAT - Catalase.
CS - Citrate synthase.
DNA - Deoxyribonucleic acid.
g - Grams.
GO - Gene ontology.
GST - Glutathione S-transferase.
HSP - Heat shock protein.
HSR - Heat shock response.
indel - Insertion or the deletion of bases in the DNA of an organism.
IPCC - Intergovernmental Panel on Climate Change.
L - Liter.
LDH - Lactate dehydrogenase.
log - Logarithm.
m - Milli-.
M - Molar.
MDA - Malondialdehyde.
min - Minutes.
mRNA - Messenger RNA.
PCR - Polymerase chain reaction.
sec - Seconds.
RNA - Ribonucleic acid.

RPC - Intergovernmental Panel on Climate Change's Representative Concentration Pathways.
ROS - Reactive oxygen species.
RT - Reverse transcriptase.
SD - Standard deviation.
SOD - Superoxide dismutase.
SRES - Intergovernmental Panel on Climate Change's Special Report on Emissions Scenarios.
Transcriptome - set of all messenger RNA expressed from the genes of a particular organism, organ or cell.
μ - Micro-.

Chapter 1

Introduction

1.1 Climate change

Climate change is undoubtedly threatening both human and natural systems, across all continents and oceans. Whether present climate change is human driven or not is still controversial, however there is little doubt that human activity is boosting climate change, with increasing emissions of CO_2 and green house gases to the atmosphere (as stated in the Fifth Assessment Report [AR5] of the Intergovernmental Panel on Climate Change [IPCC]) (Field *et al.*, 2014). Both human and natural systems are vulnerable to extreme climate events (e.g. heat waves, droughts, floods), which affect food production, ecosystem dynamics and water supply, damage infrastructures, cause morbidity and mortality, and has consequences for human health and well-being (Smith and Guégan, 2010; Füssel *et al.*, 2012a; Field *et al.*, 2014).

Past natural global climate changes have led to the extinction of many species, however they presented a slower pace than current climate change (Field *et al.*, 2014). These fast paced changes may hamper the ability of species to develop adaptation strategies to deal with climate change, through migration or by adjusting to the new local conditions, thus increasing the risk of extinction. Even though few species extinctions have been attributed to the current climate change, many terrestrial, freshwater and marine species have already shifted their distribution ranges, life-cycle (including mating, migration and other seasonal activities), abundance and interactions with other species (Field *et al.*, 2014).

1.1.1 Climate projections

The IPCC has been making projections of future climate change since the first Final Assessment Report in 1990, and afterwards, different scenarios were suggested by IPCC's future climatic projections. These scenarios are a set of predictions of the trend in several key environmental variables into the future, such as temperature and atmospheric CO_2. In the Third Assessment Report (2001), the IPCC Special Report on Emissions Scenarios (SRES) created a set of scenarios (e.g. A1B, A1T, A1FI, A2, B1, B2) (Houghton JT *et al.*, 2001; Field *et al.*, 2014). These scenarios were used in subsequent reports up to the Forth Assessment Report (2007). In 2014, however, new scenarios were created for the Fifth Assessment Report, the so called Representative Concentration Pathways (RPCs)

(Moss *et al.*, 2010; van Vuuren *et al.*, 2011; Field *et al.*, 2014). In RPCs climate mitigation procedures are also included in the projected models. RPCs are also supplemented with Extended Concentrations Pathways (ECPs), which extends climate modeling until the year 2300. RPCs are named accordingly with their approximate radiative forcing (i.e. the influence of a factor in changing the ratio of incoming and outgoing energy from Earth) that will reached by the end of the 21^{st} century (RPC2.6; RPC4.5; RCP6.0; RPC8.5) (Moss *et al.*, 2010; van Vuuren *et al.*, 2011; Field *et al.*, 2014).

These scenarios predict an increase in global mean air temperature from 0.3 °C to 4.8 °C and an increase of cumulative CO_2 emissions ranging from 140 to 1910 Gigatones of Carbon (GtC) for the 2012 to 2100 period (Table 1.1) (IPCC, 2013). Besides temperature and CO_2 emissions projections, these scenarios also project future levels of precipitation, air quality, ocean warming and acidification, sea level and cryosphere (i.e. the portions of Earth surface that is covered by water in solid state) (IPCC, 2013).

Table 1.1: Projected global mean, maximum and minimum surface temperature change (°C) and cumulative CO_2 emissions (GtC) over the 21^{st} century for the Representative Concentration Pathway (RCP) scenarios (Field *et al.*, 2014).

	Global mean air temperature change (°C)		Cumulative CO_2 Emissions (GtC)	
Scenario	Mean	Range	Mean	Range
RCP2.6	1.0	0.3 - 1.7	270	140 - 410
RPC4.5	1.8	1.1 - 2.6	780	595 - 1005
RPC6.0	2.2	1.4 - 3.1	1060	840 - 1250
RPC8.5	3.7	2.6 - 4.8	1685	1415 - 1910

1.1.2 Freshwater ecosystems

Freshwater ecosystems are deeply linked with terrestrial ecosystems. They strongly rely on the surrounding environment, which makes them particularly vulnerable to climate change. In fact, climate change is projected to have major impacts on terrestrial and aquatic ecosystems, including marine and freshwater biomes, particularly for the high-warming scenarios (RPC6.0 and RPC8.5). Regarding freshwater basins, rising water

temperatures, as a result of global air temperature increase, along with changes in precipitation, are changing river regimes and disrupting the dynamics between floods and droughts. These alterations are even worse due to the increasing occurrence of extreme events, such as heat waves and atypical rainfall (Füssel *et al.*, 2012a; Hansen *et al.*, 2012; Field *et al.*, 2014; Mantyka-Pringle *et al.*, 2014).

Recently, major focus has been given to ocean acidification, however freshwater ecosystems are also likely to suffer from this phenomenon. Both in freshwater and seawater environments, CO_2 reacts with water (H_2O) leading to the formation of carbonic acid (H_2CO_3), which dissociates into hydrogen (H^+) and bicarbonate (HCO_3^-). This addition of H^+ ions into the water decreases its pH, and represents the main cause of acidification in seawater (Feely *et al.*, 2004; Leduc *et al.*, 2013). However, in freshwater ecosystems, the main cause of water acidification is acid rains rather than atmospheric CO_2 (Leduc *et al.*, 2013; Lake *et al.*, 2000). Acid rainfall is caused by emissions of sulfur dioxides and nitrogen oxides to the atmosphere (Leduc *et al.*, 2013) and results in a decrease of water pH as well as of the buffering capacity of surrounding soils (Lake *et al.*, 2000). Altogether, these effects contribute to the further increase of the acidification of lakes and rivers (Leduc *et al.*, 2013; Field *et al.*, 2014). The increase in temperature and CO_2 concentration in water, also reduces O_2 solubility in water, which may result in hypoxic conditions for freshwater biota (Hamilton *et al.*, 1995; Beckett *et al.*, 1988).

Additionally, freshwater species may face other threats such as habitat fragmentation (e.g. dams), pollution, over-exploitation, alien species competition or predation, and exposure to new pathogens, all of which can be boosted by climate change (Bellard *et al.*, 2012; Mantyka-Pringle *et al.*, 2014).

1.1.2.1 Freshwater fish

Many studies have focused on the short term effects of temperature, pH and O_2 depletion in the physiology of freshwater fish [e.g Saint-Paul (1984); Almeida-Val *et al.* (2000); Oliveira *et al.* (2008); Almeida-Val *et al.* (2011); Eliason *et al.* (2011); Campos *et al.* (2016); Scott *et al.* (2016)]. The findings resulting from such studies are highly valuable and have greatly enhanced the knowledge of how species may respond to these climate change stressors.

Nonetheless, few studies have addressed the impacts of climate change projections on freshwater fish, i.e., the actual long-term effects of climate change stressors on fish physiology. In this sense, though many studies have exposed marine fish to climate change projections of temperature, pH and O_2 concentration (e.g. (Munday *et al.*, 2009; Bignami *et al.*, 2013; Pimentel *et al.*, 2015)), there is still a lack of knowledge on the long-term responses of freshwater fish to future climate change projections on these environmental variables.

At the beginning of this thesis, there were no studies that addressed the effects of a climate change stressors under IPCC projected scenarios on freshwater fish, however, currently, there are a few examples. Impacts on freshwater fish physiology, behavior and gene expression have been observed for a few species [e.g. *Colossoma macropomum* (G. Cuvier, 1818), *Melanotaenia duboulayi* (Castelnau, 1878) and the anadromous *Oncorhynchus gorbuscha* (Walbaum, 1792)] (Ou *et al.*, 2015; de Oliveira and Val, 2016; Mccairns *et al.*, 2016; Prado-Lima and Val, 2016).

Results may differ among species and have shown that, while extreme conditions may compromise or complicate the survival of some species, others might endure or even thrive with the changing conditions. While *Colossoma macropomum* and *Melanotaenia duboulayi* presented changes that may enable them to endure the climate change conditions simulated (de Oliveira and Val, 2016; Mccairns *et al.*, 2016), *Oncorhynchus gorbuscha* may be at great risk without mitigation measures (Ou *et al.*, 2015).

Therefore, species responses to climate change stressors neither are always clear nor are easily predicted, since the response of each species greatly depends on its environmental context.

Noteworthy, freshwater fish are ectotherms and thus their metabolism strongly depends on environmental temperature, which renders them highly susceptible to global warming (Somero, 2011). However, the knowledge on how this warming will affect their life cycles, distribution ranges or even their survival as a species is unclear. While some species are used to highly variable or extreme conditions, others live in less variable conditions (Somero, 2010, 2011). Moreover, whether eurythermic or stenothermic are more vulnerable to climate change is also not clear, since species that deal with harsher conditions often live closer to their thermal tolerance limits (Somero, 2011; Gunderson *et al.*, 2015).

1.1.3 Stress responses

Stress is an environmental or genetic factor that causes a change in a biological system, which has some consequences on the organisms' fitness (Morris *et al.*, 2013). Organisms are naturally exposed to a set of environmental stressful conditions that may pose considerable challenges to their physiology, behavior and ultimately survival (López-Maury *et al.*, 2008). To deal with stressful conditions natural populations can move to more suitable habitats, overcome them by phenotypic plasticity, or undergo evolutionary adaptation (acclimatization). Otherwise, they may become extinct (Sorensen *et al.*, 2003; Hoffmann and Sgrò, 2011). As previously demonstrated, climate change further threatens to aggravate some of these stressful conditions (e.g. increasing mean temperatures). Therefore, the study of the mechanisms by which organisms respond to stress provides important hints on how they might cope with future climate change (Somero, 2010; Tomanek, 2010; Somero, 2011; Rosner, 2013).

Cells respond to stress by initiating specific gene expression programs that help them to physiologically adjust to the new conditions and protect against cell damage, failure, or ultimately death. However, the nature of stress responses is transient, since the changes they induce are temporary. So, even when a given stressful condition persist, the stress response is only viable in the long term if the organism can achieve the previous or a new homeostatic state that allows them to survive (López-Maury *et al.*, 2008; de Nadal *et al.*, 2011).

Most stress-induced genes are related with the heat shock response, antioxidant and energy production machineries (López-Maury *et al.*, 2008; de Nadal *et al.*, 2011). On the other hand, stress-repressed genes are involved in cellular growth functions (e.g. translation and ribosome biogenesis) (López-Maury *et al.*, 2008). These inverse patterns for stress and non-stress related genes usually reflect a relocation of resources from growth functions to the stress response (López-Maury *et al.*, 2008). In fact, the balance between energy-efficient growth and the ability to maintain cellular functionality under a wide variety of environmental conditions is a driving force for evolution (López-Maury *et al.*, 2008). Stress cause phenotypic variation in response to short-term environmental changes, and also contribute to evolutionary adaptation, for instance, by affecting sexual differentiation, transposition, epigenetic changes and by favoring or spoiling new mutations (López-Maury *et al.*, 2008).

1.1.3.1 Heat shock response

The heat shock response is a major focus of this thesis, with all chapters approaching the relevance of heat shock proteins (HSPs) under thermal stress and climate change. Therefore, this subsection describes the role and importance of HSPs in organisms.

The HSPs are a ubiquitous set of highly conserved proteins present in all organisms, from bacteria to plants and animals, whose synthesis is induced in response to heat (Lindquist and Craig, 1988; Sorensen *et al.*, 2003). They were firstly discovered in *Drosophila* after exposure to high temperatures, which led to the naming of heat shock proteins(Sorensen *et al.*, 2003). However, a wide variety of HSP are also induced in response to other stressors: cold, radiation, heavy metals, pesticides, hypoxia, salinity, high density, bacterial and viral infections, parasites, physical activity, desiccation, oxidative stress and senescence (Lindquist and Craig, 1988; Sorensen *et al.*, 2003). HSPs act as molecular chaperones and are involved in the correct folding of denatured, misfolded or aggregated proteins, in the transportation of proteins and in the assembly and disassembly of protein complexes (Lindquist and Craig, 1988; Sorensen *et al.*, 2003). This mechanism is largely universal in response to several stressors and is thought to be initiated by the presence of non-native protein conformations in cells at levels above a certain threshold. Therefore, the heat shock response has a significant ecological and evolutionary role in natural populations by protecting cells against several stressors (Sorensen *et al.*, 2003; Fangue *et al.*, 2006; Van Straalen and Roelofd, 2006).

Many HSPs have been identified and grouped into families according with their molecular weight in kilo Daltons (kDa): HSP100; HSP90; HSP70 (also named DnaK); HSP60; HSP40 (also known as DnaJ) and small HSP (with molecular weight below 30 kDa) (Lindquist and Craig, 1988; Sorensen *et al.*, 2003). HSP70 is the most studied HSP and belongs to a multi-gene family, constituted by both inducible (named as HSP70) and constitutive [known as heat shock cognate 70 (HSC70)] proteins (Lindquist and Craig, 1988; Ohtsuka and Suzuki, 2000; Place and Hofmann, 2001; Sorensen *et al.*, 2003). While HSC70 is constitutively expressed during a normal cell cycle, under non-stressful conditions, HSP70 is strongly induced when the organism is exposed to several types of stress (Lindquist and Craig, 1988; Ohtsuka and Suzuki, 2000; Yamashita *et al.*, 2004). HSP70 machinery is one of the most common systems responsible for the correct folding

of many proteins and for the transportation of proteins across membranes. It can interact with unfolded nascent proteins, regulatory proteins, transcription factors, kinases, DNA replication proteins, tumor suppressing proteins, as well as, with non-native proteins (e.g. proteins denatured by heat) (Lindquist and Craig, 1988; Wegele *et al.*, 2004). After being processed by HSP70, many proteins are transferred to the HSP90 machinery. While some HSP70 substrates are fully processed by the HSP70 machinery itself, others require HSP90 for proper folding or activation. In the latter cases, the HSP70-HSP90 Organizing Protein (HOP or STIP1), as suggest by the name, helps forming the intermediate complex by which substrates are transferred between these two HSP (Wegele *et al.*, 2004). HSP90 is also capable of independent activity and is fairly abundant in normal cell function, although it can also be strongly induced in the presence of stressful conditions (Krone *et al.*, 1997; Wegele *et al.*, 2004; Mayer and Bukau, 2005; Fangue *et al.*, 2006). Furthermore, other HSP can co-operate, for example: HSP70 with HSP40 in the folding machinery of HSP70 and HSP110 with HSP70 (Lindquist and Craig, 1988; Wegele *et al.*, 2004; Polier *et al.*, 2008). Each HSP and protein complex has its own function in the folding and trafficking of proteins across membranes, for instance HSP70 is responsible for the folding of nascent proteins, while HSP90 helps folding protein kinases and regulators of transcription.

1.1.3.2 Energy metabolism and oxidative stress

Although not as well studied as the heat shock response, energy metabolism and oxidative stress are also an important subject in this thesis, and thus they are both briefly described in this subsection.

While under stressful conditions (e.g. exercise and environmental hypoxia), organisms commonly suffer metabolic readjustments, which often trigger an increase in the anaerobic metabolism (lactic acid fermentation) in order to produce Adenosine Triphosphate (ATP). In this process, animals do not use oxygen as the final acceptor of the electron transport chain, as it is used in the aerobic pathway (Nelson and Cox, 2008). In fact, anaerobic pathway starts with glycolysis, producing 2 ATP and 2 pyruvate per each molecule of glucose (Nelson and Cox, 2008). In the anaerobic metabolism pyruvate is used as the electron acceptor and converted into lactate, releasing NAD+, which is recycled to glycolysis (Nelson and Cox, 2008). However, in the presence of oxygen, the aerobic

metabolism continues the transformation/oxidation of pyruvate into Acetyl-CoA, which joins oxaloacetate and enters the citric acid cycle (or Krebs cycle), allowing the generation of reduction power to enter electric transport chain and produce a higher amount of ATP in the process (36 ATP molecules). Both citrate synthase (CS) and lactate dehydrogenase (LDH) are widely used markers to track the responses of aerobic and anaerobic metabolism, respectively [e.g. Almeida-Val *et al.* (2000); Rosa *et al.* (2016); Campos *et al.* (2016)]. CS mediates the first step of the acid citric cycle, converting Acetyl-CoA plus oxaloacetate into citrate. In skeletal muscle and often during exercise, LDH mediates the interconversion of pyruvate to lactate (fermentation), while, in liver, lactate is converted back to pyruvate (Cory cycle) (Nelson and Cox, 2008).

Furthermore, during stress conditions, the production of molecules that derive from oxygen, i.e. reactive oxygen species (ROS) is also a challenge for organisms (Sun *et al.*, 2007; Sevcikova *et al.*, 2011). ROS are chemical reactive molecules which contain oxygen, such as: superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$) (Madeira *et al.*, 2013; Patil and David, 2013). Oxidative stress occurs when the organisms' biological ratio between oxidant and antioxidant mechanisms is unbalanced, either due to the depletion of antioxidant defenses or to an excessive accumulation of ROS, or even both (Monteiro *et al.*, 2006; Patil and David, 2013). ROS, such as superoxide anion and hydrogen peroxide are responsible for damaging cellular and molecular structures (Storey and Storey, 2005; Sun *et al.*, 2007; Sevcikova *et al.*, 2011). All aerobic organisms deal with ROS and, thus, have developed mechanisms that protect them against its damaging effects (Vinagre *et al.*, 2012; Madeira *et al.*, 2013; Patil and David, 2013), which are lipid peroxidation, DNA damage, and protein damage (Monteiro *et al.*, 2006; Madeira *et al.*, 2013). This disruption in the balance between oxidant and antioxidant mechanisms may occur, for example, during thermal stress, hypoxia, pollution exposure and ultraviolet radiation (Madeira *et al.*, 2013). Interestingly, ROS increase the expression of heat shock factors (HSF1) and HSP70 in animals (Madeira *et al.*, 2013). Moreover, in order to cope with the adverse effects of ROS, cells also induce their antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione-dependent enzymes (GSH), and non-enzymatic defenses such as amino acids, tocopherol and vitamins E, K and C (Martínez-Álvarez *et al.*, 2005; Grim *et al.*, 2010; Madeira *et al.*, 2013). Antioxidant enzymes are thus commonly used to measure the level of oxidative stress that organisms are exposed to.

1.2 Transcriptome profiling

Living beings respond to climate change stressors by adjusting their physiological responses, resulting in alterations in mRNA pools, both in quantity and quality. Species responses to stressors are marked by their genetic background and by environmental stimuli. Physiological response of organisms may be conditioned by the genetic background in several ways, for instance, through modifications in gene expression (Reusch and Wood, 2007; Hansen *et al.*, 2012). Although there may be still some time for several species to adapt and evolve a particular phenotype in response to climate change, for other species with larger generation times it might be harder to adapt to the predicted time frame forecasted by IPCC. Therefore, for these species, the study of gene expression, as well as their current genetic background, are utterly important to comprehend how they are adapted to nowadays environmental conditions and to understand if they can cope with future conditions (Crozier and Hutchings, 2014; Kapsenberg and Hofmann, 2014; Rosa *et al.*, 2016).

1.2.1 Transcriptome characterization

The discovery of the specific expressed genes in a given tissue or organism is the main objective of characterizing a transcriptome. This can be achieved in a more traditional way, through Sanger sequencing, or by the use of next-generation sequencing (NGS) technologies. However, both sequencing approaches rely on the sequencing of complementary DNA (cDNA), which is synthesized from messenger RNA (mRNA), using an enzyme called reverse transcriptase (RT). cDNA sequences are encoded similarly to DNA sequences (ACGT), i.e, they are compose by adenine (A), cytosine (C), guanine (C) and thymine (T), rather then uracil (U), which replaces thymine in mRNA sequences. Though Sanger sequencing is thought to be more accurate and less error prone, it has a very reduced throughput and requires a lot of manpower and laboratory work to achieve transcriptome-wide studies, compared to NGS (Ozsolak and Milos, 2011).

In this sense, NGS revolutionized all fields of research that rely on DNA sequencing to answer its questions. This high-throughput sequencing methodology enabled the large scale sequencing of many non-model species, ranging from genomic to transcriptomic studies (Ekblom and Galindo, 2010; Goodwin *et al.*, 2016). Until recently, three

main technologies were commonly used for this type of sequencing, 454 pyrosequencing (Roche), Solexa sequencing-by-synthesis (Illumina), and Applied Biosystems sequencing by oligo ligation and detection (SOLiD), all of them have their own characteristics and have been upgraded along the years (Morozova and Marra, 2008; Ekblom and Galindo, 2010; Goodwin *et al.*, 2016).

Although very accurate, SOLiD platforms have very short read lengths [currently 75 base pairs (bp)], which difficult transcriptome and genome assembly (Goodwin *et al.*, 2016). Despite having superior read lengths, 454 and Ion Torrent struggle with the same problems: accurate indel detection and proper homopolymer sequencing (Goodwin *et al.*, 2016). On the other hand, Illumina platforms are mostly favored because they provide a wider range of applications and due to the constant innovation of their platforms, increasing read lengths and total read capacity (Goodwin *et al.*, 2016). Nowadays, Illumina is the most widely used platform due to their reasonable read lengths (150 bp), less homopolymer error prone (comparing with other technologies that have larger read lengths, such as 454 or Ion Torrent) and its price per gigabase is very affordable (Goodwin *et al.*, 2016). Currently, there is growing interest in long read sequencers (both single-molecule real-time and synthetic sequencing technologies), that can overcome the limitations of studying complex or repetitive DNA regions. However they are still very error prone (particularly for indel detection) and require high coverages (more than short read sequencers), thus increasing the costs of this sequencing methods (Goodwin *et al.*, 2016).

After the sequencing has been performed, the analysis of the resulting sequences from NGS still represents one of the major hurdles that researchers face. In Sanger sequencing, the length of the sequences may reach up to 1000 bp and their assembly into larger genes is usually a straightforward affair, that can be performed manually against a reference sequence or by comparison of multiple sequences with the appropriate software (Goodwin *et al.*, 2016). Most NGS technologies, however, produce millions of much smaller sequences, usually called *reads*, that need to be correctly assembled into larger sequences in order to produce gene sequences (Goodwin *et al.*, 2016). These larger sequences are called *contigs* and can be obtained either through alignment against a reference genome/transcriptome, a process called *mapping*, or *de novo* assembly, by stacking identical reads without the aid of any reference genome/transcriptome (Ekblom and Galindo, 2010; Robertson *et al.*, 2010; Yandell and Ence, 2012).

RNA-seq is the most used NGS technique used to sequence transcriptomes, allowing the characterization and quantification of cDNA derived from coding and non-coding RNAs (Ekblom and Galindo, 2010; Ozsolak and Milos, 2011). With RNA-seq, researchers can study a totally unknown non-model species and sequence its whole transcriptome, with much less effort than using Sanger sequencing or other gene expression quantification method (Ekblom and Galindo, 2010; Ozsolak and Milos, 2011). Moreover, it allows for the detection of novel transcripts, that have not been described in other species (Ekblom and Galindo, 2010; Ozsolak and Milos, 2011).

After obtaining gene sequences, they can be annotated, usually in two steps. First, by comparing sequences against public databases such as Ensembl (http://www.ensembl.org/) and GenBank (https://www.ncbi.nlm.nih.gov/genbank), using algorithms such as BLAST (Camacho *et al.*, 2009) (Yandell and Ence, 2012). And secondly, by adding metadata to sequences, such as gene ontology terms (using for example BLAST2GO (Conesa *et al.*, 2005) or DAVID (Huang *et al.*, 2009) programs) (Ekblom and Galindo, 2010; Yandell and Ence, 2012).

1.2.2 Transcriptome quantification

The sequencing of transcriptomes can be used to identify and characterize genes but also to investigate the gene expression levels of the specific genes. Gene expression has been long used to understand several cellular mechanisms, from normal cell functioning to organism's responses to some stimuli.

Gene expression can be quantified through the usage of polymerase chain reaction (PCR). However, RT-PCR suffers from two main problems. First, during PCR, the amount of DNA product increases exponentially until it reaches a plateau, after which the initial amount of DNA cannot be calculated. Second, it is extremely difficult to guarantee equal amounts of total RNA on each sample (Breljak and Ambriovic-Ristov, 2005). Having this in mind, researchers developed methods that enable to measure the initial amount of target mRNA in a sample. To address the first issue, measurements can be done during the exponential phase of PCR, before the plateau phase. For the second issue, a control gene, usually a housekeeping gene that does not vary for the tested experimental conditions, is added for each PCR reaction. Initially this was conducted by separating the two products in an agarose gel electrophoresis and using image densitometry to measure the intensity of

the target gene relative to the control gene (e.g. semi-quantitative PCR) (Serazin-Leroy *et al.*, 1998; Breljak and Ambriovic-Ristov, 2005). Later, real-time PCR circumvented many limitations of the previous technique, by detecting the fluorescence (using either fluorescent probes or reagents that stain DNA) of the PCR products in real-time , i.e., in each PCR cycle (Breljak and Ambriovic-Ristov, 2005). This facilitated the whole process, since there is no need to determine the PCR exponential phase and to perform the gel electrophoresis as well as image densitometry (Serazin-Leroy *et al.*, 1998; Breljak and Ambriovic-Ristov, 2005). Up to date, this technique remains the gold standard for both clinical and research assays, mainly due to its high sensitivity and specificity (Goodwin *et al.*, 2016). However, it relies on the design of species specific primers or probes, requiring prior characterization of the genes sequences, which is difficult when studying non-model species or unknown genes (Breljak and Ambriovic-Ristov, 2005; Goodwin *et al.*, 2016).

While PCR is a good approach to study some genes, it can be challenging when studying certain regulation pathways or even whole transcriptomes (Chris Tachibana, 2015; Goodwin *et al.*, 2016). The study of whole transcriptomes became possible for the first time with the development of microarrays (Chris Tachibana, 2015). This technique relies on the hybridization of probes with the sample's target DNA sequences(Chris Tachibana, 2015). Although most useful for organisms where genomes are known (and for which it is easy to obtain probes), for non-model species or unknown genes, microarrays are not well suited (Chris Tachibana, 2015; Goodwin *et al.*, 2016). Even though some homology can be achieved, particularly for species closely related to model species, there will be a potential loss of information due to the unknown nature of non-model species' genome (Chris Tachibana, 2015; Goodwin *et al.*, 2016). Moreover, background hybridization as well as probe saturation are two caveats for the detection process (Chris Tachibana, 2015; Goodwin *et al.*, 2016).

With the increasingly cheaper NGS technologies, high throughput messenger RNA sequencing (RNA-seq) became more attractive for researchers given the limitations of microarrays (Ekblom and Galindo, 2010). To quantify gene expression of RNA-seq, researchers must first decide which individuals, tissues or other biological samples that must be compared. Then, RNA libraries are built, sequenced and assembled for each sample (Ekblom and Galindo, 2010; Vijay *et al.*, 2013). After assembling, the contigs must be assigned to a transcript of origin by mapping against a reference transcriptome (regardless of being an existing transcriptome or a *de novo* assembled transcriptome) in order to

estimate transcript expression. After obtaining these estimations for each transcript, differential gene expression analysis between RNA libraries can be undertaken, using several available algorithms (e.g. EdgeR, DEseq) (Vijay *et al.*, 2013).

1.3 Characterization and quantification of proteins

1.3.1 Structure and function

While DNA sequences are encoded by a four letter code, proteins can be encoded by assemblages of 20 possible amino acids, each having its own reference letter (Buxbaum, 2007; Nelson and Cox, 2008). Amino acids differ from each other in several chemical properties (e.g. hydrophilicity or hydrophobicity, size, and functional groups), and so, different combinations of amino acids can result in different proteins with distinct physical and chemical properties, structures and functions (Nelson and Cox, 2008). The amino acid sequence of a protein is its primary structure, from which its physical and chemical parameters can be inferred (e.g. molecular weight) (Buxbaum, 2007; Nelson and Cox, 2008). Secondary structure characterizes the conformation of local segments of proteins and result from the configuration of hydrogen bonds of the protein. There are four types of structural conformations: α-*helix*, which has a spiral conformation around an imaginary axis; β-*strand*, where the chain is stretched; turns of 180^{o}, usually between two strands; and coils, which are any structure but the ones previously referred (Buxbaum, 2007; Nelson and Cox, 2008). Tertiary structure describes how the different local segments of secondary structure interact with each other in a tridimensional space to form a functional protein (Buxbaum, 2007; Nelson and Cox, 2008). Proteins can also be composed by two or more polypeptide subunits and their arrangement in space is called quaternary structure (Nelson and Cox, 2008).

Protein structure can be determined through: X-ray crystallography, electron microscopy, nuclear magnetic resonance and computer predictions (Buxbaum, 2007). Computer predictions have been widely used because they have no limitations on the amount of purified protein required, unlike the other three methods, and it greatly benefits from the vast amounts of DNA sequences produced until today (Buxbaum, 2007). These predictions are based on the principle that all information required for secondary structure

prediction are contained in primary structure, i.e, the protein sequences (Buxbaum, 2007). Together, the primary, secondary and tertiary structure enables the inference of protein domains and functions by comparison with known protein databases (such as Protein Data Bank [PDB] or UniProt) (Buxbaum, 2007).
The characterization of proteins and comparison of species living in different environments might provide some clues on which species are better adapted to deal with a given condition.

1.3.2 Quantification methods

Alongside with gene expression, protein quantification has also been widely used to study the physiological responses of species to certain stimuli, including environmental stressors related to climate change [e.g. Sorensen (2010); Aurélio *et al.* (2013); Rosa *et al.* (2016)]. Although gene expression results from the cellular state, whether it is an homeostatic state or a physiological state resulting from a response to a given stimulus (e.g. stressful condition), protein quantity or enzyme activity may differ from what would be expected from gene expression due to post-transcriptional and translation mechanisms, as well as to protein degradation (Vogel and Marcotte, 2012).

Proteins can be quantified basically with two main methods: colorimetric assays, where amino acids interact either with dyes or cooper, and ultraviolet (UV) absorbance. In the first type, amino acids interact with cooper, emitting a blue color (e.g. Lowry and Bicinchoninic Acid protein assays) or amino acids interact with dyes (e.g. Bradford protein assays) also resulting in a blue color (Noble and Bailey, 2009; Kurien and Scofield, 2012). Other method that allows for better measurements of protein quantity is the ultraviolet absorbance method. This method is based on the absorbance of light by amino acids at 280 nm, mainly by tryptophan and tyrosine (Noble and Bailey, 2009; Kurien and Scofield, 2012).

Enzymatic activity is a particular case in protein quantification since it does not measure the direct quantity of a given enzyme but rather the consumption of subtract or production of product, resulting from the anabolic or catalytic activity of the enzyme, over time. Quantification can also be carried out by a spectrophotometer since it allows for the quantification of proteins, nucleic acids and also metabolites, through the measurement of light (UV or visible) absorbed or reflected by a specific compound (Bisswanger, 2013;

Cornish-bowden, 2013). Also, enzyme-linked immunosorbent assay (ELISA) may be used to quantify proteins that interact with a specific antibody or antibodies, resulting in a color, fluorescent or electrochemical signal. Enzyme levels are more easily estimated than other proteins since they can be identified by their catalytic reaction rather than through direct quantification. However, for proper enzyme activity quantification, the pH, temperature and other conditions such as nature and strength of ions and substrate saturation must be well controlled and specific for each reaction (Cornish-bowden, 2013).

1.4 Iberian Cyprinids

Fishes are the richest vertebrate group, representing more than half of all vertebrate species. Estimates point to a total of more than 32,000 described fish species. The Cyprinidae family (Order Cypriniforms) is the species-richest freshwater fish family, only surpassed by Gobiidae as the largest vertebrate family (Nelson *et al.*, 2016). Cyprinids are distributed throughout North America, Africa, Europe and Asia, totalizing 3,006 species (Nelson *et al.*, 2016). Cyprinids have countless types of diet and they are important fish for food industry, ornamental fish market and biological research (Nelson *et al.*, 2016). The three better-known species of this family are: the Common Carp *Cyprinus carpio* Linnaeus, 1758, the Goldfish *Carassius auratus* (Linnaeus, 1758), and the Zebra fish *Danio rerio* (F. Hamilton, 1822) (Nelson *et al.*, 2016). The latter is widely used as a model species for research purposes (Nelson *et al.*, 2016).

The sub-family Leuciscinae (Cyprinidae) is distributed throughout North America and Eurasia. In the Iberian Peninsula, Leuciscins are represented by the former *Chondrostoma* s.l. (comprising 6 genera: *Achondrostoma*, *Iberochondrostoma*, *Parachondrostoma*, *Protochondrostoma* and *Pseudochondrostoma*), *Anaecypris* and *Squalius* (formerly known as *Leuciscus*) genera (Robalo *et al.*, 2007; Perea *et al.*, 2010). The *Squalius* genus currently has 51 well recognized species (Froese and Editors, 2016), widely distributed across Europe, with a remarkable diversity in the the circum-Mediterranean region(Sanjur *et al.*, 2003; Perea *et al.*, 2010), which is considered one of the 25 global hotspots of biodiversity (Figure 1.1) (Myers *et al.*, 2000). The south of the Iberian Peninsula is included in this region, being characterized by the presence of a high number of endemic vertebrates, including freshwater cyprinid fish. Especially, the Iberian Peninsula possesses many unique

species from the *Squalius* genus (Sanjur *et al.*, 2003; Perea *et al.*, 2010).

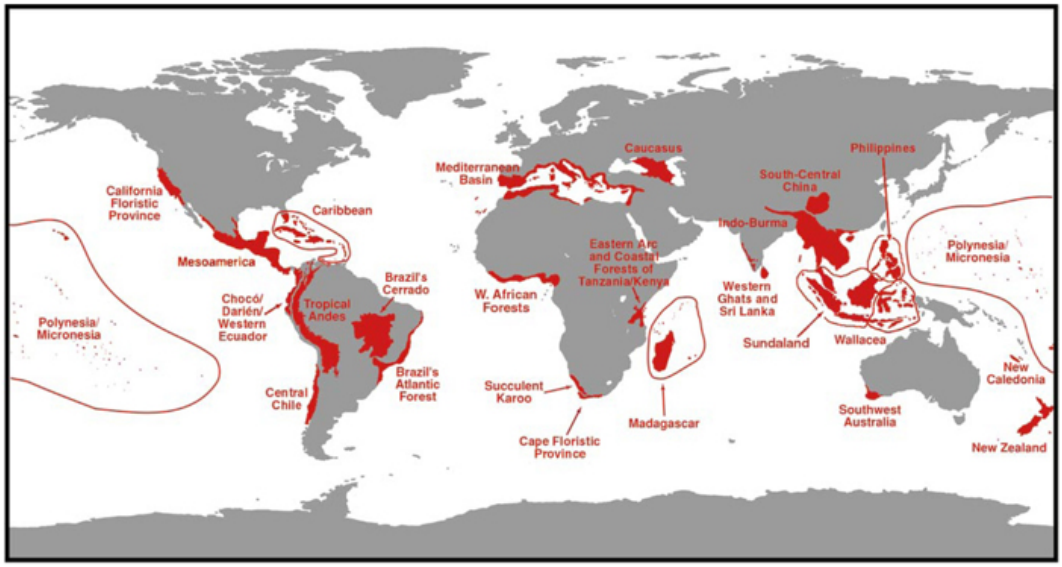

Figure 1.1: Geographical location of the 25 hotspots for biodiversity described by Myers *et al.* (2000). Figure was retrieved from Myers *et al.* (2000).

1.4.1 *Squalius* genus in Portuguese inland waters

In Portuguese inland waters, the *Squalius* genus is represented by four endemic species: *S. carolitertii* (Doadrio, 1988), *S. pyrenaicus* (Günther, 1868), *S. torgalensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira, 1998), *S. aradensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira, 1998) and the hybrid allopolyploid complex *S. alburnoides* (Steindachner, 1866). The former four species live in allopatry along a latitudinal cline. *S. carolitertii* inhabits the northern region, followed by *S. pyrenaicus* which inhabits the central and southern regions, and finally by *S. torgalensis* and *S. arandensis* which live in the southwestern region of Portugal (Figure 1.2). *S. alburnoides* co-occurs in some of the same river basins as the species with which it hybridizes: *S. carolitertii*, *S. pyrenaicus* and *S. aradensis* (Robalo *et al.*, 2006; Sousa-Santos *et al.*, 2007). The southwestern region of Portugal is believed to held the oldest isolated rivers within Iberia, which led to the higher differentiation of both *S. torgalensis* and *S. arandensis* when compared with the remaining *Squalius* present in the Iberian Peninsula (Mesquita *et al.*, 2007). Regarding

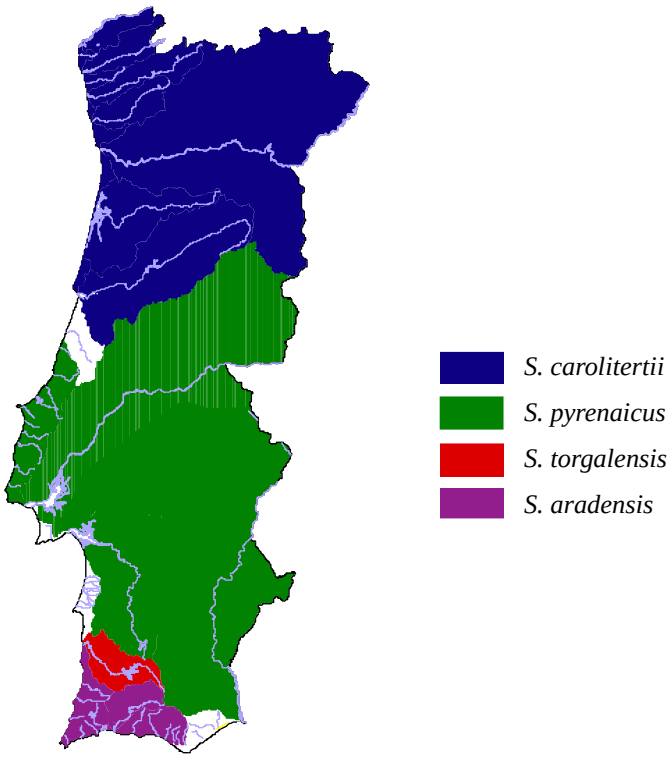

Figure 1.2: Geographical distribution of non-hybrid *Squalius* in Portuguese territory.

the conservation status of Portuguese *Squalius*, *S. torgalensis* and *S. aradensis* are currently critically endangered, while *S. carolitertii* and *S. pyrenaicus* are least concerned and endangered species, respectively.

The high rate of endemism of Leuciscins in Iberia has been related to historical factors, such as the establishment of river drainages. Besides the establishment of the current river drainages system, climate may also have led (and continue to lead) to the differentiation of extant Leuciscinae species, including *Squalius* species. For instance, during Pleistocene glaciations, the Iberian Peninsula constituted an important refugium to northern and central European fauna (Almaça, 1995; Carvalho *et al.*, 2010). Glaciations also influenced the distribution of Leuciscins inhabiting in the most affected regions in the Iberian Peninsula (Brito *et al.*, 1997; Almada and Sousa-Santos, 2010; Sousa *et al.*, 2010; Perea *et al.*, 2010). Within the Iberian Peninsula only northern rivers and streams were covered by ice, which may also reflect its reduced number of endemisms compared with the Iberian southern region (Filipe *et al.*, 2009).

Nowadays, the Iberian climate is mainly divided into two types: Atlantic, affecting northern regions; and Mediterranean, present in southern regions. Atlantic climate is mainly observed north of the Tagus River, including the major mountain systems of Iberia. On the other hand, Mediterranean climate is the dominant type of climate and is mostly observed in southern regions (Carvalho *et al.*, 2010).

Due to the heterogeneous nature of Iberian climate, species have adapted throughout evolutionary history to cope with contrasting environmental characteristics. The northern species, *S. carolitertii*, is adapted to temperatures ranging from 3 °C to 31 °C (Carvalho *et al.*, 2010; SNIRH, 2010) (Atlantic climate type). On the other hand, central and southern species (*S. pyrenaicus* [in some streams of its distribution range], *S. torgalensis* and *S. aradensis*) deal with a marked interchange between floods and droughts (Magalhães *et al.*, 2003; Carvalho *et al.*, 2010; Henriques *et al.*, 2010) (Mediterranean climate type). Southern rivers have a higher temperature variation both in a daily basis and globally along the year, ranging from 4 °C to 38 °C, and lower oxygen concentrations during the dry season as a result of droughts (Carvalho *et al.*, 2010; SNIRH, 2010). This river regime might have left signatures of adaptation to more extreme conditions on *S. torgalensis* and *S. aradensis*, since they were isolated in southwestern Portugal for much longer (Coelho *et al.*, 1998; Mesquita *et al.*, 2007). However, whether these past adaptations will help dealing with the current and upcoming climate changes is still unknown.

1.5 Objectives and structure of the thesis

European climate change reports point to an ongoing process that already diminished river flow and increased mean water temperature between 1 °C to 3 °C over the last decades (Füssel *et al.*, 2012b; Field *et al.*, 2014). This issue is particularly noticeable for many European rivers during summer season, with special emphasis within the southern European rivers where the severity and frequency of droughts has significantly increased (Füssel *et al.*, 2012b).

The main goal of this thesis is to comprehend the mechanisms by which Iberian freshwater fish of the *Squalius* genus inhabiting two distinct environmental conditions (with Atlantic and Mediterranean climates) may cope with future climate change. To this end, two endemic fish species from the Iberian region were chosen as representatives of these

distinct environments, *S. carolitertii* from the northern region and *S. torgalensis* from the southern region. To address this issue, four specific objectives were established:

1. To identify differences in gene expression between both species in response to acute thermal stress.

2. To characterize the transcriptomic responses of *S. carolitertii* and *S. torgalensis* exposed to acute thermal stress conditions.

3. To characterize genes suitable to be studied under other thermal stress conditions.

4. To assess the effects of climate change projections in the gene expression of genes of interest and in biochemical and physiological response of both species.

This thesis is comprised of four chapters: (i) the Introduction; (ii) and (iii) two chapters in which the results of five publications, three of which are already published in peer-reviewed journals, one is submitted and another one is in preparation; and (iv) the Discussion and final remarks. For the first objective, we used conventional thermal stress markers (*hsp70* and *hsc70*) to investigate gene expression changes in representatives of *S. carolitertii* and *S. torgalensis* exposed to acute thermal stress (Chapter 2). Given that thermal stress responses often involve other hsp genes and mechanisms (Lindquist and Craig, 1988; Sorensen *et al.*, 2003), in the second and third publications we intended to evaluate the transcriptome-wide responses of both species after acute thermal stress. This study resulted in the characterization of the transcriptomes of both species (addressing the second objective) and in the differential gene expression analysis of the transcriptome of both species in response to thermal stress, resulting in the characterization of several target genes for accessing thermal stress responses in fish (third objective) (Chapter 2).

Although heat shock experiments gave clues about the acclimation potential of species to future warming, climate change is more complex than just warming (Field *et al.*, 2014), and will certainly last longer than any heat shock situation. Thus, for the fourth objective, fish of both species were exposed to a scenario combining 3°C higher temperature with acidic conditions (ΔpH = -0.4) considering as the control conditions of summer average freshwater temperatures and pH. Gene expression and protein modeling of fourteen target genes involved in key pathways were evaluated for both species after exposure to three conditions (higher temperature, acidic water, and the two conditions combined) in order to

address objective 4 (Chapter 3, section 3.1). Furthermore, we also evaluated the activity of key metabolic enzymes, heat shock proteins and antioxidant enzymes of both species exposed to the same experimental conditions (Chapter 3, section 3.2).

With this study we aimed to comprehend how these two species will deal with the future climate change and how they are currently adapted to deal with distinct environmental conditions, and finally to contribute for the adoption of proper conservation measures for these species, safeguarding the endangered species, such as *S. torgalensis*.

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Chapter 2

Acute thermal stress responses

2.1 Different levels of *hsp70* and *hsc70* mRNA expression in Iberian fish exposed to distinct river conditions

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Tiago F. Jesus, Ângela Inácio and Maria M. Coelho

Centro de Biologia Ambiental, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016, Lisbon, Portugal.

Abstract

Comprehension of the mechanisms by which ectotherms, such as fish, respond to thermal stress is paramount for understanding the threats that environmental changes may pose to wild populations. Heat shock proteins are molecular chaperones with an important role in several stress conditions such as high temperatures. In the Iberian Peninsula, particularly in Portugal, freshwater fish of the genus *Squalius* are subject to daily and seasonal temperature variations. To examine the extent to which different thermal regimes influence the expression patterns of *hsp70* and *hsc70* transcripts we exposed two species of *Squalius* (*S. torgalensis* and *S. carolitertii*) to different temperatures (20, 25, 30 and 35 °C). At 35 °C, there was a significant increase in the expression of *hsp70* and *hsc70* in the southern species, S. torgalensis, while the northern species, *S. carolitertii*, showed no increase in the expression of these genes; however, some individuals of the latter species died when exposed to 35 °C. These results suggest that *S. torgalensis* may cope better with harsher temperatures that are characteristic of this species´ natural environment; *S. carolitertii*, on the other hand, may be unable to deal with the extreme temperatures faced by the southern species.

Keywords: Cyprinidae, heat shock proteins, *Squalius*, thermal stress.

Introduction

Many organisms are frequently exposed to stressful environmental conditions, such as temperature variations, that pose substantial challenges to their survival and reproduction (López-Maury *et al.*, 2008). Stressful conditions may limit the geographical distribution of organisms by causing them to move to more suitable locations (Hoffmann and Sgrò, 2011). Organisms can also deal with stressful conditions by adapting to them, either through changes in the genetic composition of populations as a result of selection, and/or by phenotypic plasticity; without this adaptability many species would become extinct (Sorensen *et al.*, 2003; Dahlhoff and Rank, 2007; Berg *et al.*, 2010; Hoffmann and Sgrò, 2011). Most animal species (>99%), including fish, are ecthoterms that cannot regulate their body temperature and this ultimately affects their metabolism (Berg *et al.*, 2010).

2. ACUTE THERMAL STRESS RESPONSES

Since increases in temperature are one of the major consequences of climate change it is important to know how organisms, particularly ecthoterms, respond to high temperatures.

Heat shock proteins (HSP) are part of an important mechanism that helps organisms to cope with adverse environmental conditions such as thermal stress. This mechanism has a significant ecological and evolutionary role in natural populations (Sorensen *et al.*, 2003; Fangue *et al.*, 2006; Van Straalen and Roelofd, 2006). In addition to thermal stress, other factors such as insecticides, heavy metals, desiccation, diseases and parasites can also induce HSP (Lindquist and Craig, 1988; Sorensen *et al.*, 2003; Fangue *et al.*, 2006). Heat shock proteins are vital for proper cell functioning since they facilitate the folding and refolding of proteins and the degradation of misfolded, aggregated or denaturated proteins (Lindquist and Craig, 1988; Ohtsuka and Suzuki, 2000; Sorensen *et al.*, 2003; Wegele *et al.*, 2004).

Several closely related *hsp* genes have been identified and grouped into families based on their evolutionary relationships (Lindquist and Craig, 1988). The extensively studied 70-kDa heat shock protein (*hsp70*) belongs to a multi-gene family and its gene expression varies under different physiological conditions (Lindquist and Craig, 1988). The genes that encode the HSP70 proteins (*hsp70s*) are considered the major *hsp* gene family and consist of exclusively inducible (*hsps*), exclusively constitutive [Heat shock cognates (*hscs*)] and even simultaneously inducible and constitutive genes (Lindquist and Craig, 1988; Ohtsuka and Suzuki, 2000; Place and Hofmann, 2001; Sorensen *et al.*, 2003). The *hsp70* genes and the genes that encode the HSC70 protein (*hsc70*) belong to the *hsp70* gene family. Whereas *hsp70* genes are induced by several types of stress, *hsc70* genes are mainly constitutively expressed under normal (non-stress) conditions (Lindquist and Craig, 1988; Ohtsuka and Suzuki, 2000; Yamashita *et al.*, 2004).

Members of the *hsp70* gene family have been widely studied in many organisms and distinct expression patterns have been found. Several studies have reported a relationship between the expression patterns of *hsp70* and environmental variations throughout a species' range (Sorensen *et al.*, 2003; Fangue *et al.*, 2006; Karl *et al.*, 2009; Sorensen *et al.*, 2009; Blackman, 2010; Sarup and Loeschcke, 2010). For example, Fangue *et al.* (2006) detected significant differences in the gene expression levels of *hsp70* between northern and southern populations of *Fundulus heteroclitus* in North America, with the latter being exposed to higher temperatures. Similarly, Sorensen *et al.* (2009) found that southern

populations of *Rana temporaria* from Sweden, when exposed to higher temperatures, had the highest levels of HSP70 protein expression.

The *hsc70* gene was initially described as being constitutively expressed under normal and stressful conditions (Lindquist and Craig, 1988; Place and Hofmann, 2001; Yeh and Hsu, 2002; Yamashita *et al.*, 2004). Fangue *et al.* (2006) reported that individuals from southern populations of *F. heteroclitus* showed enhanced expression of this gene at higher temperatures. This finding demonstrates the importance of studying the expression of *hsp70* genes in closely related species or populations exposed to different temperature regimes in their natural habitats. These findings also suggest that HSPs play an important role in thermal tolerance and that, despite being occasionally paradoxical, the expression patterns of these genes must be interpreted according to the ecological context of each species (Sorensen *et al.*, 2003).

In the Iberian Peninsula, particularly in Portugal, the congeneric freshwater fish species, *Squalius carolitertii* (Cyprinidae) (Doadrio, 1988), a species of least concern (Cabral *et al.*, 2006), and *Squalius torgalensis* (Coelho *et al.*, 1998), a critically endangered species (Cabral *et al.*, 2006) , inhabit distinct regions. *S. carolitertii* inhabits the northern region whereas *S. torgalensis* is restricted to a small river basin (the Mira river) in the southwestern region (Figure 2.1) (Cabral *et al.*, 2006). In these areas, the two species are exposed to different environmental conditions with distinct seasonal and even daily water temperature variations. The northern rivers of Portugal have lower temperatures and fewer temperature fluctuations than the southern rivers (Henriques *et al.*, 2010; SNIRH, 2010). In northern rivers, the maximum temperature usually does not exceed 31 °C (range: 3-31 °C), whereas southern rivers are characterized by an intermittent regime of floods and droughts in which, during the dry season, freshwater fish are trapped in small pools in which temperatures can reach 38 °C (range: 4-38 °C) (Magalhães *et al.*, 2003; Henriques *et al.*, 2010; SNIRH, 2010).

The main goal of this study was to gain insights into the potentially important molecular mechanism involved in the response of *S. carolitertii* and *S. torgalensis* to thermal stress, particularly since these species inhabit regions with distinct environmental regimes. Specifically, we examined the *hsp70* and *hsc70* gene transcription patterns for each species exposed to different temperatures and compared the patterns between the two species; we also tried to correlate our findings with the ecological context of each species. Finally, we examined whether the patterns of transcript expression (for the genes of interest) were

similar to those of muscle, which is the most frequently used tissue in such studies (Yamashita *et al.*, 2004). The results described here provide useful insights into the roles of *hsp70* and *hsc70* gene expression in the response of Iberian *Squalius* to thermal stress.

Methods

Sampling and maintenance of fish

Adult fish (6-8 cm long) of *S. carolitertii* and *S. torgalensis* were collected from Portuguese rivers by electro-fishing (300 V, 4 A) (Figure 2.1). The pulses used were of low duration to avoid killing juveniles. Sampling was done during the spring, when the water temperature in the southern and northern rivers is 18-22 °C. Fish of both sexes were used since there is no sexual dimorphism in either species. *Squalius torgalensis* individuals were sampled in the Mira river basin since this species is endemic to this region and individuals of *S. carolitertii* were collected in the Mondego, Vouga and Douro river basins of the northern region. The fish were maintained in 30 L aquaria at 20 °C (mean temperature observed during sampling) on a 12 h photoperiod and were fed daily with commercial flake fish food.

Experimental design

After two weeks of acclimation (to reduce the stress caused by fishing and confinement), individuals of each species were subjected to four temperature regimens: 20 °C (control temperature) and increases in temperature from 20 °C to 25 °C, 30 °C and 35 °C (testing temperatures). These increases in temperature were achieved with gradual increments of 1 °C per day and, once the testing temperature was reached, individuals were kept at this temperature for 24 hours. Six to seven individuals of each species were exposed to each experimental condition, with each individual being exposed to only one experimental condition. After acclimation at the desired test temperature, fish were anesthetized with 300 mg/L tricaine mesylate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) and fin clips were collected from the pectoral, pelvic and upper caudal fins. The fin clips from each fish were pooled and stored at -80 °C until RNA extraction. To compare the expression patterns of fins and muscle and determine whether fin clips could be used instead of muscle

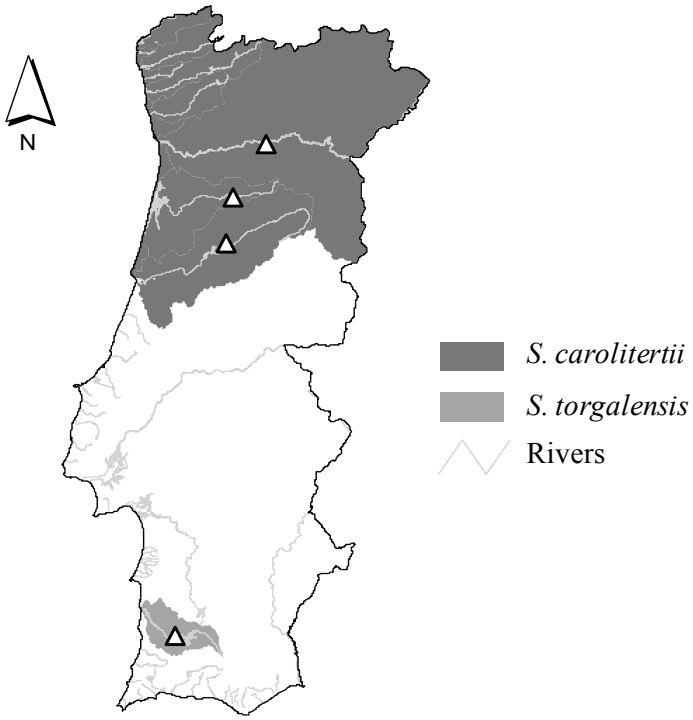

Figure 2.1: Geographical distribution of S. torgalensis and S. carolitertii in Portugal, with the respective sampling sites marked with triangles.

to assess transcript expression, four individuals of *S. torgalensis* (one per test temperature) and 16 individuals of *S. carolitertii* (four per test temperature) were euthanized with MS-222 and muscle tissue was collected. Since *S. torgalensis* is a critically endangered species, our study was designed to minimize the number of individuals euthanized.

RNA extraction and cDNA synthesis

For RNA extraction, TRI Reagent (Ambion, Austin, TX, USA) was added to fin clips and muscle samples. After homogenization with an Ultra-Turrax homogenizer (IKA, Staufen, Germany), RNA was extracted according to the manufacturer´s protocol and TURBO DNase (Ambion) was used to degrade any remaining genomic contaminants, followed by phenol/chloroform purification and LiCl precipitation (Cathala *et al.*, 1983). Glycogen was used as a co-precipitant in RNA precipitation (Sigma-Aldrich). The quality of

the samples was checked using a Nanodrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) based on the 260/280 nm and 260/230 nm absorbance ratios. The concentrations of the samples were determined to ensure a sufficient amount of homogeneous RNA for complementary DNA (cDNA) synthesis. cDNA was synthesized using a RevertAid H Minus First Strand cDNA synthesis kit (Fermentas Inc., Glen Burnie, MD, USA), according to the manufacturer's instructions and stored at -20 °C.

Semi-quantitative RT-PCR

Sixty-one individuals (31 *S. torgalensis* and 30 *S. carolitertii*) were used for quantification of the target transcripts. The *hsp70*-specific primers GGCCCTCATCAAACGC (forward) and TTGAAGGCGTAAGACTCCAG (reverse) and the *hsc70*-specific primers GTTCAAGCAGCCATCTTAGC (forward) and TGACCTTCTCCTTCTGAGC (reverse) were designed using PerlPrimer software v.1.1.19 (Marshall, 2004). The resulting amplicons were sequenced and the sequences then checked manually for errors using SEQUENCHER v.4.2 (Gene Codes Corporation, Ann Arbor, MI, USA). The identities of the genes of interest were confirmed by BLAST searches (Zhang *et al.*, 2000).

Multiplex PCRs were used to amplify the glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) serving as internal control and the gene of interest, which allowed normalized quantification of the mRNAs of interest (*hsp70* or *hsc70*). The primers used to amplify *gapdh* were ATCAGGCATAATGGTTAAAGTTGG (forward) (Pala et al., 2008) and GGCTGGGATAATGTTCTGAC (reverse) (Matos IM, unpublished). *Gapdh* has been extensively used as an internal control in several studies and has been validated as a good reference gene for gene expression studies in different experimental conditions (Aoki et al., 2000; Zhou et al., 2010), including those involving temperature changes (Liu *et al.*, 2012). Semi-quantitative RT-PCRs were optimized to ensure the amplification of both cDNAs in the exponential phase (Serazin-Leroy *et al.*, 1998; Breljak and Ambriovic-Ristov, 2005). The amplification conditions for the pair *hsp70*/*gapdh* were those described in the manufacturer´s instructions (QIAGEN multiplex PCR kit, Qiagen Inc., Valencia, CA, USA) (final concentration: 1× PCR master mix with 3 mM MgCl2, 0.5× of Q-solution and 0.2 μM of each primer), with an initial denaturation step at 95 °C for 15 min, followed by 30 cycles at 95 °C for 1 min, 58 °C for 1 min and 30 sec and 72 °C for 1 min, with a final extension at 72 °C for 10 min. For the gene pair *hsc70*/*gapdh*, the PCR conditions

were: 1 unit of GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA) with 0.3 μM of each primer, 0.25 mM of each dNTP and 2 mM of MgCl2. The cycling conditions included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 58 °C for 45 sec and 72 °C for 1.5 min, with a final extension at 72 °C for 10 min. Controls without template and without RT (reverse transcriptase) were included to check for PCR contamination and genomic DNA contamination, respectively.

For transcript quantification, 4 μL of each PCR product was loaded onto a 1% agarose gel stained with RedSafe (Chembio Ltd, Hertfordshire, England) and the gels were photographed with a DC290 Kodak digital camera for subsequent image densitometry using ImageJ 1.43u software (Abràmoff *et al.*, 2004). An uncalibrated optical density was used (Abràmoff *et al.*, 2004) and the band of interest was quantified and normalized against the internal control band (*gapdh*) present in the same lane.

Real-time RT-PCR

To assess whether the results obtained with semi-quantitative PCR corresponded to valid transcript expression patterns, an experiment with real-time PCR was done. In this experiment, for both species, three individuals from each experimental condition were analyzed with two PCR replicates. The primer pairs AATTCCACCTGCACCACG (forward) and TCTCCTCTTTGCTCAGTCTG (reverse) and TTTGCTGTTGGATGTCACTC (forward) and GTGGGAATGGTGGTGTTC (reverse) were used to amplify the *hsp70* and *hsc70* genes, respectively. These specific primers were designed based on the sequences previously obtained from semi-quantitative PCR. The relative expression levels of the genes of interest were measured against *gapdh* (reference gene). The primers used to amplify the *gapdh* gene were GTACAAGGGTGAGGTTAAGGC (forward) and GTGATGCAGGTGCTACATACGT (reverse). All pairs of primers used were designed using PerlPrimer software v.1.1.19 (Marshall, 2004).

Real-time PCRs were done in a final volume of 15 μL containing 7.5 μL of SsoFas EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) and 0.6 μL of each primer (with a concentration of 0.4 μM). The assay conditions included an initial denaturation step at 95 °C for 30 sec, followed by 40 cycles at 95 °C for 5 sec and 55 °C for 5 sec. The reactions were done in a Bio-Rad CFX96 system (Bio-Rad). Controls without template and without RT were included to check for PCR contamination and genomic DNA contamination,

respectively. The identities of the amplicons were confirmed by melting curve analysis and Sanger sequencing. The PCR efficiency for each sample was assessed using LinRegPCR 11.1 software, which fits a regression line to a subset of data points in the log-linear phase (Ruijter *et al.*, 2009). PCR efficiency ranged from 1.91 to 2 for all primer pairs (1.91 for *hsp70* primers and 2 for *gapdh* and *hsc70* primers). The relative amount of the genes of interest was calculated by the comparative threshold cycle (CT) method with efficiency correction, using the mean PCR efficiency for each amplicon (Ruijter *et al.*, 2009).

Statistical analyses

In the semi-quantitative PCR analysis, arbitrary values for quantification of the band of interest (*hsp70* or *hsc70*) were divided by the corresponding value for the control band (*gapdh*) to obtain a *hsp70*/*gapdh* or *hsc70*/*gapdh* ratio.

In graphs of the fold change in expression for each transcript a temperature of 20 °C was considered the control condition and assigned a value of 1. The fold variation in the other treatments, relative to the control condition, was calculated as follows: $I_i = \sum x_i / n\overline{x}_{20}$, where I_i is the mean fold increase in expression, x_i is the observed value, $\overline{x}_{20}$ is the mean value of observations at 20 °C for each species and n is the number of individuals of each species per tested temperature.

The data were log transformed [$log_{10}(x+1)$] for analysis of variance (ANOVA) in order to test for differences in transcript expression patterns across the experimental conditions for both genes. Whenever the assumptions of homoscedasticity and normality were not met, non-parametric Kruskal-Wallis analyses were done and the results from both analyses were compared. Post-hoc parametric and non-parametric comparisons were performed, using the Tukey test and Dunn's test, respectively. The real-time PCR data were analyzed in a manner similar to that used for semi-quantitative PCR, except that the fold change was calculated by the method of Pfaffl (2001). Prior to analysis, the real-time PCR data were transformed as described by Willems et al. (2008); the statistical tests used were the same as those used for semi-quantitative PCR. In all cases, a value of $p<0.05$ indicated significance. All statistical comparisons were done using Statistica 9.0 software (StatSoft, 2009).

Results

Survival in the experiments

Two of seven *S. carolitertii* individuals did not reach the 35 °C experimental condition because they died during the increase from 34 °C to 35 °C. In contrast, none of the *S. torgalensis* individuals died or showed signs of loss of equilibrium during the experiments. In the experiment to compare gene expression in muscle and fins, all individuals of *S. carolitertii* died at 34 °C, before reaching 35 °C.

Expression pattern of the *hsp70* gene

Initially, the identity of each amplicon was confirmed by sequencing. This showed that the *hsp70* primers amplified a fragment with high homology to the inducible form of *hsp70* from other cyprinids, including *Megalobrama amblycephala* (96.5% identity; accession number: EU884290), *Tanichthys albonubes* (96% identity; HQ007352), *Cyprinus carpio* (95.4% identity; AY120894), *Carassius auratus* (94.3% identity; AB092839) and *Danio rerio* (91.7% identity; BC056709). The sequences of the *hsp70* genes of *S. torgalensis* and *S. carolitertii* were deposited in GenBank under accession numbers JQ608477 and JQ608476, respectively.

In both species, the levels of *hsp70* gene expression in muscle and fin clips with increasing water temperature were similar in both tissues (Figure 2.6, Supplementary material). Consequently, in all subsequent analyses fin clips were used in order to avoid euthanasia of the fish.

In *S. torgalensis* exposed to 35 °C there was a 59-fold increase in the *hsp70* mRNA levels when compared with 20 °C (control condition) and an 53-fold increase when compared with 30 °C. In contrast, in *S. carolitertii* the corresponding expression increased by no more than three-fold, even at the highest temperature (Figure 2.2). Statistical analyses indicated a significant difference in *hsp70* mRNA expression among *S. torgalensis* exposed to different temperatures ($F = 29.486$, $df = 3$, $p < 0.001$), with post-hoc comparisons showing that *S. torgalensis* exposed to 30 °C and 35 °C had a significant increase in hsp70 levels compared with those observed at 20 °C and 25 °C (Table 2.1, Supplementary material). Post-hoc comparisons also demonstrated a significant difference between fish exposed to 30 °C and 35 °C (Table 2.1, Supplementary material). There were no

significant differences in the mRNA levels among the groups of *S. carolitertii* exposed to different temperatures ($H = 3.086$, $df = 3$, $p > 0.300$). As this latter dataset violated the assumption of homoscedasticity the results were also compared with a non-parametric test but the outcome was the same, i.e, there were no differences in the expression of *hsp70* in *S. carolitertii* exposed to different temperatures ($F = 1.220$, $df = 3$, $p > 0.300$).

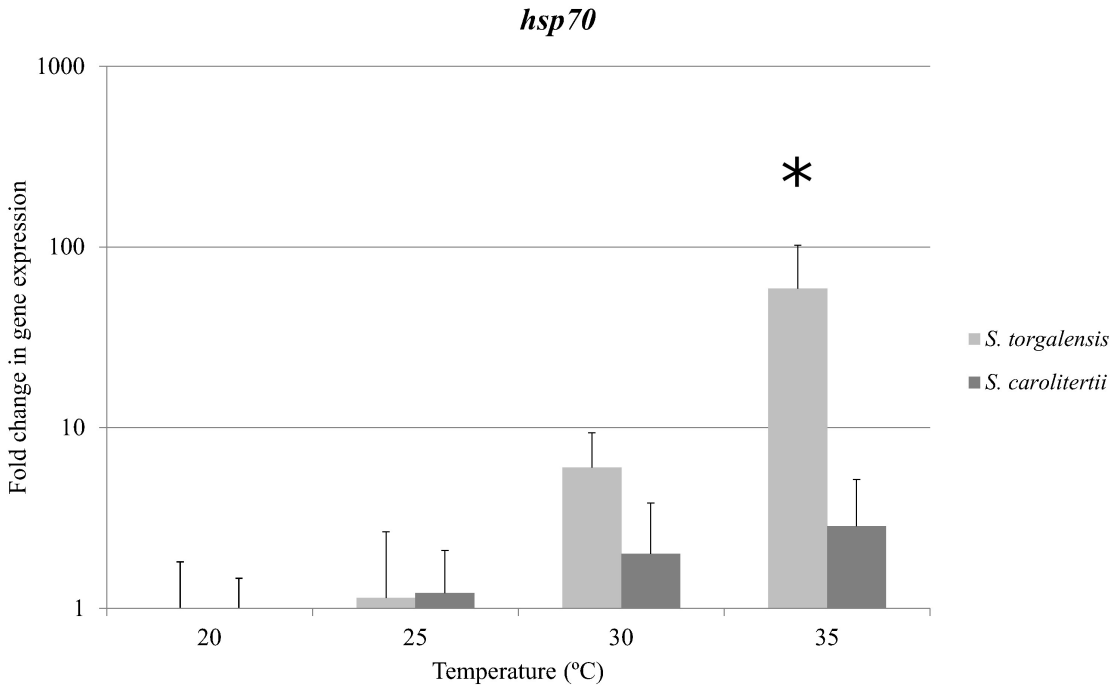

Figure 2.2: Fold change in *hsp70* transcript expression in *S. torgalensis* and *S. carolitertii* compared to 20 °C (control condition), as assessed by semi-quantitative PCR. The columns are the mean ± SD of 6 or 7 fish. $p < 0.05$ compared to all other treatments.

In general, the real-time PCR results showed similar patterns to those obtained with semi-quantitative PCR for both species, although for *S. torgalensis* the expression pattern of the *hsp70* gene obtained with real-time PCR differed significantly ($F = 92.356$, $df = 3$, $p < 0.001$) among the experimental conditions (Figure 2.3; Table 2.2, Supplementary material). Since this dataset did not satisfy the assumption of homogeneity of variances

a non-parametric test was also applied and showed a significant difference in the mRNA expression levels between 20 °C and 35 °C ($H = 9.974$, $df = 3$, $p < 0.050$) (Table 2.2, Supplementary material).

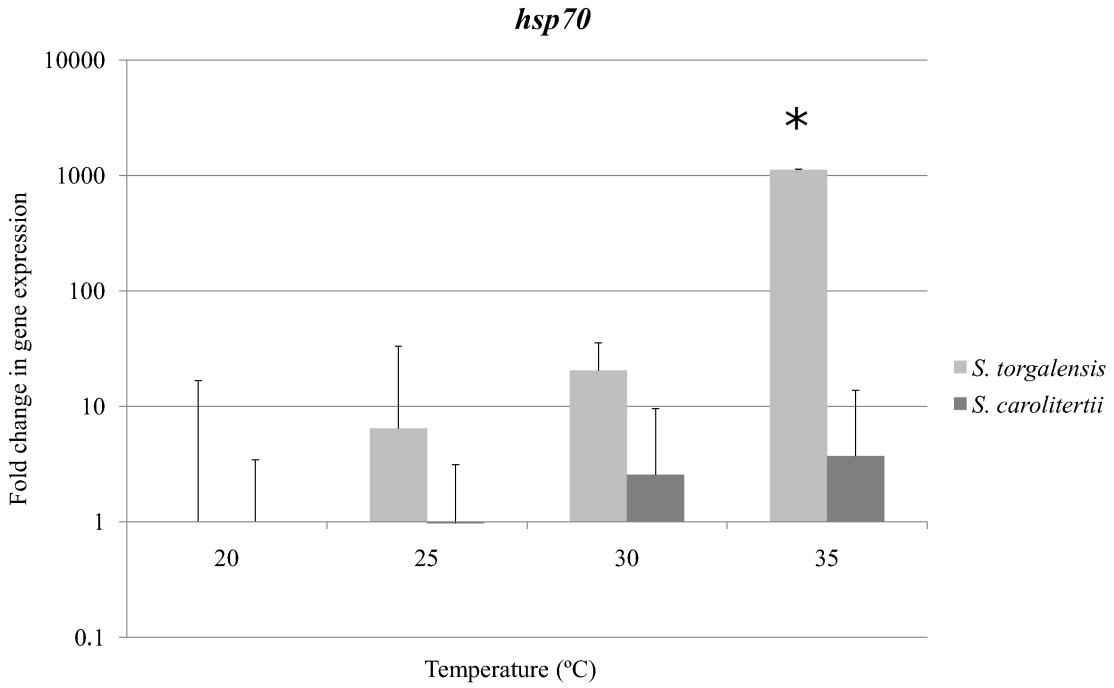

Figure 2.3: Fold change in *hsp70* transcript expression in *S. torgalensis* and *S. carolitertii* compared to 20 °C (control condition), as assessed by real-time PCR. The columns are the mean ± SD of 3 fish. $p < 0.05$ compared to all other treatments.

Expression pattern of the *hsc70* gene

The pair of *hsc70* primers amplified a fragment with high homology to the *hsc70-1* gene from *C. carpio* (78.2% identity; AY120893), followed by *hsc70* from *D. rerio* (81.5% identity; L77146), *M. amblycephala* (80.9% identity; EU623471) and *Ctenopharyngodon idella* (80.1% identity; EU816595). The *hsp70* gene sequences of *S. torgalensis* and *S.*

carolitertii were deposited in GenBank under accession numbers JQ608475 and JQ608474, respectively. The levels of *hsc70* gene expression in muscle and fin clips from S. carolitertii were similar in both tissues, but this was not the case for *S. torgalensis* (Figure 2.6, Supplementary material); the latter species showed higher expression in the fins compared to muscle and all subsequent analyses were done with fins. Individuals of *S. torgalensis* exposed to 35 °C showed a 14-fold increase in *hsc70* mRNA levels compared to 20 °C (control condition) and an 12-fold increase compared to 30 °C (Figure 2.4). One-way ANOVA indicated significant differences in the expression levels of the *hsc70* gene among the four temperatures ($F = 12.504$, $df = 3$, $p < 0.001$) and post-hoc comparisons identified a difference between the 35 °C treatment and the other three temperatures (Table 2.3, Supplementary material). Kruskal-Wallis analysis confirmed the presence of significant differences among the experimental conditions ($H = 15.351$, $df = 3$, $p < 0.005$). Although the non-parametric post-hoc test showed no significance between the 30 °C and 35 °C treatments, a significant difference was still observed between the 20 °C and 35 °C groups (Table 2.3, Supplementary material). In contrast, the increase in mRNA levels in *S. carolitertii* was not greater than three-fold, with the greatest increase occurring at 30 °C, although this was not statistically significant (F =1.439, df = 3, p > 0.200; Figure 2.4).

Real-time PCR confirmed the significant increase in *hsc70* expression in *S. torgalensis* at 35 °C ($F = 4.481$, $df = 3$, $p < 0.050$), whereas *S. carolitertii* showed no significant differences among the experimental conditions ($F = 1.391$, $df = 3$, $p > 0.300$) (Figure 2.5; Table 2.4, Supplementary material).

Discussion

In this study, we used fin samples (instead of other organs) to measure *hsp70* transcript expression, thereby avoiding the euthanasia of animals, which is a particularly relevant consideration when studying endangered species. Our finding agree with those of Yamashita *et al.* (2004) who found similar patterns of HSP70 expression in muscle and in fibroblasts cultured from caudal fin tissue of Xyphophorus maculatus. In *S. carolitertii*, fin clips and muscle showed similar patterns of *hsc70* expression, but this similarity was not so evident for *S. torgalensis*. However, this result needs to be interpreted with caution given the small number of muscle samples used from the latter species. Nevertheless,

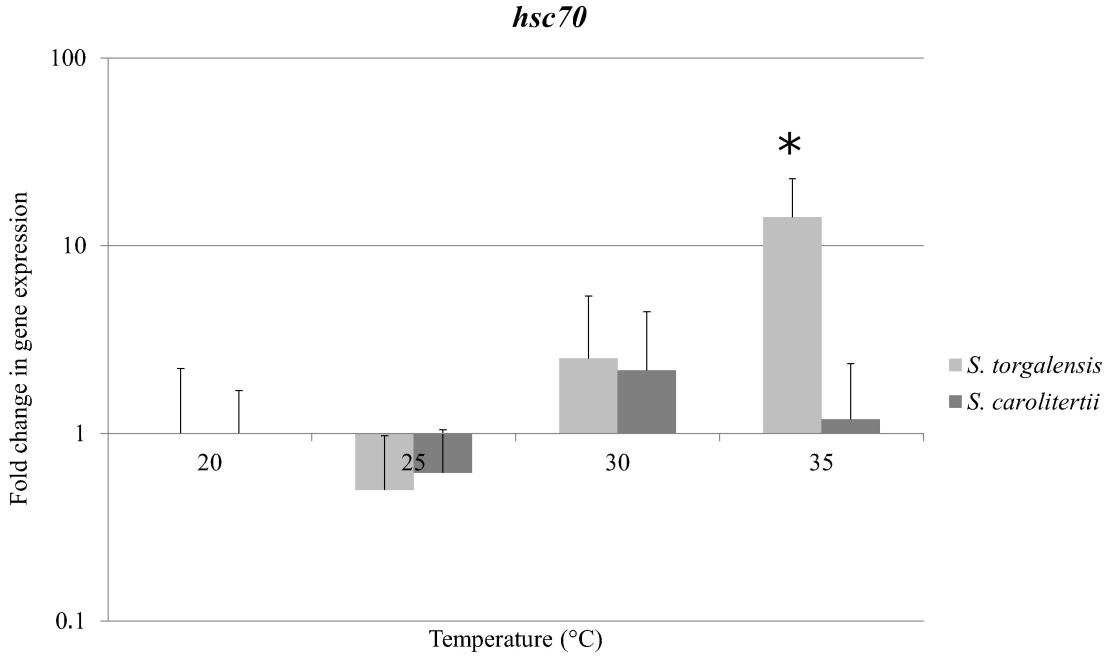

Figure 2.4: Fold change in *hsc70* transcript expression in *S. torgalensis* and *S. carolitertii* compared to 20 °C (control condition), as assessed by semi-quantitative PCR. The columns are the mean ± SD of 6 or 7 fish. $p < 0.05$ compared to all other treatments.

there was an increase in *hsc70* mRNA expression in fins of *S. torgalensis* in response to higher temperatures.

As shown here, there was an increase in *hsp70* mRNA levels in *S. torgalensis* individuals exposed to higher temperatures, as also reported for *hsp70s* in other species (Buckley *et al.*, 2001; Yeh and Hsu, 2002; Yamashita *et al.*, 2004; McMillan *et al.*, 2005; Fangue *et al.*, 2006; Karl *et al.*, 2009; Sorensen *et al.*, 2009; Sarup and Loeschcke, 2010; Waagner *et al.*, 2010). There were significant differences in the expression of this gene between *S. torgalensis* exposed to 20 °C and those exposed to other temperatures, particularly 35 °C. This result was somewhat expected since *S. torgalensis* inhabits an environment that is susceptible to extreme conditions (such as small ponds that can reach high temperatures

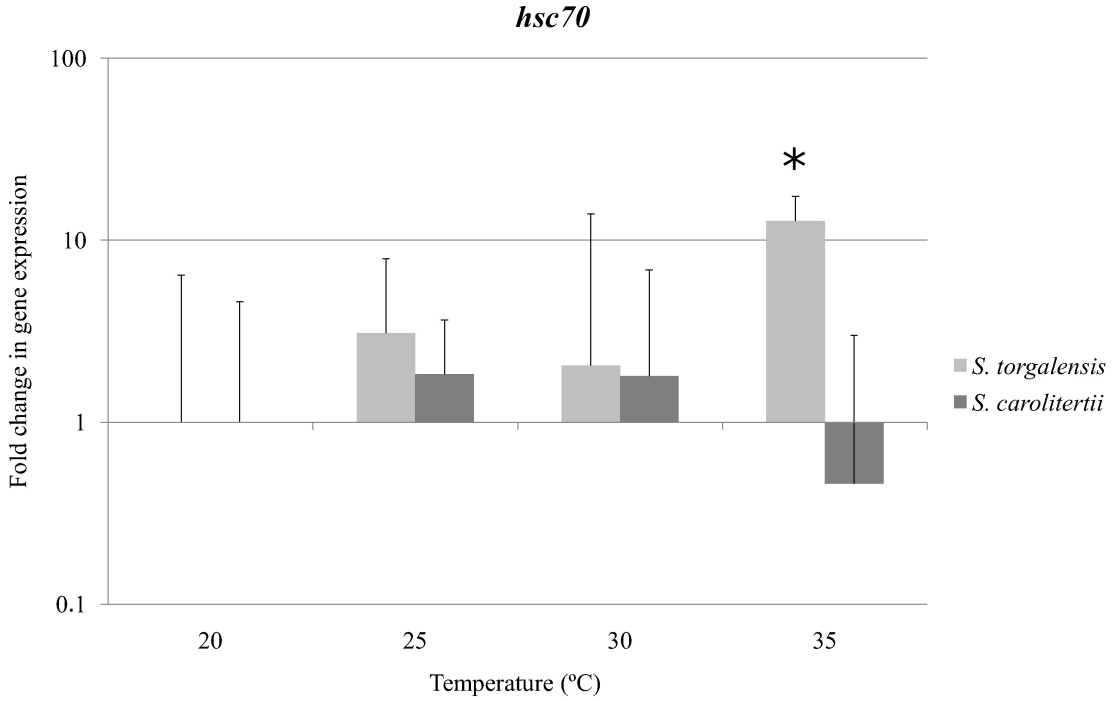

Figure 2.5: Fold change in *hsc70* transcript expression in *S. torgalensis* and *S. carolitertii* compared to 20 °C (control condition), as assessed by real-time PCR. The columns are the mean ± SD of 3 fish. $p < 0.05$ compared to all other treatments.

during the dry season) and should therefore be able to deal with protein denaturation. In contrast, *S. carolitertii* showed no significant increase in *hsp70* expression levels, which suggests that this species is unable to respond to stressful conditions associated with elevations in temperature. Unlike *S. torgalensis*, which showed the largest induction of *hsp70*, some individuals of *S. carolitertii* died at 35 °C, possibly because of this species' inability to adjust to thermal stress. The failure of *S. carolitertii* to increase the expression of *hsp70* may reflect its poor ability to adapt to 35 °C; this conclusion agrees with the fact that in its natural environment this species never experiences temperatures >31 °C (SNIRH, 2010).

However, other mechanisms may also be involved in the responses to thermal stress,

including the hormone cortisol, heat shock factors (involved in the regulation of the heat shock response), other *hsps* and even transcripts that encode other proteins (such as the protein WAP65) (Tomanek and Somero, 2002; Frydenberg *et al.*, 2003; Kassahn *et al.*, 2007; Sarropoulou *et al.*, 2010; Vandersteen Tymchuk *et al.*, 2010; Celi *et al.*, 2012). To clarify the molecular mechanisms involved, future experiments should examine how temperature influences cortisol levels in both species since interactions between HSP and cortisol are known to be involved in stress responses (Celi *et al.*, 2012). The divergent response between the two species may also reflect the more stable environment, with less severe temperature variations, in northern rivers compared to southern rivers (SNIRH, 2010).

The *hsc70* gene is often considered to be part of constitutive cell functions in non-stress situations such that an increase in temperature may either decrease or have no effect on the expression of this gene (Yeh and Hsu, 2002; Yamashita *et al.*, 2004; López-Maury *et al.*, 2008). As shown here, there was no significant variation in *hsc70* mRNA expression in *S. carolitertii* at the different temperatures. In contrast, *S. torgalensis* showed a significant increase in *hsc70* expression in fins at 35 °C when compared with the other temperatures. Thus, *S. torgalensis* can enhance the mRNA expression of inducible *hsp70* and constitutive *hsc70* in response to increases in temperature. The latter finding is similar to that of Fangue *et al.* (2006) who reported an increase in *hsc70* mRNA levels during heat stress in *F. heteroclitus* from southern North America. In addition, ATPase activity has been observed in *Gillichthys mirabilis* HSC70 at high temperatures, suggesting that this protein can function even at extreme temperatures (Place and Hofmann, 2001). With regard to our findings, the lack of an increase in mRNA expression levels in muscle makes it difficult to conclude that *hsc70* expression confers protection against thermal stress, although the enhanced expression in fins may indicate that the extensive contact surface of this tissue with the external environment might favor this response. Another possible explanation for the variation in mRNA levels between these tissues could be the existence of negative feedback (between HSP and mRNAs) in the regulation of *hsp* gene expression (Celi *et al.*, 2012).

The increase in *hsp70* expression seen at higher temperatures in *S. torgalensis* may be important in the degradation and re-folding of denatured proteins and suggests that these fish are adapted to deal with high temperatures when they are trapped in ponds during the dry season; in contrast, *S. carolitertii* is unable to deal with such high temperatures.

Magalhães *et al.* (2003) stated that *S. torgalensis* has traits typical of species adapted to harsh environments (short life span, earlier spawning age and small body size compared to other *Squalius* that inhabit more stable environments). In addition, species living closer to their thermal tolerance limits may be particularly prone to small changes in their thermal regime (Dahlhoff and Rank, 2007; Reusch and Wood, 2007; Sorensen *et al.*, 2009; Somero, 2010; Tomanek, 2010; Hoffmann and Sgrò, 2011). In this regard, intermittent systems such as that of the Mira river basin are particularly vulnerable to environmental changes. Changes in the seasonal regime of floods and droughts, with the increasing occurrence of severe droughts, may pose new challenges to these fish. Hence, to preserve this species, it would be advisable to promote habitat conservation with a particular emphasis on the conservation of refuges (pools) during the dry season (Sousa-Santos *et al.*, 2009; Henriques *et al.*, 2010).

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Supplementary material

The following material is only available online as supplementary material of the manuscript.

Table 2.1: Semi quantitative PCR post hoc comparisons for *hsp70* gene expression between treatments for *S. torgalensis*, using Tukey HSD test statistics. Each cell represents the *p-value* in each pairwise comparison. Significant differences ($p < 0.050$) are marked with *.

	20 °C	25 °C	30 °C	35 °C
20 °C		0.958	0.007*	0.000*
25 °C			0.002*	0.000*
30 °C				0.002*
35 °C				

Table 2.2: Real-time PCR post hoc comparisons for *hsp70* gene expression between treatments for *S. torgalensis*, using Tukey HSD test statistics (upper diagonal) and Dunn's test (lower diagonal). Each cell represents the p-value in each pairwise comparison. Significant differences ($p < 0.050$) are marked with *.

	20 °C	25 °C	30 °C	35 °C
20 °C		0.004*	0.000*	0.000*
25 °C	1.000		0.046*	0.000*
30 °C	0.249	1.000		0.000*
35 °C	0.013*	0.249	1.000	

Table 2.3: Semi quantitative PCR post hoc comparisons for *hsc70* gene expression between treatments for *S. torgalensis*, using Tukey HSD test statistics (upper diagonal) and Dunn's test (lower diagonal). Each cell represents the p-value in each pairwise comparison. Significant differences ($p < 0.050$) are marked with *.

	20 °C	25 °C	30 °C	35 °C
20 °C		0.960	0.593	0.000*
25 °C	1.000		0.309	0.000*
30 °C	1.000	1.000		0.001*
35 °C	0.007*	0.004*	0.139	

Table 2.4: Real-time PCR post hoc comparisons for *hsc70* gene expression between treatments for *S. torgalensis*, using Tukey HSD test statistics. Each cell represents the p-value in each pairwise comparison. Significant differences ($p < 0.050$) are marked with *.

	20 °C	25 °C	30 °C	35 °C
20 °C		0.958	0.007*	0.000*
25 °C			0.002*	0.000*
30 °C				0.002*
35 °C				

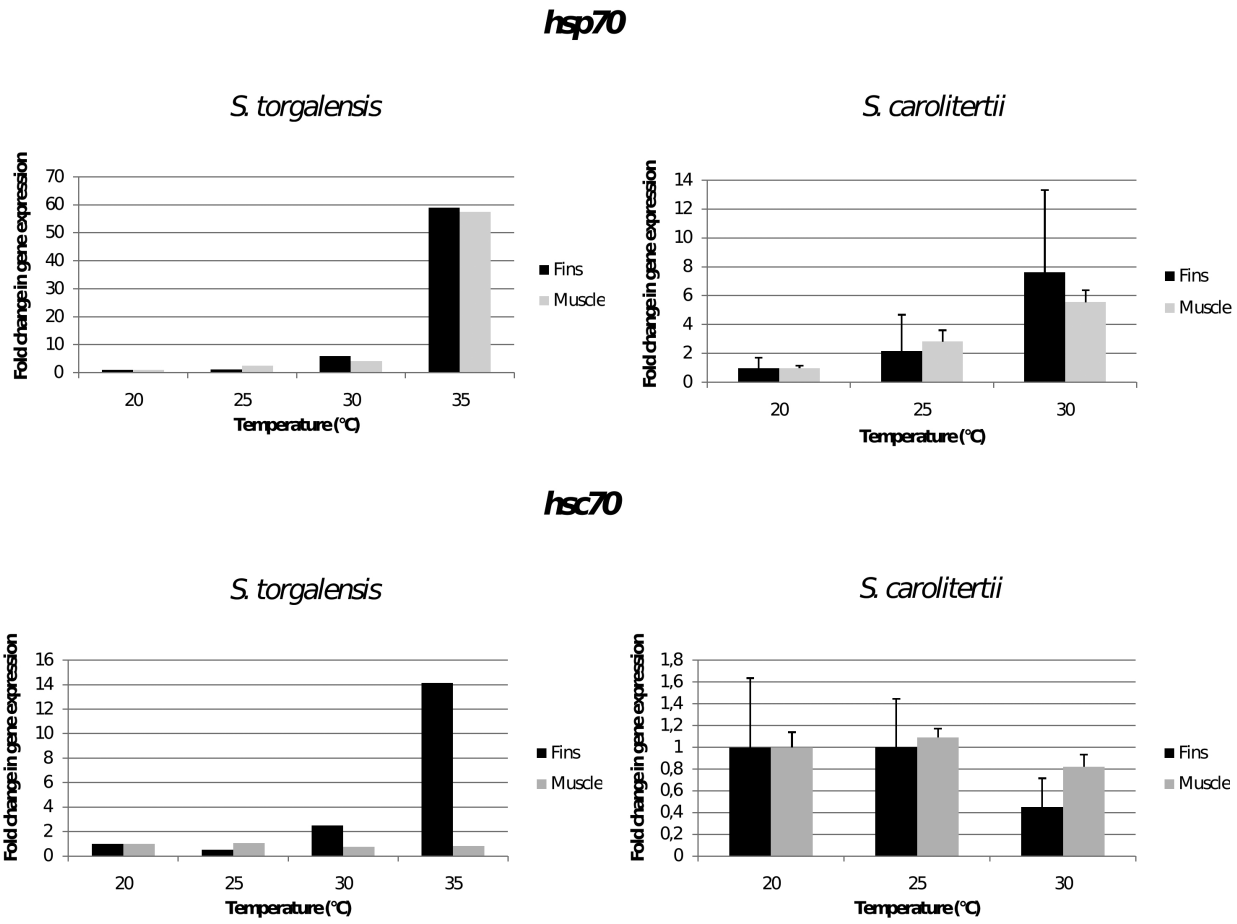

Figure 2.6: *hsp70* and *hsc70* transcript abundance in fin clips and muscle of *S. carolitertii* and *S. torgalensis*.

2.2 Transcriptome characterization of *S. carolitertii* and *S. torgalensis*

2.2.1 Genomic Resources Development Consortium

This section describes the sequencing, assembly and annotation of the transcriptomes of S. carolitertii and S. torgalensis. However, the original work was published as resources note (Genomic Resources Development Consortium, Almeida-Val V., Boscari E., Coelho M.M., Congiu L., Grapputo A., Grosso A.R., Jesus T.F., Luebert F., Mansion G., Muller L.A.H., Tore D., Vidotto M., Zane L. (2016). Genomic Resources Notes accepted 1 April 2015 - 31 May 2015. Molecular Ecology Resources, 15:1256–1257.), in which these transcriptomes were published in a consortium, together with other organism's transcriptomes from other authors. Therefore, first, in section 2.2, I present the PDF of the resources note and then the supporting information that is the result of my work on the assembly and annotation of both species transcriptomes. This supporting information is a form that was sent to the Molecular Ecology Resources journal, thus many fields are standard and different from other research papers.

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GENOMIC RESOURCES NOTE

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GENOMIC RESOURCES DEVELOPMENT CONSORTIUM,[1] VERA MARIA FONSECA ALMEIDA-VAL,[2] E. BOSCARI,[3] MARIA MANUELA COELHO,[4] L. CONGIU,[3] A. GRAPPUTO,[3] ANA RITA GROSSO,[5] TIAGO FILIPE JESUS,[4] FEDERICO LUEBERT,[6] GUILHEM MANSION,[7] LUDO A. H. MULLER,[8] DEMET TÖRE,[7] M. VIDOTTO[9,10] and L. ZANE[3]

[1]*Molecular Ecology Resources Editorial Office, 6270 University Blvd, Vancouver, BC V6T 1Z4, Canada,* [2]*Laboratório de Ecofisiologia e Evolução Molecular, Instituto Nacional de Pesquisas da Amazônia (INPA), Av. André Araújo 2.936, Petrópolis, CEP 69067-375 Manaus, AM, 2223, Brazil,* [3]*Department of Biology, University of Padova, Via G. Colombo 3, 35131 Padova, Italy,* [4]*CE3C – Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, Edifício C2 3o Piso, Campo Grande, 1749-016 Lisboa, Portugal,* [5]*Instituto de Medicina Molecular, Av. Prof. Egas Moniz, Edf. Egas Moniz, Sala P3B-34, 1649-028 Lisboa, Portugal,* [6]*Nees-Institut für Biodiversität der Pflanzen, Universität Bonn, Meckenheimer Alle 170, 53115 Bonn, Germany,* [7]*Freie Universität Berlin, Institut für Biologie – Botanik, Altensteinstraße 6, 14195 Berlin, Germany,* [8]*Botanischer Garten und Botanisches Museum, Freie Universität Berlin, Königin-Luise-Straße 6-8, 14195 Berlin, Germany,* [9]*Department of Agricultural and Environmental Sciences, University of Udine, via delle Scienze 206, Udine, Italy,* [10]*Institute of Applied Genomics, Via J. Linussio, 51, 33100 Udine, Italy*

Abstract

This article documents the public availability of transcriptomic resources for (i) the stellate sturgeon *Acipenser stellatus*, (ii) the flowering plant *Campanula gentilis* and (iii) two endemic Iberian fish, *Squalius carolitertii* and *Squalius torgalensis*.

Table 1 contains information on the focal species, data type and resource developed, as well as access details for the data. The authors responsible for each genomic resource are listed in the final column. Full descriptions of how each resource was developed and tested are uploaded as (Appendix S1–S3, Supporting

Correspondence: Genomic Resources Development Consortium,
E-mail: editorial.office@molecol.com

Table 1 Information on the focal species, data type and resource developed, as well as access details for the data. The authors responsible for each genomic resource are listed in the final column

Species (no. of individuals)	Data type	Resources	Authors
Acipenser stellatus (2)	Transcriptome sequencing, assembly, annotation, and SNP and INDEL discovery	Transcriptome sequence data: NCBI Sequence Read Archive PRJNA278747 Contig assembly: Dryad doi:10.5061/dryad.kj4mh Contigs annotation: Dryad doi:10.5061/dryad.kj4mh KEGG pathways annotation: Dryad doi:10.5061/dryad.kj4mh Putative SNP and INDEL data: Dryad doi:10.5061/dryad.kj4mh Scripts: Dryad doi:10.5061/dryad.kj4mh	Vidotto M., Grapputo A., Boscari E., Zane L., Congiu L.
Campanula gentilis (1)	Transcriptome sequencing, assembly, ORF prediction, annotation and expression levels	Transcriptome sequence data: European Nucleotide Archive: PRJEB7897 Contig assembly: Dryad DOI doi:10.5061/dryad.1hj3m Putative Open Reading Frames (ORFs): Dryad DOI doi:10.5061/dryad.1hj3m Contig and ORF annotation: Dryad DOI doi:10.5061/dryad.1hj3m Relative expression levels: Dryad DOI doi:10.5061/dryad.1hj3m	Demet Töre, Federico Luebert, Guilhem Mansion, Ludo A. H. Muller
Squalius carolitertii (14) and *Squalius torgalensis* (14)	Transcriptome sequencing, assembly and annotation	Transcriptome sequence data: NCBI Sequence Read Archive SRP049801 and SRP049802 Assembled contigs: Dryad doi:10.5061/dryad.fm28d Blast hits: Dryad doi:10.5061/dryad.fm28d Gene ontology annotations Dryad doi:10.5061/dryad.fm28d	Tiago Filipe Jesus, Ana Rita Grosso, Vera Maria Almeida-Val, Maria Manuela Coelho

Information) with the online version of this manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Transcriptomic resources for the critically endangered stellate sturgeon *Acipenser stellatus*.

Appendix S2. Transcriptome sequences for *Campanula gentilis*.

Appendix S3. Characterization of two Iberian freshwater fish transcriptomes, *Squalius carolitertii* and *Squalius torgalensis*, living in distinct environmental conditions.

2.2.2 Supporting information - Appendix S3. Characterization of two Iberian freshwater fish transcriptomes, *Squalius carolitertii* and *Squalius torgalensis*, livingin distinct environmental conditions

Authors: Tiago Filipe Jesus[1], Ana Rita Grosso[2], Vera Maria Fonseca Almeida-Val[3] and Maria Manuela Coelho[1]

[1] - CE3C – Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, Edifício C2, 3º Piso, Campo Grande, 1749-016 Lisboa, Portugal.

[2] - Instituto de Medicina Molecular, Av. Prof. Egas Moniz, Edf. Egas Moniz, Sala P3B-34, 1649-028 Lisboa, Portugal.

[3] - Laboratório de Ecofisiologia e Evolução Molecular, Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM, Brasil.

Abstract

The advance of NGS technologies opened exciting research avenues, as for example expanding the study of the mechanisms underlying adaptation from model organisms to natural systems. We used NGS technologies to sequence 12 RNA-seq libraries, and provide the first transcriptomes of two endemic Iberian Cyprinids. The species *Squalius carolitertii* and *S. torgalensis* inhabit different regions of Portugal with distinct climate types, Atlantic in the North and Mediterranean in the South, respectively. While northern regions present mild temperatures, in southern regions fish are often under harsh temperatures and droughts. Herein, we sequenced the transcriptome from three tissues (skeletal muscle, liver and fins) in an Illumina HiSeq2000 of fish exposed to different temperatures: 18^{o}C (control) and 30^{o}C (test). Around 200 million raw reads were generated for each species, with similar number of reads per library (approximately 30 million), rendering de novo assemblies with a total of 145975 and 137303 contigs, for *S. carolitertii* and *S. torgalensis*, respectively. Gene ontology showed that around 60% of the annotated genes belonged to four biological processes and approximately 75% to two molecular functions. Besides, this study provides, for the first time, the transcriptome characterization of two endemic fish from Iberian freshwater basins, *S. carolitertii* and *S. torgalensis*, and constitutes a valuable resource for understanding environmental adaptations of Iberian Cyprinids.

Introduction

The Iberian Peninsula presents a remarkable endemic biodiversity, typical of the circum Mediterranean areas. Cyprinids are among the richest freshwater fish families in endemic representatives in the Iberian Peninsula, some of which inhabit in just one river basin (Coelho *et al.*, 1998; Sousa-Santos *et al.*, 2007). This pattern of high endemism is presumably related with the role of the Peninsula as refugia during the Pleistocene glaciations (Filipe *et al.*, 2009). However, the observed biodiversity can also be the result of climatic heterogeneity (Filipe *et al.*, 2009; Carvalho *et al.*, 2010). The Iberian Peninsula presents two distinct types of climates, Atlantic in the north and Mediterranean, in the south (Carvalho *et al.*, 2010). The *Squalius* genus (Cyprinidae family) presents an opportunity to study closely related species as a proxy of these distinct types of climates, because some species are restricted to certain river basins or regions. For example, *S.*

carolitertii (Doadrio, 1988) inhabits the northern region (Atlantic climate) whereas *S. torgalensis* (Coelho *et al.*, 1998), a critically endangered species (Cabral *et al.*, 2006), has a restricted distribution to the Mira river basin in the southwestern region (Coelho *et al.*, 1998) (Mediterranean climate). So, the two species are acclimatized to different environmental conditions with distinct seasonal and even daily water temperature variations (Magalhães *et al.*, 2003; Jesus *et al.*, 2013). In northern rivers lower temperatures and fewer temperature fluctuations are observed, ranging from 3 to 31 °C throughout the year. On the other hand, southern rivers are characterized by an intermittent regime of floods and droughts in which freshwater fish are exposed to higher temperatures, ranging from 4 to 38 °C, what results in lower oxygen concentrations (Magalhães *et al.*, 2003; Henriques *et al.*, 2010; Jesus *et al.*, 2013).

Despite fairly studied from the phylogenetic and conservation genetics point of view, both *S. carolitertii* and *S. torgalensis* as other *Squalius* relatives suffer from a massive lack of genomic resources, resulting in some unresolved taxonomic relationships between species (Gante *et al.*, 2010; Almada and Sousa-Santos, 2010; Waap *et al.*, 2011). This limitation was evident in a previous study that attempted to understand how these two species cope with different temperatures (Jesus *et al.*, 2013). In that study, it was observed that *S. carolitertii* showed no significant changes in the expression of genes related to thermal stress, *hsp70* and *hsc70*, while *S. torgalensis* presented a significant up regulation of both genes. These results suggest that *S. torgalensis* is better adapted to harsher temperatures than *S. carolitertii*. Nevertheless, the thermal stress response is far more complex and other genes are most probably involved (Lindquist and Craig, 1988; Murtha, 2003; López-Maury *et al.*, 2008; de Nadal *et al.*, 2011).

The development of "next-generation" sequencing technologies facilitated the sequencing of large amounts of genes, including for non-model species, allowing comprehensive studies of unknown genomes (Ekblom and Galindo, 2011; Kawakami *et al.*, 2014; Lamanna *et al.*, 2014). In the present study, we present the first transcriptomes of two endemic Iberian freshwater fish, *S. carolitertii* and *S. torgalensis*, encompassing 12 RNA-seq libraries and sequence information from Illumina HiSeq 2000 for three different tissues (fins, liver and skeletal muscle) and two temperatures (control and test). We aimed to (i) characterize the transcriptomes of liver, muscle and fins from these two species exposed to different temperature conditions; and (ii) obtain sequence resources to be used in future studies, in particular on environmental adaptation of these freshwater fish.

Data Access

NGS raw sequence files: Raw sequences are available from NCBI SRA (projects accession number SRP049802 and SRP049801). Individual SRA numbers are provided in Table 2.5.

Assembled contigs: Assemblies in fasta format (.fas) are available from Dryad entry doi:10.5061/dryad.fm28d. Blast hits (with NCBI nonredundant protein (nr) database): The files in txt format (.txt), containing the top blast hits, are accessible on Dryad: doi:10.5061/dryad.fm28d.

Gene ontology annotations: The files are in txt format (.annot) and contains the gene ontology annotations retrieved by Blast2GO program. Available on Dryad: doi:10.5061/dryad.fm28d.

Meta Information

Sequencing center – Bgi Tech Solutions CO., Limited (Shenzhen, China, http://www.genomics.cn/).

Platform and model – Illumina HiSeq™ 2000.

Design Description- Adult fish (6 -7 cm) of *S. carolitertii* and *S. torgalensis* were captured, by electrofishing (300V, 4A), in Mondego and Mira rivers, respectively. Sampling was carried out during spring, when water temperature varied from 18 °C to 22 °C, approximately (Jesus *et al.*, 2013). Fish were maintained in groups of seven fish in four aquariums of 30 L, two for each species. Temperature was kept constant at 18 °C with a 12 h photoperiod and fish were fed once a day with commercial flake food, for two weeks. After these two weeks of acclimation, temperature was raised 1 °C/h until 30 °C in one aquarium for each species, where fish were kept for 1 h and euthanized. Temperature was kept constant at 18 °C in the remaining aquaria, and fish were maintained at acclimation conditions and euthanized at the same time of the test group. In both cases euthanasia was carried out with tricaine mesylate (400 ppm of MS-222; Sigma-Aldrich, St. Louis, MO, USA) and promptly decapitate previously to the organs harvesting to guarantee the death. In all aquariums oxygen was kept in normoxic conditions (6 – 8 mg/L of O_2). Samples from skeletal muscle, liver and fins were collected in RNAlater (Ambion, Austin, TX, USA) and stored according with manufacturer' instructions.

Analysis type – RNA.

Run date – 2013/02/04.

Library

Strategy – RNA-seq (Illumina).

Taxon – *Squalius torgalensis* and *Squalius carolitertii*.

Sample details – 7 adult individuals per species per temperature treatment with unknown sexes.

Tissue – Skeletal muscle, liver and fins.

Location – Mira River (37.633198, -8.624536) and Mondego River (40.136077, -8.144272).

Sample handling – n/a.

Additional sample information – n/a.

Selection – n/a.

Layout – Paired-end reads (2 × 90 bp).

Library Construction Protocol - RNA was extracted from skeletal muscle, liver and fin clips, as in Jesus *et al.* (2013), using the seven individuals from each treatment. Samples were homogenized with a TissueRuptor (Qiagen, Valencia, CA, USA) and RNA was extracted using TRI Reagent (Ambion, Austin, TX, USA) and TURBO DNase (Ambion) was used to degrade any remaining genomic contaminants. Quality and quantity of samples were checked using a Nanodrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Equal amounts of RNA from seven samples of each organ, were pooled into one library and quality was accessed with an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Twelve pools (3 tissues × 2 species × 2 temperature treatments) with at least 5 g of RNA were submitted to BGI Tech Solutions CO., Limited (BGI, Shenzhen, China) for sequencing. At BGI, beads with Oligo(dT) were used to isolate poly(A) mRNA. Fragmentation buffer was added for breaking mRNA to short fragments. Random hexamer-primers were used to synthesize the first-strand cDNA. The second-strand cDNA was synthesized using buffer, dNTPs, RNaseH and DNA polymerase I. Short fragments were purified with QiaQuick PCR extraction kit, resolved with EB buffer for end reparation and added poly(A). Short fragments were ligated to sequencing adapters and, after agarose gel electrophoresis, suitable fragments were selected for PCR amplification as templates. Finally, libraries were sequenced using Illumina HiSeq™ 2000 (Paired-end, 90 bp).

Processing

After sequencing, the quality of the resulting raw sequence files (fastq) was checked using FastQC v0.10.1 (Andrews, 2010) and adapter sequences and reads containing "N" characters were removed using PRINSEQ-lite 0.19.5 (Schmieder and Edwards, 2011). Then, the first 5' end nucleotide of all reads were removed given its low quality and at the 3' end, nucleotides with phred quality score lower than 20, were removed (both performed in PRINSEQ-lite 0.19.5). These filters improved the quality of reads for posterior applications, enhancing the accuracy of the assembly (Vijay *et al.*, 2012; Garcia *et al.*, 2012; Schliesky *et al.*, 2012).

Trinity (Grabherr *et al.*, 2011) was used to perform de novo assembly for both species. First, Trinity partitions the sequence data into many individual de Bruijn graphs, then each graph extracts the full-length splicing isoforms and group them in clusters and, finally, assigns transcripts derived from paralogous genes (Grabherr *et al.*, 2011). Each organ was assembled separately and posteriorly a draft transcriptome, containing all three tissues, was constructed using cd-hit-est [from the program CD-HIT version 4.6 (Li *et al.*, 2006)], with redundancy removal.

Assembled contigs (for both transcriptomes) were searched against NCBI nonredundant protein (nr) database using blastx [BLAST 2.2.28+ (Camacho *et al.*, 2009)], using an e-value cut-off of 1-6 and 3 blast hits were stored in the resulting xml file. The top blast hit (highest e-value) was held for each blast query and Gene Ontology (GO) terms were assigned utilizing Blast2GO (Conesa *et al.*, 2005) (E-value cut-off = 1-6 and HSP = 55).

Results

Total number of reads ranged from 32,032,530 to 34,396,772 (Table 2.5) and, after filtering, read length ranged from 47 to 89 nt for both *S. carolitertii* and *S. torgalensis*, while the total number of reads remained equal. Read quality was in general poorer in fin clips with a drop in quality of the 3' end of reads, particularly in fastq files of the 2nd sequencing end, retrieving shorter read lengths, which are reflected in the average read length (Table 2.5).

After redundancy removal, a total number of 145,975 and 137,303 contigs were obtained for *S. carolitertii* and *S. torgalensis*, respectively (Table 2.6). From these contigs,

38.94% and 40.38% had blast hits for *S. carolitertii* and *S. torgalensis* (Table 2.7), with 73.55% and 75.23% first hits corresponding to protein-coding genes known for *Danio rerio*, respectively (Figure 2.7).

Gene ontology analysis revealed 83,435 and 85,118 biological processes and 33,468 and 34,011 molecular functions for *S. carolitetii* and *S. torgalensis*, respectively, with both species showing similar proportions of each gene ontology category (Figure 2.8). For both species, over 60% of the genes were assigned to four biological processes (cellular process, single-organism process, metabolic process and biological regulation) and over 75% to two molecular functions (binding and catalytic activity) (Figure 2.8).

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Tables

Table 2.5: Total number of reads sequenced and average length of the sequences after quality filters for the 1st and 2nd end sequenced.

	Tissue	Condition	Number of reads	Average read lenght (1st end)	Average read lenght (2nd end)
Squalius carolitertii	Liver	18 °C	34353812	88.00 ± 3.63 nt	87.37 ± 4.55 nt
		30 °C	34273118	87.69 ± 4.28 nt	87.60 ± 4.30 nt
	Muscle	18 °C	34353812	87.84 ± 4.10 nt	87.89 ± 3.84 nt
		30 °C	34396772	87.94 ± 3.90 nt	87.90 ± 3.83 nt
	Fins	18 °C	32121586	88.08 ± 3.51 nt	85.75 ± 6.44 nt
		30 °C	32032530	88.07 ± 3.50 nt	85.75 ± 6.44 nt
Squalius torgalensis	Liver	18 °C	34141975	87.96 ± 3.74 nt	87.42 ± 4.51 nt
		30 °C	32789234	87.75 ± 4.13 nt	87.54 ± 4.37 nt
	Muscle	18 °C	32304376	87.88 ± 4.00 nt	87.92 ± 3.78 nt
		30 °C	33891437	87.92 ± 3.94 nt	87.91 ± 3.82 nt
	Fins	18 °C	33405759	88.05 ± 3.55 nt	85.85 ± 6.33 nt
		30 °C	33762905	88.05 ± 3.52 nt	85.65 ± 6.50 nt

Table 2.6: *de novo* assembly statistitcs for each tissue and for the total transcriptome.

Species	Tissue	Number of contigs	Average contig lenght	N50	GC content (%)
Squalius carolitertii	Liver	96430	898.47	1592	45.83
	Muscle	80981	806.16	1332	46.83
	Fins	105297	963.24	1778	45.60
	Total	145975	801.96	1454	44.96
Squalius torgalensis	Liver	66206	786.29	1277	46.01
	Muscle	82050	857.74	1460	46.82
	Fins	111360	883.96	1586	45.44
	Total	137303	796.21	1340	44.98

Table 2.7: Annotation statistics for whole transcriptome draft.

	Number of contigs	no blast hits (%)	blast hits (%)	
			unknown function	known function
Squalius carolitertii	145975	61.06	19.39	19.55
Squalius torgalensis	137303	59.62	19.34	21.03

Figures

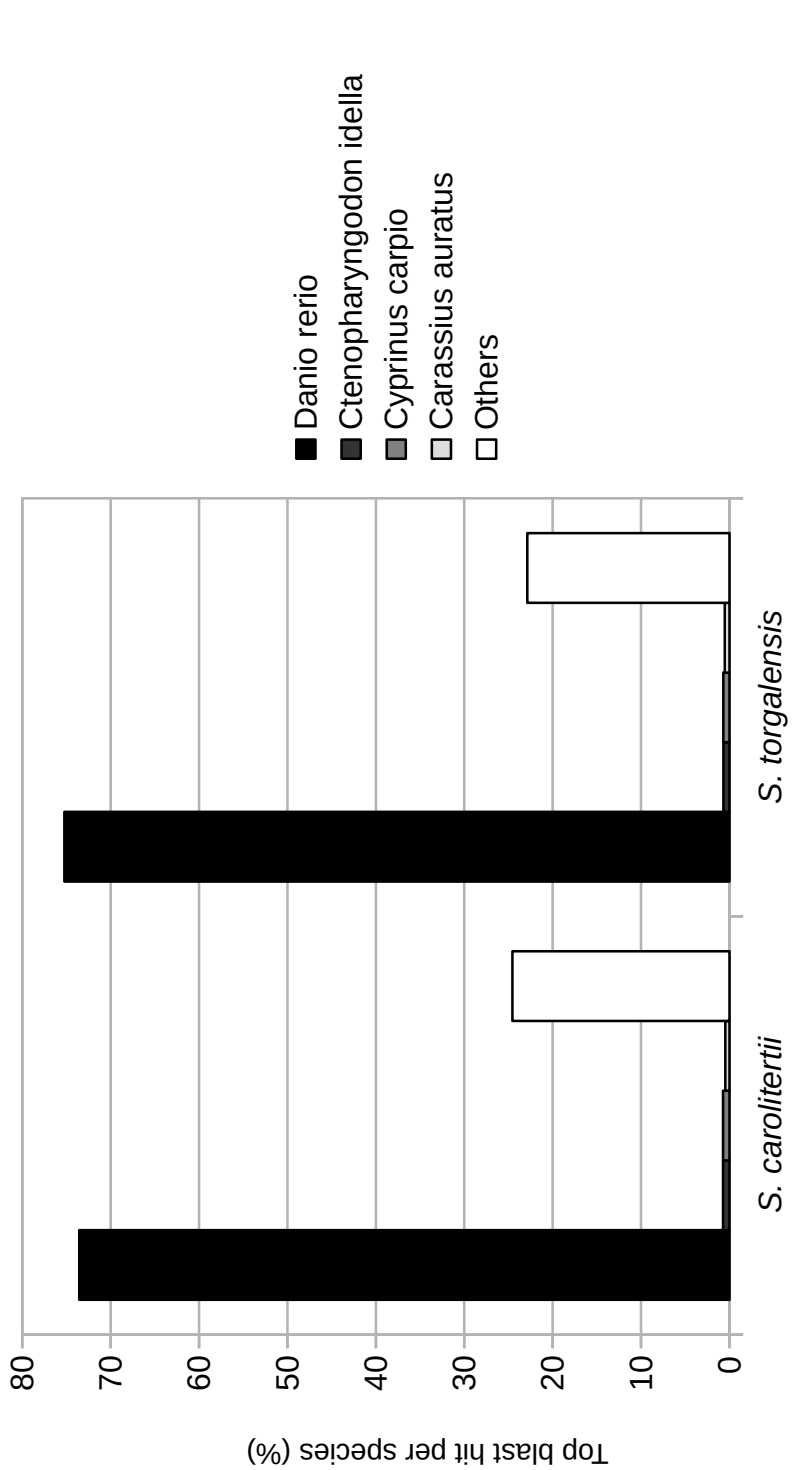

Figure 2.7: Species distribution of top blast hits for both transcriptomes, with focus on four Cyprinidae species.

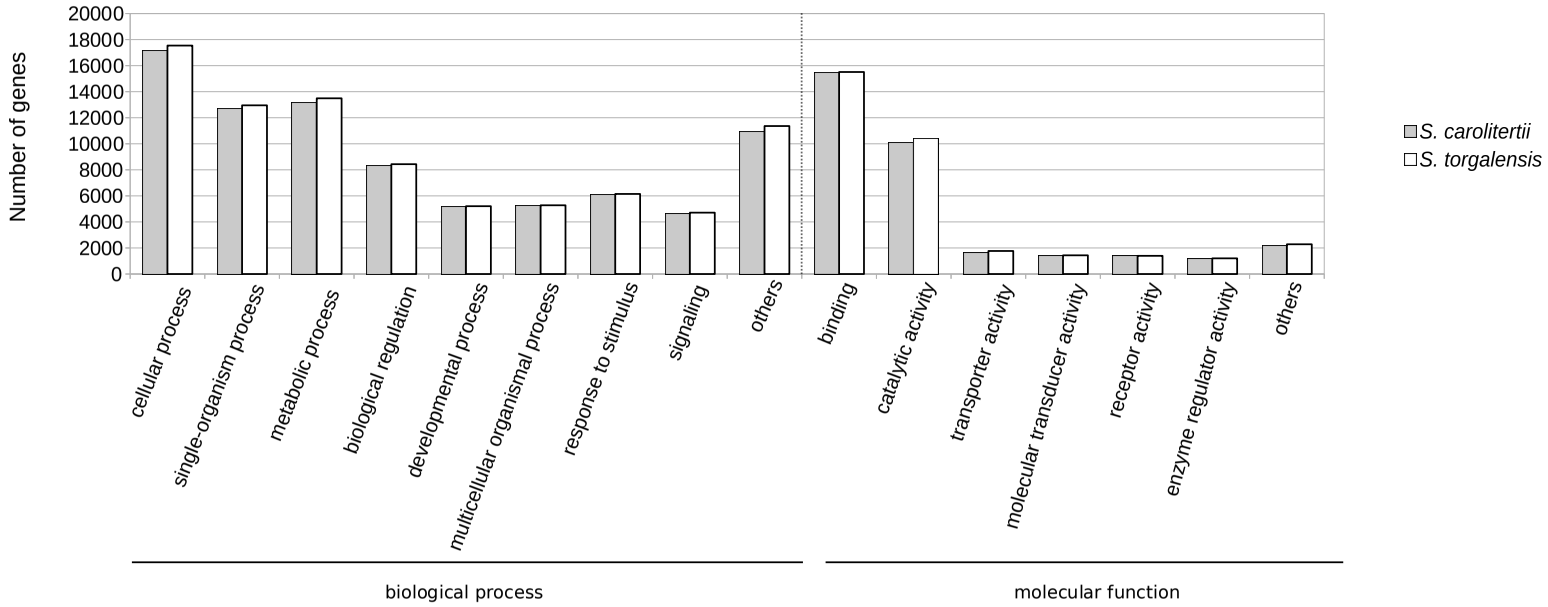

Figure 2.8: Number of genes for the most common gene ontology categories (biological process and molecular functions) for *S. carolitertii* (grey) and *S. torgalensis* (white).

2.3 Transcriptome profiling of two Iberian freshwater fish exposed to thermal stress

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Tiago Filipe Jesus[1], Ana Rita Grosso[2], Vera Maria Fonseca Almeida-Val[3] and Maria Manuela Coelho[1]

[1] - CE3C – Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, Edifício C2, 3º Piso, Campo Grande, 1749-016 Lisboa, Portugal

[2] - Instituto de Medicina Molecular, Av. Prof. Egas Moniz, Edf. Egas Moniz, Sala P3B-34, 1649-028 Lisboa, Portugal

[3] - Laboratório de Ecofisiologia e Evolução Molecular, Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM, Brasil.

Abstract

The congeneric freshwater fish *Squalius carolitertii* and *S. torgalensis* inhabit different Iberian regions with distinct climates; Atlantic in the North and Mediterranean in the South, respectively. While northern regions present mild temperatures, fish in southern regions often experience harsh temperatures and droughts. Previous work with two *hsp70* genes suggested that *S. torgalensis* is better adapted to harsher thermal conditions than *S. carolitertii* as a result of the different environmental conditions. We present a transcriptomic characterization of these species' thermal stress responses. Through differential gene expression analysis of the recently available transcriptomes of these two endemic fish species, comprising 12 RNA-seq libraries from three tissues (skeletal muscle, liver and fins) of fish exposed to control (18 °C) and test (30 °C) conditions, we intend to lay the foundations for further studies on the effects of temperature given predicted climate changes. Results showed that *S. carolitertii* had more upregulated genes, many of which are involved in transcription regulation, whereas *S. torgalensis* had more downregulated genes, particularly those responsible for cell division and growth. However, both species displayed increased gene expression of many *hsps* genes, suggesting that they are able to deal with protein damage caused by heat, though with a greater response in *S. torgalensis*. Together, our results suggest that *S. torgalensis* may have an energy saving strategy during short periods of high temperatures, re-allocating resources from growth to stress response mechanisms. In contrast, *S. carolitertii* regulates its metabolism by increasing the expression of genes involved in transcription and promoting the stress response, probably to maintain homeostasis. Additionally, we indicate a set of potential target genes for further studies that may be particularly suited to monitoring the responses of Cyprinidae to changing temperatures, particularly for species living in similar conditions in the Mediterranean Peninsulas.

Keywords: Cyprinidae; gene expression; RNA-seq; *Squalius*; temperature

Introduction

Temperature is crucial to survival, and thermal adaptation is increasingly of interest given the growing threat of climate change. Freshwater ecosystems are particularly prone to

the effects of climate change, such as shifts in thermal, precipitation and flow regimes (Field *et al.*, 2014). Often, this is coupled with an increase in the severity and frequency of droughts, ultimately resulting in an increase in mean water temperature and a decrease in oxygen concentration (Field *et al.*, 2014). Such changes in natural freshwater systems directly influence survival and persistence of extant populations. Ectotherms, such as fish, are especially vulnerable to environmental temperature changes since their body temperature strongly relies on it (Berg *et al.*, 2010). Therefore, to cope with these changes, fish must either exhibit phenotypic plasticity or adapt through micro-evolution, since migration to a more suitable river is often not possible or easily achieved (Bellard *et al.*, 2012).

The Iberian Peninsula presents two distinct types of climate, the Atlantic in the north and Mediterranean in the south (Carvalho *et al.*, 2010). Northern rivers present lower temperatures and fewer temperature fluctuations, ranging from 3-31 °C throughout the year. In contrast, southern rivers are characterized by an intermittent regime of floods and droughts in which freshwater fish are exposed to higher temperatures, ranging from 4-38 °C, which also results in lower oxygen concentrations (Magalhães *et al.*, 2003; Henriques *et al.*, 2010; Jesus *et al.*, 2013). These southern rivers are also more likely to be exposed to extreme temperatures and more extended drought periods (Füssel *et al.*, 2012).

The *Squalius* genus (Cyprinidae family) presents an opportunity to study closely related species under distinct climate scenarios because some species are endemic to certain river basins or regions. *S. carolitertii* (Doadrio, 1988) inhabits the northern region of the Iberian Peninsula (Atlantic climate), whereas *S. torgalensis* (Coelho *et al.*, 1998), a critically endangered species (Cabral *et al.*, 2006), is restricted to the Mira river basin in the southwestern region (Coelho *et al.*, 1998) (Mediterranean climate) (Figure 2.9). Hence, the two species are adapted to different environmental conditions, with distinct seasonal and even daily water temperature variations (Magalhães *et al.*, 2003; Jesus *et al.*, 2013).

From a physiological point of view, little is known about the responses of these two species to thermal stress, with only one study characterizing changes in gene expression of two Heat Shock Proteins (HSPs) in response to thermal stress (Jesus *et al.*, 2013). In that study, fish of both species were exposed to four temperature treatments (20 °C, 25 °C, 30 °C and 35 °C), with increments of 1 °C per day, and, after reaching the test temperature, fin clips were collected for gene expression. *S. carolitertii* presented no significant changes in the expression of *hsp70* and *hsc70*, whereas both genes were significantly upregulated

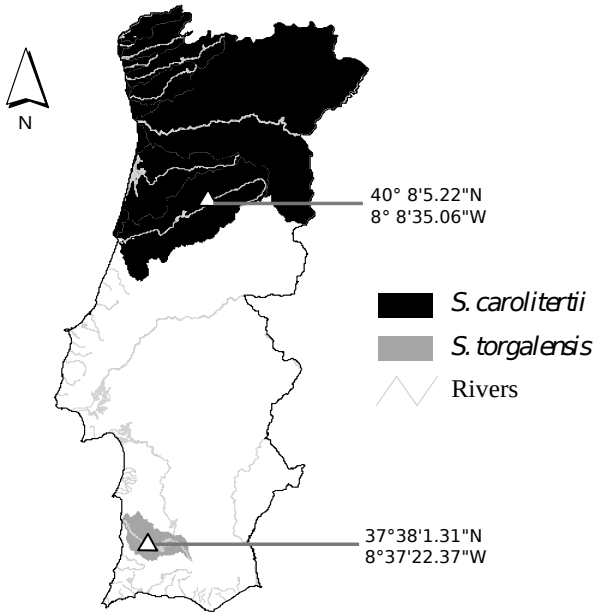

Figure 2.9: Species distribution map. Sampling sites are marked with a triangle.

in *S. torgalensis* when exposed to a higher temperature (35 °C). Also, two out of seven individuals of *S. carolitertii* did not survive at 35 °C, whereas all *S. torgalensis* individuals survived all treatments. Based on those results, it was suggested that *S. torgalensis* is better adapted to harsher thermal conditions than *S. carolitertii*. However, thermal stress responses are more complex and certainly involve the regulation of other genes (Lindquist and Craig, 1988; Murtha, 2003; López-Maury *et al.*, 2008; de Nadal *et al.*, 2011).

The recent availability of the transcriptomes of both these species, *S. carolitertii* and *S. torgalensis*, (Genomic Resources Development Consortium, Almeida-Val *et al.*, 2015), comprising 12 RNA-seq libraries and sequence information from three different tissues (fins, liver and skeletal muscle) at two temperatures (18 °C and 30 °C), made it possible for us to perform a more comprehensive analysis of their responses to increasing temperatures. Here, we take advantage of these transcriptomes to profile the gene expression responses to thermal stress in three different tissues of these two species, thereby extending our previous research (Jesus *et al.*, 2013). Specifically, we aimed to (i) characterize the transcriptomic

responses of both species to heat stress, both quantitatively and qualitatively; and (ii) search for a set of target genes involved in relevant functional categories for thermal stress responses in fish.

Methods

Data Acquisition

The recently available transcriptomes of *S. carolitertii* and *S. torgalensis* were obtained from Dryad (entry doi:10.5061/dryad.fm28d) and raw sequences were accessed in NCBI SRA (project accession numbers SRP049802 and SRP049801). For these transcriptomes, adult fish (6 -7 cm) of *S. carolitertii* and *S. torgalensis* were captured, by electrofishing (300V, 4A), in Mondego and Mira rivers, respectively (Figure 2.9). Sampling was carried out during spring, when water temperature varied from 18 °C to 22 °C, approximately. Fish were maintained in groups of seven fish in four aquariums of 30 L, two for each species. Temperature was kept constant at 18 °C with a 12 h photoperiod and fish were fed once a day with commercial flake food, for two weeks. After these two weeks of acclimation, the temperature was raised 1 °C/h until 30 °C in one aquarium for each species, where fish were kept for 1 h before being euthanized. Temperature was kept constant at 18 °C in the remaining aquaria, and the fish they contained were maintained at acclimation conditions and euthanized at the same time as the test group. In both cases, euthanasia was carried out with tricaine mesylate (400 ppm of MS-222; Sigma-Aldrich, St. Louis, MO, USA), followed by decapitation to guarantee death prior to organ harvesting. In all aquariums, normoxic conditions were maintained (6 – 8 mg/L of O_2).

RNA was extracted as described in Genomic Resources Development Consortium, Almeida-Val *et al.* (2015) and samples of the same tissue were pooled prior to sequencing, comprising 12 RNA-seq libraries (7 pooled individuals per library), with 6 libraries per species. For each species, there are two libraries per tissue (fins, liver and skeletal muscle); one from a control condition of 18 °C, and another from a test condition of 30 ^{o}C (the temperature was raised 1 °C/h from 18 °C up to 30 °C). The detailed experimental design, as well as the transcriptome assembly procedure, can be found at Genomic Resources Development Consortium, Almeida-Val *et al.* (2015).

Differential gene expression

Abundance estimation was performed by aligning the raw reads of a given library against the respective species transcriptome (available at Dryad entry doi:10.5061/dryad.fm28d) using bowtie 0.12.9 (Langmead *et al.*, 2009). Then, RSEM 1.2.8 (Li and Dewey, 2011) was used to compute expression, both in read counts and fragments per kilobase of exon per million fragments mapped (FPKMs) (Trapnell *et al.*, 2010).

In order to assess similarity between tissues, samples were grouped based on hierarchical clustering (Euclidean distance) using expression values ($\log_2$ FPKM) of the 4,000 most variable contigs across all samples of each species.

Differential gene expression analyses were performed in EdgeR, Bioconductor R package (Robinson *et al.*, 2010), using the $run_D E_a nalysis.pl$ script from the Trinity package (Grabherr *et al.*, 2011). For these analyses, we compared two temperatures for each tissue and each species (e.g. Liver 18 °C vs Liver 30 °C). Transcripts with a sum of read counts < 10 in both conditions were discarded in further analyses and we used the statistical cut-off of a false discovery rate (FDR) $< 5\times10^{-4}$, together with a cut-off |Fold Change| $\approx$ 1.5 ($|\log_2(\text{Fold Change})| > 0.58$) to select differentially expressed (DE) transcripts. Transcripts with significant variations were searched against the NCBI non-redundant protein (nr) database using blastx (BLAST 2.2.28+ (Camacho *et al.*, 2009)), using an e-value cut-off of 1×10^{6} and storing 10 blast hits. The top blast hit (highest e-value) was held for each blast query and Gene Ontology (GO) terms were assigned utilizing Blast2GO (Conesa *et al.*, 2005) (e-value cut-off = 1×10^{-6} and Highest Scoring Pair = 55). Contigs that corresponded to blast hits were renamed as the accession number, thus allowing us to directly compare contigs with the same accession number in both species. Contigs with no accession numbers maintained their original names (obtained by the assembly).

A list of accession numbers per tissue was constructed for the top blast hits as an input list for the DAVID functional annotation tool (Huang *et al.*, 2009a,b). Two other lists of accession numbers (per tissue) were provided as an input to DAVID: one with the upregulated genes, and another with the downregulated genes. We used a minimum number of counts ≤ 2 and an EASE score < 0.05 for all functional analyses performed in DAVID. Through this approach, we intended to find enriched GO terms separately among upregulated and downregulated genes. We then plotted the most significant enriched GO terms for Biological Process and Molecular Function and KEGG Pathways

using a threshold for adjusted *p-values* (Benjamini) of 0.05 for all DE contigs. To provide a comprehensive picture of thermal responses through transcriptome alterations, we produced a list of genes that show expression variations under increasing temperatures, common to our data and previous works (Buckley *et al.*, 2006; Kassahn *et al.*, 2007; Lewis *et al.*, 2010; Smith *et al.*, 2013). Furthermore, other DE genes in this study involved in three main biological processes (protein folding, immune response and oxidative stress response) were added to this list Table 2.12.

We used python and R scripts to parse files and generate graphics in several steps of the analyses.

Results

Clustering analysis of the 4,000 most variable contigs for *S. carolitertii* showed that both 18 °C and 30 °C treatments grouped well in skeletal muscle, while the fins and liver of fish subjected to 30 °C were more alike than the same tissue from fish subjected to 18 °C (Figure 2.13, Supplementary material). A similar clustering analysis for *S. torgalensis* generated a clustering pattern in which both treatments of the same tissue were grouped, generating three main clusters by tissue (Figure 2.13, Supplementary material).

Differential expression analysis between 18 °C and 30 °C revealed 1,409 to 6,597 DE genes for *S. carolitertii* and 493 to 10,044 DE genes for *S. torgalensis* (Table 2.8 and Figure 2.14, Supplementary material). Since 70-71% of DE genes had at least one blast hit (Table 2.8, Supplementary material) and the general pattern of gene expression of all tissues did not change with the inclusion of non-annotated contigs (Figure 2.10 and Fig. 2.14, Supplementary material), only the annotated protein-coding genes were considered in downstream analysis. Also, through this procedure we ensure that genes are the same when comparing both species in downstream analysis, while for non-annotated contigs comparisons are difficult between species.

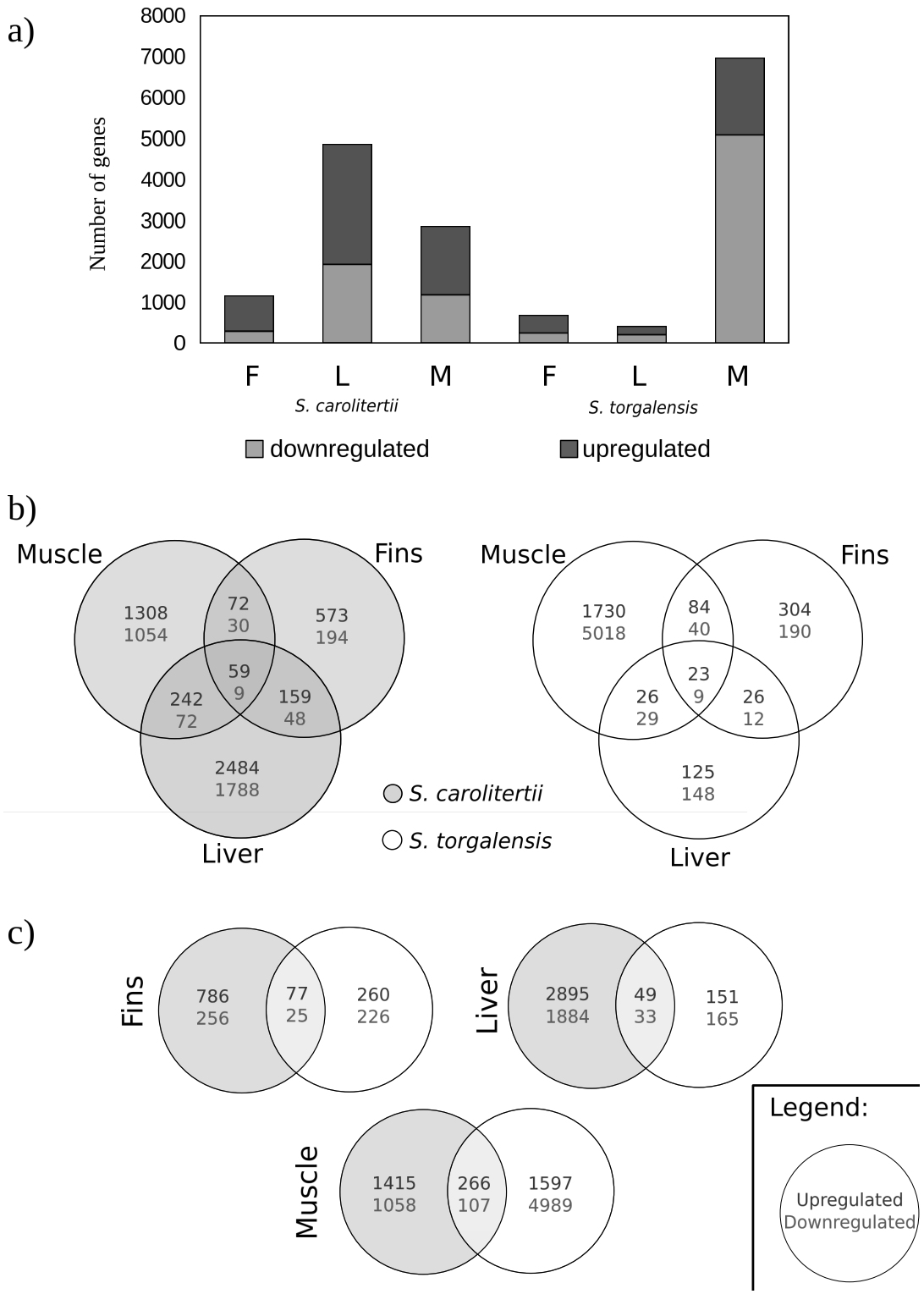

Figure 2.10: *Continues on next page.*

Figure 2.10: Number of DE genes up (dark grey) and downregulated (light grey), for both species – *S. carolitertii* (grey) and *S. torgalensis* (white). a) Total number of up- and downregulated genes, in relation to the control condition, per organ of each species. F corresponds to fins, L to liver and M to skeletal muscle. b) Genes commonly expressed between tissues represented in a Venn diagram. c) DE genes common to both species in the same tissue represented in a Venn diagram. In both Venn diagrams, the above number represent the number of upregulated genes and the bottom number the number of downregulated genes.

S. carolitertii presented a higher number of DE genes for liver and more upregulated genes for all tissues (Figure 2.10a). In contrast, *S. torgalensis* presented a much greater number of DE genes in skeletal muscle, with similar numbers of upregulated and downregulated genes in fins and liver (around 40% and 50% of downregulated genes, respectively) and a high proportion of downregulated genes in muscle (over 70%) (Fig. 2.10a).
S. carolitertii displayed a larger number of genes shared by at least two tissues than *S. torgalensis*, particularly among upregulated genes (Figure 2.10b). In turn, *S. torgalensis* presented few shared DE genes between tissues, which was to be expected given the reduced number of DE genes in fins and liver (Figure 2.10b). Pairwise comparisons between both species showed a higher number of common genes in skeletal muscle (around 10%), with 2% (107 of 6154 genes) of downregulated genes shared between both species, contrasting with 8% (266 of 3278 genes) of upregulated genes (Figure 2.10c). Moreover, fins and liver presented 7% and 2% of shared DE genes, respectively, as a result of the reduced number of DE genes in these tissues, particularly in liver, for *S. torgalensis*. However, overall gene expression of all these tissues revealed no difference in the number of DE genes in each species, from which 2109 are common between them (Fig. 2.15, Supplementary material).
Results of gene ontology analysis of DE genes did not differ much from the complete transcriptomes of both species, with similar proportions of gene ontology categories between species and tissues (Fig. 2.15, Table 2.9 and Table 2.10, supplementary material). Approximately 61% of the genes were assigned to four biological processes [cellular process (20%), single-organism process (14%), metabolic process (17%) and biological regulation (10%)] and around 78% to two molecular functions [binding (47%) and catalytic

activity (31%)], for all tissues and species (Fig. 2.16, Supplementary material). Regarding cellular components, there are three major GO terms for all libraries (membrane, cell and cell junction), representing more than 75% of all GO terms (Figure 2.16 and Table 2.11, Supplementary material).

For up- and downregulated genes, the most represented functional categories (biological processes, molecular functions and cellular components) are the same as for all DE genes (Figure 2.16, Supplementary material). Also, when we consider the DE genes shared between species or exclusive to one species (for each tissue), the same GO terms are the most represented among these genes (Figure 2.10c and Figure 2.17, Supplementary material).

Enrichment analysis of the functionally annotated genes showed that upregulated genes in *S. carolitertii* liver were essentially related to neural crest cell differentiation/development, regulation of transcription, biological adhesion, regulation of the RNA metabolic process, the transmembrane receptor protein tyrosine kinase signaling pathway, cell motility, embryonic morphogenesis and skeletal system development (Figure 2.11). Other categories presented a predominance of downregulated genes including those involved in the amine catabolic process, liver development, embryonic hematopoiesis, the organic acid metabolic process, regulation of body fluid levels and oxidation-reduction. However, *S. torgalensis* skeletal muscle only revealed enriched categories for downregulated genes, such as those involved in the cofactor biosynthetic process, protein localization, microtubule-based processes, cell division, the RNA metabolic process, organelle fission, ribonucleoprotein complex biogenesis, ribosome biogenesis, cellular response to stress, chromosome organization, cell cycle and the DNA metabolic process (Figure 2.11).

Also, both KEGG Pathways and Molecular Functions (Figure 2.18, Supplementary material) presented a predominance of downregulated genes in the enriched categories for *S. torgalensis* skeletal muscle, with several terms being related to those described above. *S. carolitertii* muscle presented enrichment in circadian rhythm functions for downregulated genes, while *S. torgalensis* fins were enriched in circadian rhythm functions for upregulated genes (Fig. 2.18, Supplementary material).

Figure 2.11: Enriched biological processes of up- and downregulated genes, in relation to the control condition, with adjusted p-value (Benjamini) < 0.05. F corresponds to fins, L to liver and M to skeletal muscle.

We generated a list of 70 candidate DE genes between both treatments, with 38 recovered genes from other transcriptomic studies in fish in (Buckley et al., 2006; Kassahn et al., 2007; Lewis et al., 2010; Smith et al., 2013) and 32 new genes involved in 3 target biological processes (protein folding, immune and oxidative stress responses) (Figure 2.12 and Table 2.12, Supplementary material). From this list we observed that genes for heat shock proteins (*hsp40s/DnaJs*, *hsp70s* and *hsp90s*) were upregulated in several tissues - the *hsp70* gene had the highest induction under thermal stress, particularly for *S. torgalensis* (Figure 2.12). Target genes involved in immune responses presented several expression changes in *S. carolitertii*, with many upregulated genes in liver tissue, whereas only two annotated genes of this category presented different expression profiles for *S. torgalensis* (Figure 2.12). In contrast, genes involved in transport, responses to oxidative stress and glutamine biosynthesis were downregulated in *S. torgalensis* (particularly in skeletal muscle) but not in *S. carolitertii*. The six genes with no functional annotation information in gene ontology databases (Figure 2.12), were also reported as responding to temperature in other studies (Table 2.12, supplementary material) and therefore we can assume that they are sensitive to thermal stress.

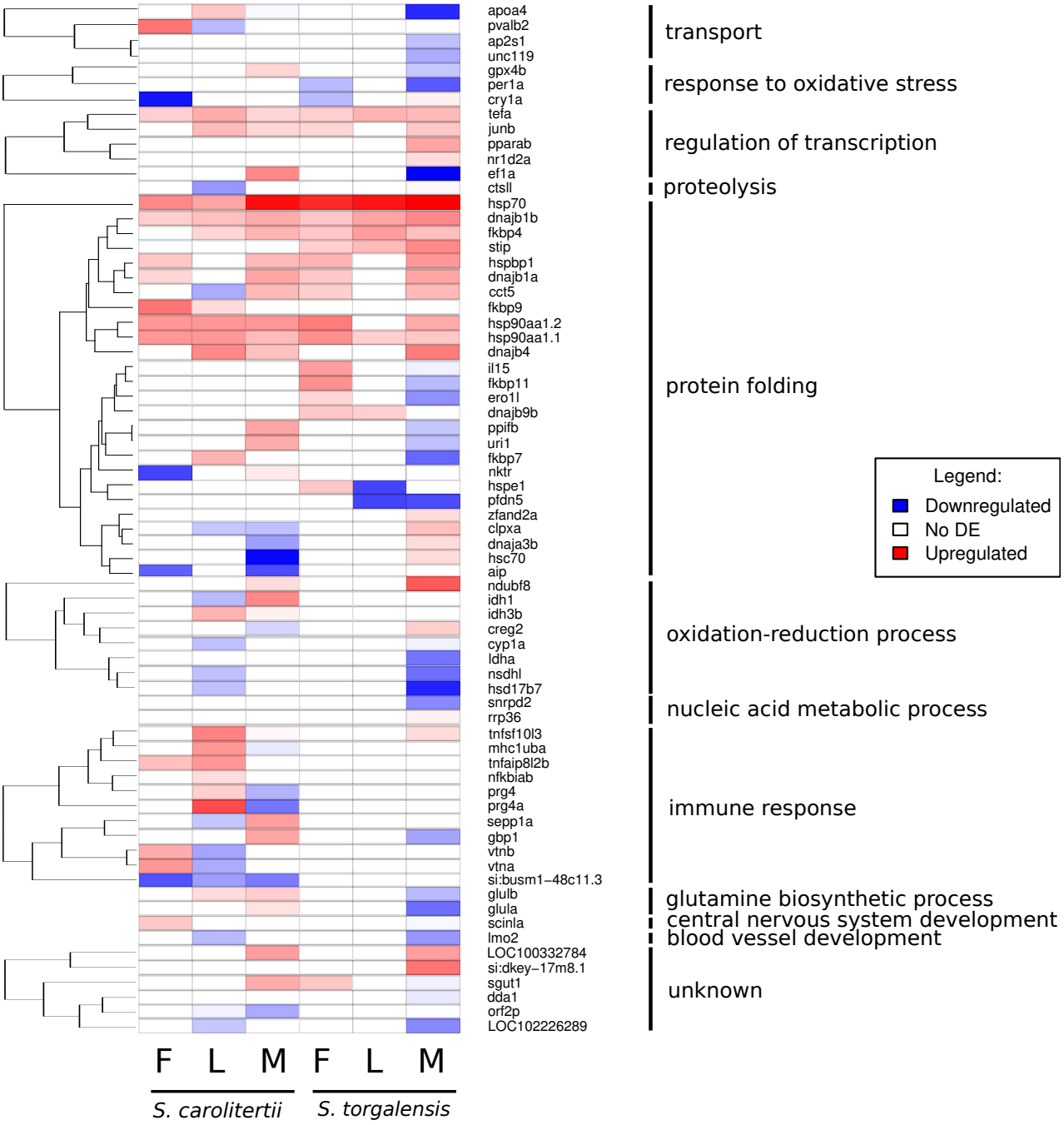

Figure 2.12: Heatmap showing the $\log_2$ (fold change), for which in red are represented the upregulated genes and in green the downregulated genes, in relation to the control condition, with colour intensity indicating the degree of gene expression change. F corresponds to fins, L to liver and M to skeletal muscle.

Discussion

Next generation sequencing technologies have provided a cost-effective way to generate genomic resources for non-model species (Ekblom and Galindo, 2011). Transcriptomes constitute a good resource for identifying gene expression profiles, and for non-model species their power surpasses microarrays since they do not rely on hybridization-based technology so, in theory, every species may benefit from the same accuracy and reliability (Stapley *et al.*, 2010; Ozsolak and Milos, 2011; Alvarez *et al.*, 2014). We took advantage of the recently available *S. carolitertii* and *S. torgalensis* transcriptomes to perform a comprehensive study that increases our knowledge on the thermal stress responses of these species.

In general, gene expression is tissue specific (Krueger and Morison, 2008; Xiong *et al.*, 2010; de Nadal *et al.*, 2011) and the heat shock response in fish, including for *Danio rerio*, is also known to be tissue specific (Lele *et al.*, 1997; Råbergh *et al.*, 2000; Buckley *et al.*, 2006; Currie *et al.*, 2010; Madeira *et al.*, 2014). Tissue specificity is, in fact, largely evident in our study both at the whole transcriptome or specific gene levels (see Figure 2.11 and 2.12). Supporting this, the same tissues were more alike, irrespective of the treatment. However, fins and liver tissues tended to be more similar in both species, although it should be noted that fins consist of an element of skeletal muscle. Therefore, despite the relevance of using fins for transcriptome-wide studies in endangered species like *S. torgalensis*, they may not be a suitable tissue for drawing general conclusions about thermal responses. Thus, we recommend the use of other tissues, such as skeletal muscle and liver, given the difficulty in interpreting patterns obtained from fins.

Regarding the DE analysis, in general *S. carolitertii* presented more upregulated genes and *S. torgalensis* more downregulated genes, suggesting a less costly response in the latter since upregulation of more genes would lead to greater energy consumption (Sorensen *et al.*, 2003; López-Maury *et al.*, 2008; de Nadal *et al.*, 2011). However, whether this difference represents a benefit in the cost-efficiency trade-off depends on the type of genes that are up- or downregulated.

Yet, for enriched categories, we observed that the biological processes contrast somewhat in both species. *S. carolitertii* shows several upregulated biological processes in liver, such as regulation of transcription and the RNA metabolic process, suggesting that this species responds by increasing the mRNA levels of genes, probably to maintain homeostasis. In

contrast, *S. torgalensis* displays exclusively downregulated-enriched categories in skeletal muscle, which suggests a shutdown of several pathways and mainly those involved in cell division and growth (e.g. nuclear division, cell cycle, chromosome organization). This decreased expression of genes involved in growth has been described as a mechanism to save energy during heat stress, channeling energy towards the repair and replacement of damaged molecules (e.g. proteins and membranes) (Sorensen *et al.*, 2003; Buckley *et al.*, 2006; López-Maury *et al.*, 2008). Similar results were also observed for *Saccharomyces cerevisiae* in response to heat, with resources being redirected from growth to stress functions and where the degree of stress resistance is inversely correlated with growth rate (López-Maury *et al.*, 2008). Also, the fish *Gillichthys mirabilis* presents a similar response, i.e. repressing many genes involved in growth and proliferation for muscle tissue, and an induction of stress-related genes in response to heat (Buckley *et al.*, 2006). In this sense, *S. torgalensis* actually conserves energy by shutting down these pathways, which may result from being acclimatized to a warmer environment during summer. Conversely, *S. carolitetii* is not usually exposed to such high temperature fluctuations and thus its response might be maladapted to this condition.

We also attempted to characterize the response of all genes present in the gene ontology analysis belonging to the three biological processes previously reported as being biologically significant during thermal stress: protein folding, and the immune and oxidative stress responses (Kassahn *et al.*, 2007; Lewis *et al.*, 2010; Smith *et al.*, 2013). Therefore, we identified in our dataset a set of DE genes, from previously reported genes and from three biological significant functions (protein folding, immune and oxidative stress responses), which can be used as markers of thermal stress in future studies (Figure 2.12 and Table 2.12, Supplementary material).

Among these genes are the heat shock proteins, *hsp90a*, *hsp70* and *hsp40*, which may play a major role during harsh temperature events [reviewed in Lindquist and Craig (1988) and in Sørensen et al. (2003)]. *Hsp70* was upregulated in both species and all three tissues, but was generally more upregulated in *S. torgalensis*. This corroborates the general trends observed in Jesus *et al.* (2013), with a greater increase in *hsp70* expression for *S. torgalensis* than *S. carolitertii*, and *hsc70* being upregulated in *S. torgalensis* and downregulated in *S. carolitertii*. Though, in the present study, we cannot reveal if the stronger upregulation of *hsps* in *S. torgalensis* is an adaptive response or if it is simply more stressed than *S. carolitertii*, in Jesus *et al.* (2013) not all *S. carolitertii* survived

at the highest temperature, which supports the first hypothesis. Moreover, fin tissue seems to be suitable for measuring several *hsps* but, as previously mentioned, its limited effectiveness for general conclusions might inhibit its use. However, the usefulness of *hsps* as biomarkers of environmental stress is limited since they respond to several stressors, such as temperature, hypoxia, heavy metals and inbreeding, and even the cell cycle can produce changes in their expression levels (Sorensen, 2010; Morris *et al.*, 2013).
Other genes with known interactions with *hsps*, e.g. aryl-hydrocarbon receptor-interacting protein (*aip*), FK506 binding protein (*fkbps*) and open reading frame 2 encoded protein (*orf2p*) (Wegele *et al.*, 2004; John *et al.*, 2011; Linnert *et al.*, 2013) were also DE in at least one of the treatments (see Figure 2.12). However, despite protein folding being the most represented GO category among the target genes, it does not explain the differences found between *S. carolitertii* and *S. torgalensis*. Therefore, both species appear to deal with protein denaturation/degradation, although *S. torgalensis* presented a stronger induction of these genes, which also suggests a better capacity to deal with periods of high temperatures.
Many other genes present differential expression between tissues and species, however, it is noteworthy that there are two genes among the list of DE genes that play pivotal roles in the maintenance of circadian rhythms (*cry1a* and *per1a*). Furthermore, differences in enrichment analysis (KEGG pathways) were also found for circadian rhythms. Changes in circadian rhythms may have significant impacts on fish that evolved a periodic gene expression program to deal with expected environmental fluctuations (López-Maury *et al.*, 2008). Alterations in the biological clock might disrupt fish behaviours such as feeding and reproduction, as well as physiological aspects, including their metabolism (Idda *et al.*, 2012).

Conclusions

In summary, our results suggest that *S. torgalensis* may have an energy saving strategy during short periods of exposure to high temperatures, by redirecting resources from growth to stress response mechanisms. On the other hand, *S. carolitertii* regulates its metabolism by increasing the expression of genes involved in transcription and promoting the stress response, probably to maintain homeostasis. Furthermore, *S. torgalensis* present several characteristics that may favor them to live in a harsher environment, such

as shorter life span, earlier spawning age and smaller body size compared to *S. carolitertii* (Magalhães et al., 2003). In previous experiments some *S. carolitertii* individuals were unable to cope with temperatures as high as 35 °C, whereas all *S. torgalensis* individuals survived (Jesus *et al.*, 2013). Hence, the latter seems fitter to deal with extreme temperature fluctuations for short periods of time.

However, for medium- and long-term exposures to high temperatures, the response is unlikely to be similar, since the interruption of growth and the continuous maintenance of a stress response might be deleterious (López-Maury *et al.*, 2008). Moreover, climate change can create new challenges for species, particularly those living closer to their thermal tolerance limits and prone to small changes in environmental temperatures (Reusch and Wood, 2007; Dahlhoff and Rank, 2007; Sorensen *et al.*, 2009; Somero, 2010; Tomanek, 2010; Hoffmann and Sgrò, 2011). In this regard, species living in intermittent systems, such as the rivers characterized by the Mediterranean regime, are particularly vulnerable to environmental change, since an increase in the occurrence of severe droughts may considerably challenge their ability to persist. Additionally, we indicate a set of potential target genes for further studies that may be particularly suited to monitoring the responses of these and other Iberian freshwater cyprinids to increasing temperatures.

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Supplementary material

Tables

Table 2.8: EdgeR results and annotation statistics of DE genes with FDR $< 5\times10^{-4}$.

	S. carolitertii			*S. torgalensis*		
	Fins	Liver	Muscle	Fins	Liver	Muscle
total number of contigs	1409	6597	4460	922	493	10044
annotated contigs	1144	4861	2846	688	398	6959
annotated contigs upregulated	863	2944	1681	437	200	1863
annotated contigs downregulated	281	1917	1165	251	198	5096
non annotated contigs	265	1736	1614	234	95	3085
non annotated contigs upregulated	152	858	809	141	56	1247
non annotated contigs downregulated	113	878	805	93	39	1838
all	1409	6597	4460	922	493	10044
all upregulated	1015	3802	2490	578	256	3110
all downregulated	394	2795	1970	344	237	6934

Table 2.9: Number of DE annotated genes belonging to main Biological Processes in each tissue. *Continues on next page.*

GO	GO description	*S. carolitertii*								
		Fins			Liver			Muscle		
		ALL	UP	DOWN	ALL	UP	DOWN	ALL	UP	DOWN
GO:0009987	cellular process	543	388	155	2384	1492	892	1699	1025	674
GO:0044699	single-organism process	398	285	113	1875	1249	626	1135	664	471
GO:0008152	metabolic process	574	444	130	2130	1087	1043	1412	891	521
GO:0065007	biological regulation	319	227	92	1373	952	421	820	496	324
GO:0032502	developmental process	151	114	37	844	630	214	468	279	189
GO:0032501	multicellular organismal process	171	128	43	884	628	256	476	283	193
GO:0050896	response to stimulus	249	184	65	983	673	310	567	341	226
GO:0023052	signaling	137	96	45	704	527	177	406	250	156
GO:0051179	localization	119	92	23	638	380	258	354	188	166
GO:0071840	cellular component organization or biogenesis	73	51	22	344	244	100	335	174	161
GO:0002376	immune system process	28	21	7	123	76	47	72	37	35
GO:0022610	biological adhesion	21	12	9	139	120	19	59	36	23
GO:0051704	multi-organism process	24	20	4	43	15	28	30	19	11
GO:0040011	locomotion	14	8	6	175	144	31	84	39	45
GO:0040007	growth	10	7	3	97	74	23	39	27	12
GO:0000003	reproduction	7	5	2	37	20	17	15	13	2
GO:0048511	rhythmic process	8	1	7	7	3	4	8	8	0
GO:0001906	cell killing	0	0	0	2	1	1	2	0	2

Table 2.9: *Continuation of the table from previous page.*

GO	GO description	*S. torgalensis*								
		Fins			Liver			Muscle		
		ALL	UP	DOWN	ALL	UP	DOWN	ALL	UP	DOWN
GO:0009987	cellular process	455	294	161	247	124	123	4601	1295	3306
GO:0044699	single-organism process	339	214	125	175	86	89	3212	916	2296
GO:0008152	metabolic process	385	246	139	211	102	109	3841	1069	2772
GO:0065007	biological regulation	263	157	106	141	61	80	2103	656	1447
GO:0032502	developmental process	167	102	65	78	33	45	1255	386	869
GO:0032501	multicellular organismal process	168	104	64	76	38	38	1300	399	901
GO:0050896	response to stimulus	202	128	74	126	57	69	1449	452	997
GO:0023052	signaling	125	78	53	69	26	43	947	308	639
GO:0051179	localization	112	72	34	52	27	25	1169	329	840
GO:0071840	cellular component organization or biogenesis	101	61	40	59	26	33	1048	261	787
GO:0002376	immune system process	30	26	4	14	6	8	227	71	156
GO:0022610	biological adhesion	17	8	9	10	5	5	145	59	86
GO:0051704	multi-organism process	18	16	2	6	3	3	100	30	70
GO:0040011	locomotion	34	24	10	14	4	10	208	80	128
GO:0040007	growth	26	17	9	14	5	9	120	38	82
GO:0000003	reproduction	21	10	11	5	1	4	170	43	127
GO:0048511	rhythmic process	8	4	4	3	1	2	30	14	16
GO:0001906	cell killing	0	0	0	0	0	0	9	2	7

Table 2.10: Number of DE annotated genes belonging to main Molecular Functions in each tissue. *Continues on next page.*

GO	GO description	*S. carolitertii*								
		Fins			Liver			Muscle		
		ALL	UP	DOWN	ALL	UP	DOWN	ALL	UP	DOWN
GO:0005488	binding	542	388	154	2382	1514	868	1651	1023	628
GO:0003824	catalytic activity	376	304	72	1619	735	884	973	617	356
GO:0005215	transporter activity	56	49	7	232	105	127	114	62	52
GO:0030234	enzyme regulator activity	75	61	14	223	127	96	105	66	39
GO:0001071	nucleic acid binding transcription factor activity	53	34	19	263	198	65	116	76	40
GO:0060089	molecular transducer activity	53	35	18	268	196	72	114	77	37
GO:0004872	receptor activity	46	36	10	306	227	79	91	60	31
GO:0009055	electron carrier activity	20	19	1	65	10	55	9	5	4
GO:0005198	structural molecule activity	9	8	1	121	95	26	82	56	26
GO:0000988	protein binding transcription factor activity	6	3	3	14	11	3	21	6	15
GO:0016209	antioxidant activity	3	3	0	19	3	16	10	9	1
GO:0016247	channel regulator activity	0	0	0	1	1	0	7	5	2
GO:0042056	chemoattractant activity	0	0	0	1	1	0	0	0	0
GO:0016530	metallochaperone activity	0	0	0	2	0	2	0	0	0
GO:0045499	chemorepellent activity	0	0	0	0	0	0	0	0	0
GO:0030545	receptor regulator activity	0	0	0	0	0	0	0	0	0
GO:0045182	translation regulator activity	0	0	0	1	0	1	3	1	2
GO:0045735	nutrient reservoir activity	0	0	0	1	0	1	0	0	0

Table 2.10: *Continuation of the table from previous page.*

GO	GO description	*S. torgalensis*								
		Fins			Liver			Muscle		
		ALL	UP	DOWN	ALL	UP	DOWN	ALL	UP	DOWN
GO:0005488	binding	402	248	154	250	122	128	3882	1115	2767
GO:0003824	catalytic activity	238	161	77	140	67	73	2827	705	2122
GO:0005215	transporter activity	24	19	5	18	12	6	363	100	263
GO:0030234	enzyme regulator activity	26	18	8	16	7	9	239	72	167
GO:0001071	nucleic acid binding transcription factor activity	49	27	22	25	13	12	229	92	137
GO:0060089	molecular transducer activity	39	17	22	17	7	10	213	71	142
GO:0004872	receptor activity	26	11	15	14	6	8	187	68	119
GO:0009055	electron carrier activity	2	2	0	8	2	6	36	7	29
GO:0005198	structural molecule activity	8	3	5	4	3	1	197	68	129
GO:0000988	protein binding transcription factor activity	7	4	3	0	0	0	78	24	54
GO:0016209	antioxidant activity	0	0	0	2	0	2	28	5	23
GO:0016247	channel regulator activity	2	2	0	0	0	0	8	2	6
GO:0042056	chemoattractant activity	0	0	0	0	0	0	4	4	0
GO:0016530	metallochaperone activity	0	0	0	0	0	0	5	2	3
GO:0045499	chemorepellent activity	0	0	0	0	0	0	2	1	1
GO:0030545	receptor regulator activity	0	0	0	0	0	0	3	3	0
GO:0045182	translation regulator activity	0	0	0	0	0	0	2	1	1
GO:0045735	nutrient reservoir activity	0	0	0	3	0	3	0	0	0

Table 2.11: Number of DE annotated genes belonging to main Cellular Components in each tissue. *Continues on next page.*

GO	GO description	*S. carolitertii*								
		Fins			Liver			Muscle		
		ALL	UP	DOWN	ALL	UP	DOWN	ALL	UP	DOWN
GO:0016020	membrane	181	149	32	1108	711	397	536	306	230
GO:0005623	cell	355	235	120	1586	1031	555	1227	765	462
GO:0043226	organelle	221	147	74	913	582	331	840	524	316
GO:0005576	extracellular region	82	75	7	377	225	152	80	52	28
GO:0032991	macromolecular complex	98	64	34	362	242	120	366	213	153
GO:0019012	virion	8	0	8	7	4	3	16	10	6
GO:0031974	membrane-enclosed lumen	25	18	7	57	41	16	99	53	46
GO:0031012	extracellular matrix	3	2	1	113	106	7	27	15	12
GO:0030054	cell junction	12	12	0	85	60	25	38	28	10
GO:0045202	synapse	0	0	0	22	17	5	23	9	14
GO:0009295	nucleoid	0	0	0	0	0	0	0	0	0
GO:0055044	symplast	0	0	0	2	0	2	0	0	0

Table 2.11: *Continuation of the table from previous page.*

GO	GO description	*S. torgalensis*								
		Fins			Liver			Muscle		
		ALL	UP	DOWN	ALL	UP	DOWN	ALL	UP	DOWN
GO:0016020	membrane	157	106	51	70	39	31	1695	436	1259
GO:0005623	cell	346	213	133	207	104	103	3586	929	2657
GO:0043226	organelle	256	163	93	144	80	64	2649	695	1954
GO:0005576	extracellular region	31	20	11	26	14	12	214	74	140
GO:0032991	macromolecular complex	92	49	43	48	27	21	1294	335	959
GO:0019012	virion	6	5	1	1	0	1	25	5	20
GO:0031974	membrane-enclosed lumen	46	29	17	18	9	9	521	116	405
GO:0031012	extracellular matrix	6	4	2	4	4	0	42	18	24
GO:0030054	cell junction	14	10	4	8	1	7	99	26	73
GO:0045202	synapse	6	2	4	6	4	2	63	21	42
GO:0009295	nucleoid	0	0	0	0	0	0	7	1	6
GO:0055044	symplast	0	0	0	3	0	3	0	0	0

Table 2.12: List of candidate genes, with their annotation and matching contigs. *Continues on next page.*

gene description	gene name	GO description	Species	Reference	putative functional annotation
Previousy reported candidate genes					
heat shock protein HSP 90-alpha 1 [Danio rerio]	*hsp90aa1.1*	protein folding	*Oncorhynchus mykiss* *Gillichthys mirabilis*	Buckley *et al.* (2006) Lewis et al. (2010)	
heat shock protein HSP 90-alpha [Danio rerio]	*hsp90aa1.2*	protein folding			
T-complex protein 1 subunit epsilon [Danio rerio]	*cct5*	protein folding	*Oncorhynchus mykiss*	Lewis *et al.* (2010)	
Jun B protein [Ctenopharyngodon idella]	*junb*	regulation of transcription	*Oncorhynchus mykiss*	Lewis *et al.* (2010)	
PREDICTED: stress-induced-phosphoprotein 1-like [Oryzias latipes]	*stip*	-	*Oncorhynchus mykiss*	Lewis *et al.* (2010)	sensitive to thermal stress
Apolipoprotein A-IV, partial [Danio rerio]	*apoa4*	transport	*Oncorhynchus mykiss*	Lewis *et al.* (2010)	
rhombotin-2 [Danio rerio]	*lmo2*	blood vessel development	*Oncorhynchus mykiss*	Lewis *et al.* (2010)	
Zgc:55259 protein [Danio rerio]	*hspbp1*	-	*Oncorhynchus mykiss*	Lewis *et al.* (2010)	
PREDICTED: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial-like [Astyanax mexicanus]	*nudbf8*	oxidation-reduction process	*Oncorhynchus mykiss*	Lewis *et al.* (2010)	

Table 2.12: *Continues on next page.*

gene description	gene name	GO description	Species	Reference	putative functional annotation
ORF2-encoded protein, partial [Danio rerio]	*orf2p*	-	*Oncorhynchus mykiss*	Lewis *et al.* (2010)	sensitive to thermal stress
3-keto-steroid reductase [Danio rerio]	*hsd17b7*	oxidation-reduction process	*Melanotaenia duboulayi*	Smith *et al.* (2013)	
sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating [Danio rerio]	*nsdhl*	oxidation-reduction process	*Melanotaenia duboulayi*	Smith *et al.* (2013)	
PREDICTED: catechol O-methyltransferase domain-containing protein 1-like [Xiphophorus maculatus]	*LOC102226289*	-	*Melanotaenia duboulayi*	Smith *et al.* (2013)	sensitive to thermal stress
scinderin like a [Danio rerio]	*scinla*	central nervous system development	*Melanotaenia duboulayi*	Smith *et al.* (2013)	
PREDICTED: lysosomal alpha-glucosidase isoform X1 [Danio rerio]	*LOC100332784*	-	*Melanotaenia duboulayi*	Smith *et al.* (2013)	sensitive to thermal stress
protein CREG2 precursor [Danio rerio]	*creg2*	oxidation-reduction process	*Melanotaenia duboulayi*	Smith *et al.* (2013)	
thyrotrophic embryonic factor [Danio rerio]	*tefa*	regulation of transcription	*Melanotaenia duboulayi*	Smith *et al.* (2013)	

Table 2.12: *Continues on next page.*

gene description	gene name	GO description	Species	Reference	putative functional annotation
Elongation factor 1 a	*ef1a*	regulation of transcription	*Gillichthys mirabilis*	Buckley *et al.* (2006)	
PREDICTED: cathepsin L, like isoform X1 [Danio rerio]	*ctsll*	Proteolysis	*Gillichthys mirabilis*	Buckley *et al.* (2006)	
suppressor of G2 allele of SKP1 homolog [Danio rerio]	*sgut1*	-	*Gillichthys mirabilis*	Buckley *et al.* (2006)	sensitive to thermal stress
isocitrate dehydrogenase [NAD] subunit beta, mitochondrial [Danio rerio]	*idh3b*	oxidation-reduction process	*Gillichthys mirabilis*	Buckley *et al.* (2006)	
isocitrate dehydrogenase [NADP] cytoplasmic [Danio rerio]	*idh1*	oxidation-reduction process	*Gillichthys mirabilis*	Buckley *et al.* (2006)	
glutamine synthetase 1 [Danio rerio]	*glula*	glutamine biosynthetic process	*Gillichthys mirabilis*	Buckley *et al.* (2006)	
Glutamate-ammonia ligase (glutamine synthase) b [Danio rerio]	*glulb*	glutamine biosynthetic process	*Gillichthys mirabilis*	Buckley *et al.* (2006)	
RecName: Full=Parvalbumin beta; AltName: Full=Parvalbumin V [Squalius cephalus]	*pvalb2*	Transport	*Gillichthys mirabilis*	Buckley *et al.* (2006)	
heat shock 70 kDa protein [Ctenopharyngodon idella]	*hsp70*	protein folding	*Gillichthys mirabilis*	Buckley *et al.* (2006)	
uncharacterized protein LOC393586 [Danio rerio]	*hsc70*	protein folding	*Gillichthys mirabilis*	Buckley *et al.* (2006)	

Table 2.12: *Continues on next page.*

gene description	gene name	GO description	Species	Reference	putative functional annotation
AN1-type zinc finger protein 2A [Danio rerio]	*zfand2a*	protein folding	*Oncorhynchus mykiss*	Lewis *et al.* (2010)	
NF-kappa-B inhibitor alpha [Danio rerio]	*nfkbiab*	immune response	*Oncorhynchus mykiss*	Lewis *et al.* (2010)	
protein unc-119 homolog B [Danio rerio]	*unc119*	transport	*Oncorhynchus mykiss*	Lewis *et al.* (2010)	
DET1- and DDB1-associated protein 1 [Danio rerio]	*dda1*	-	*Oncorhynchus mykiss*	Lewis *et al.* (2010)	sensitive to thermal stress
AP-2 complex subunit sigma [Danio rerio]	*ap2s1*	transport	*Melanotaenia duboulayi*	Smith *et al.* (2013)	
ribosomal RNA processing protein 36 homolog [Danio rerio]	*rrp36*	nucleic acid metabolic process	*Melanotaenia duboulayi*	Smith *et al.* (2013)	
small nuclear ribonucleoprotein Sm D2 [Danio rerio]	*snrpd2*	nucleic acid metabolic process	*Melanotaenia duboulayi*	Smith *et al.* (2013)	
PREDICTED: C-Jun-amino-terminal kinase-interacting protein 4-like isoform X2 [Danio rerio]	*si:dkey-17m8.1*	-	*Melanotaenia duboulayi*	Smith *et al.* (2013)	sensitive to thermal stress
peroxisome proliferator activated receptor alpha b [Ctenopharyngodon idella]	*pparab*	regulation of transcription	*Melanotaenia duboulayi*	Smith *et al.* (2013)	
cytochrome P450 1a [Gobiocypris rarus]	*cyp1a*	oxidation-reduction process	*Melanotaenia duboulayi*	Smith *et al.* (2013)	

Table 2.12: *Continues on next page.*

gene description	gene name	GO description	Species	Reference	putative functional annotation
nuclear receptor subfamily 1, group D, member 2a [Danio rerio]	*nr1d2a*	regulation of transcription	*Melanotaenia duboulayi*	Smith *et al.* (2013)	
L-lactate dehydrogenase A chain [Danio rerio]	*ldha*	oxidation-reduction process	*Gillichthys mirabilis*	Buckley *et al.* (2006)	
New candidate genes					
DnaJ (Hsp40) homolog, subfamily B, member 1a [Danio rerio]	*dnajb1a*	protein folding			
DnaJ (Hsp40) homolog, subfamily B, member 1 [Danio rerio]	*dnajb1b*	protein folding			
DnaJ homolog subfamily B member 4 [Danio rerio]	*dnajb4*	protein folding			
Major histocompatibility complex class I UBA gene [Danio rerio]	*mhc1uba*	immune response			
PREDICTED: NK-tumor recognition protein isoform X1 [Danio rerio]	*nktr*	protein folding			
Zgc:123307 protein [Danio rerio]	*ppifb*	protein folding			
tumor necrosis factor (ligand) superfamily, member 10 like 3 [Danio rerio]	*tnfsf10l3*	immune response			
aryl hydrocarbon receptor interacting protein [Danio rerio]	*aip*	protein folding			
uncharacterized protein LOC368614 precursor [Danio rerio]	*si:busm1-48c11.3*	immune response			

Table 2.12: *Continues on next page.*

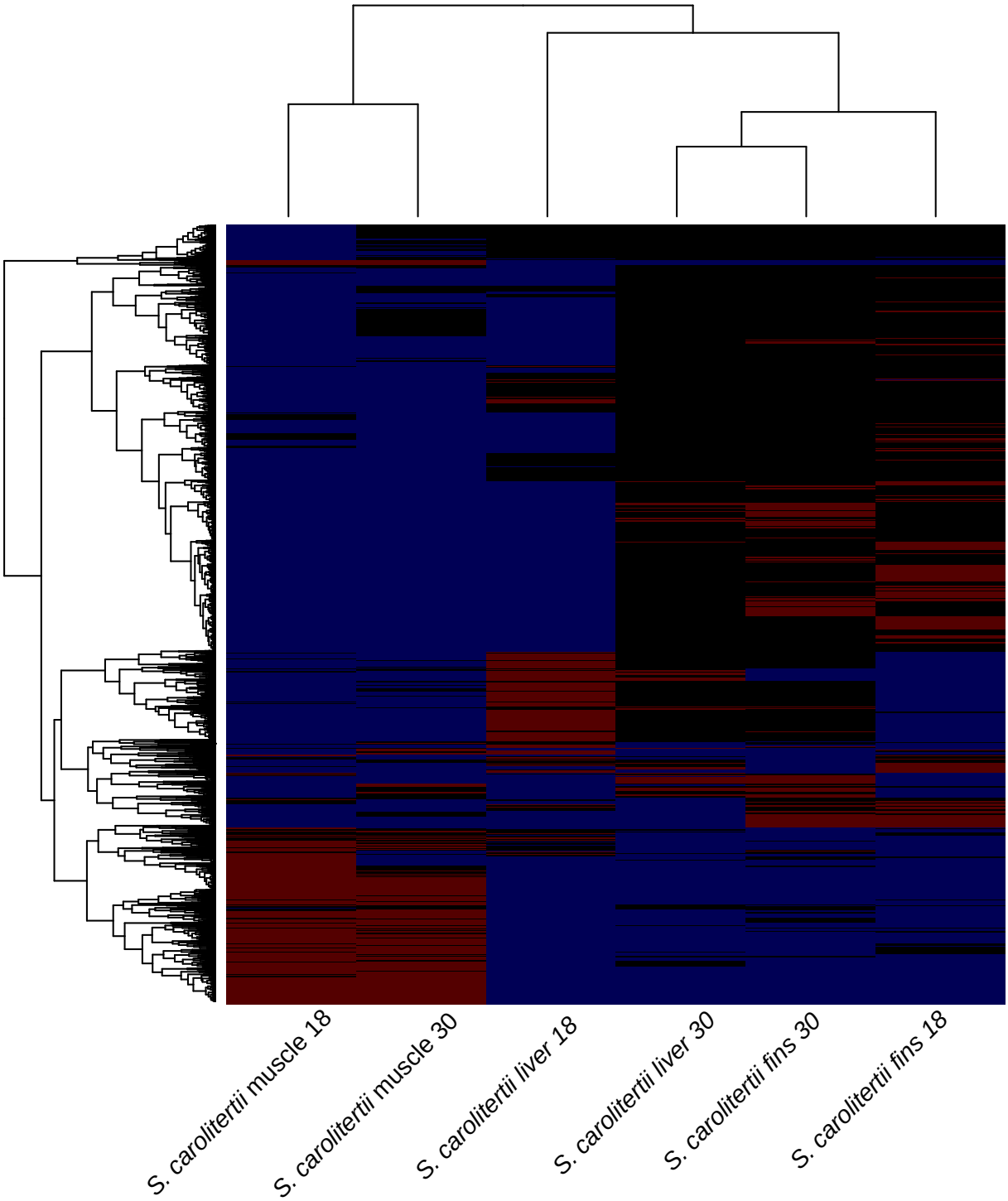
gene description	gene name	GO description	Species	Reference	putative functional annotation
peptidyl-prolyl cis-trans isomerase FKBP9 precursor [Danio rerio]	*fkbp9*	protein folding			
tumor necrosis factor, alpha-induced protein 8-like protein 2 B [Danio rerio]	*tnfaip8l2b*	immune response			
proteoglycan 4 precursor [Danio rerio]	*prg4a*	immune response			
Prg4 protein, partial [Danio rerio]	*prg4*	immune response			
selenoprotein 1a [Danio rerio]	*sepp1a*	immune response			
vitronectin a precursor [Danio rerio]	*vtna*	immune response			
Vtnb protein, partial [Danio rerio]	*vtnb*	immune response			
Cryptochrome 1a [Danio rerio]	*cry1a*	response to oxidative stress			
peptidyl-prolyl cis-trans isomerase FKBP4 [Danio rerio]	*fkbp4*	protein folding			
period 1 [Danio rerio]	*per1a*	response to oxidative stress			
prefoldin subunit 5 [Danio rerio]	*pfdn5*	protein folding			
10 kDa heat shock protein, mitochondrial [Danio rerio]	*hspe1*	protein folding			

Table 2.12: *Continuation of the table from previous page.*

gene description	gene name	GO description	Species	Reference	putative functional annotation
uncharacterized protein LOC554091 precursor [Danio rerio]	*dnajb9b*	protein folding			
ERO1-like protein alpha precursor [Danio rerio]	*ero1l*	protein folding			
peptidyl-prolyl cis-trans isomerase FKBP11 precursor [Danio rerio]	*fkbp11*	protein folding			
interleukin 15 [Danio rerio]	*il15*	protein folding			
ATP-dependent Clp protease ATP-binding subunit clpX-like, mitochondrial [Danio rerio]	*clpxa*	protein folding			
DnaJ (Hsp40) homolog, subfamily A, member 3B [Danio rerio]	*dnaja3b*	protein folding			
glutathione peroxidase 4b [Danio rerio]	*gpx4b*	response to oxidative stress			
guanylate binding protein 1 [Danio rerio]	*gbp1*	immune response			
unconventional prefoldin RPB5 interactor [Danio rerio]	*uri1*	protein folding			
peptidyl-prolyl cis-trans isomerase FKBP7 precursor [Danio rerio]	*fkbp7*	protein folding			

Figures

A)



Continues on next page.

B)

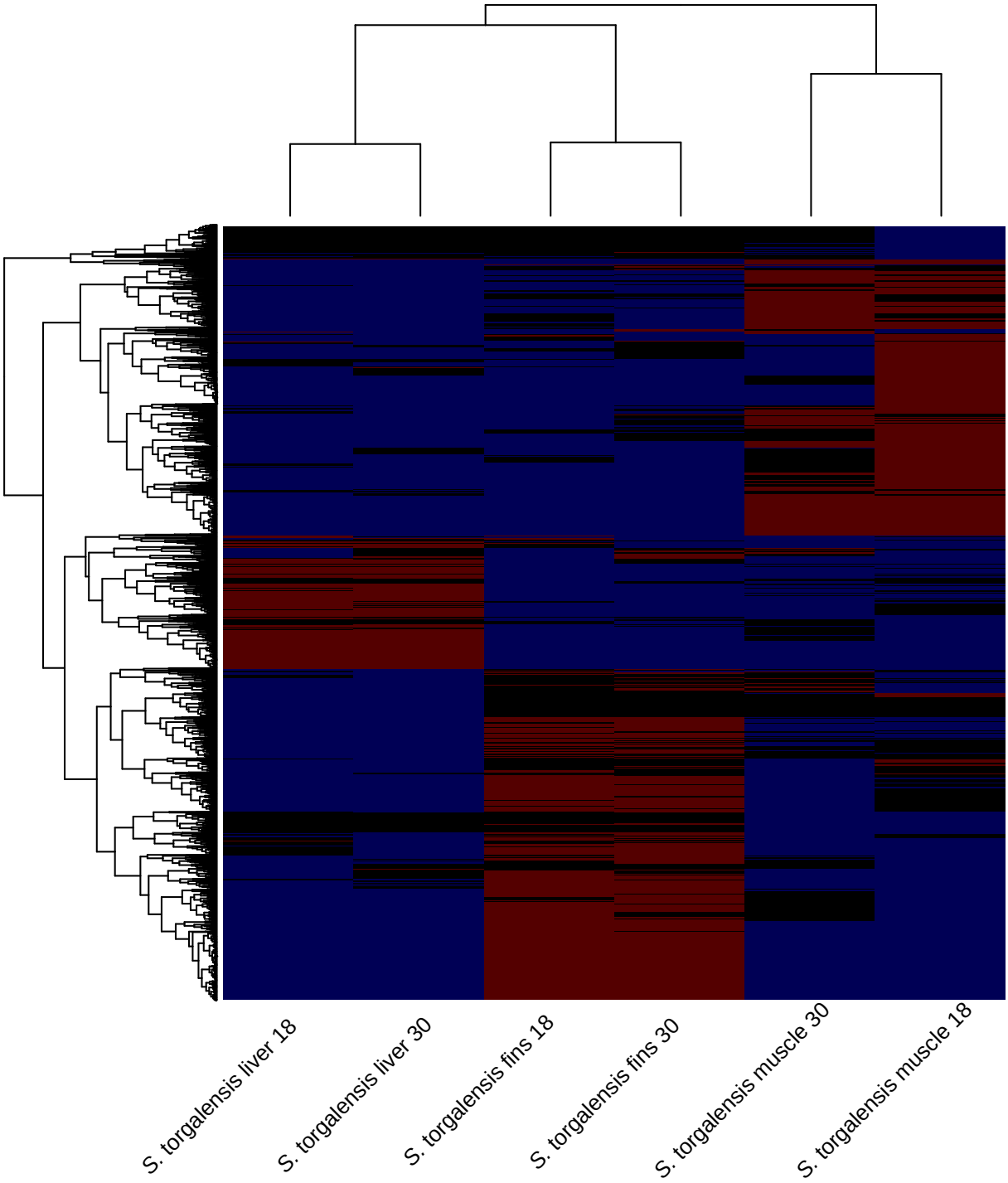


Figure 2.13: Unbiased clustering analysis of the 4,000 FPKMs with higher variance, for A) *S. carolitertii* and B) *S. torgalensis*. In the heatmaps columns 18 refers to the 18 °C treatment and 30 refers to the 30 °C treatment.

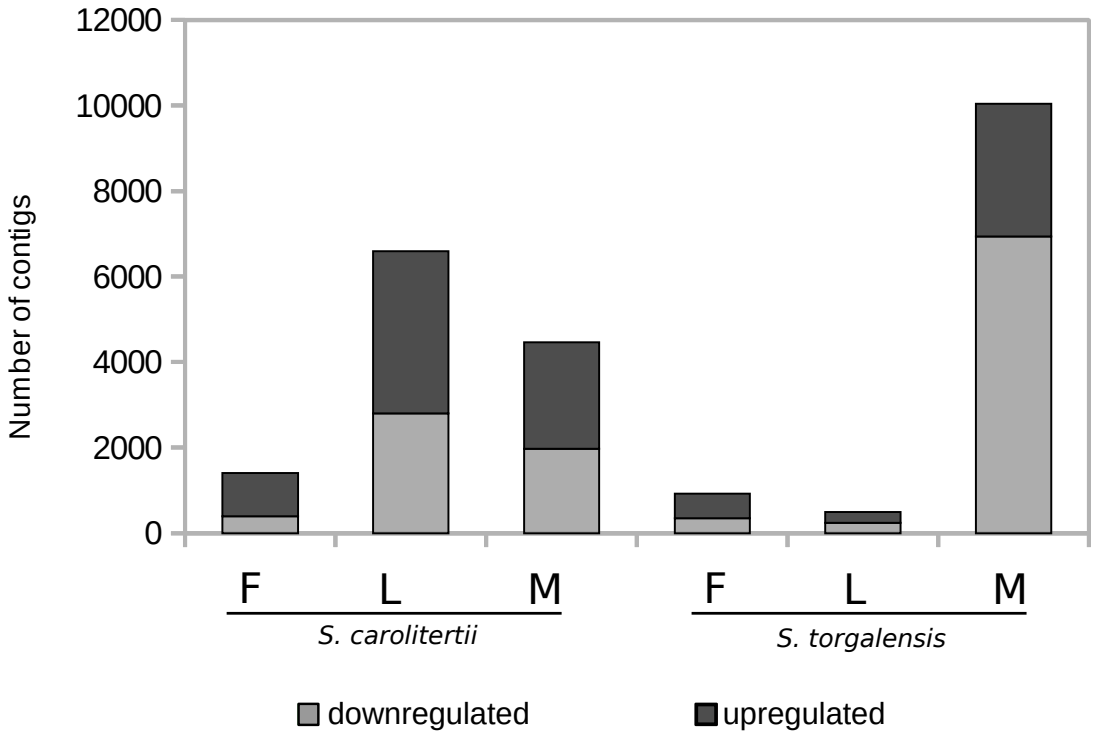


Figure 2.14: Number of all DE contigs (with and without blast hits) up and downregulated in all organs, for all DE contigs identified. F correspond to fins, L to liver and M to skeletal muscle.

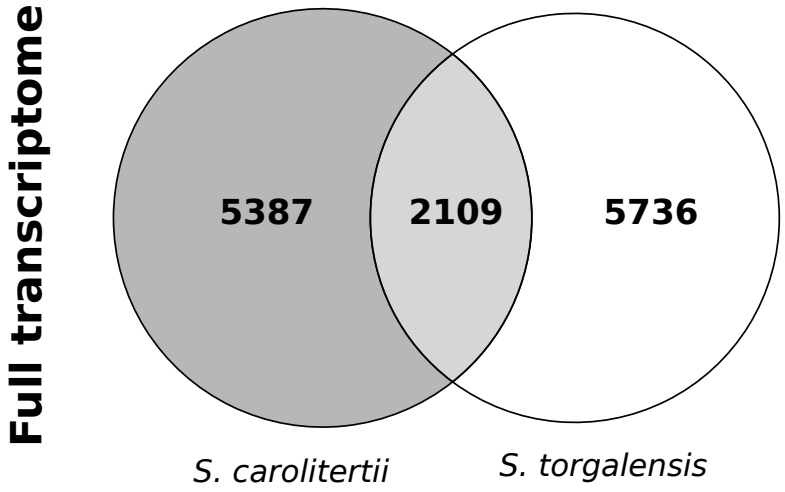


Figure 2.15: Shared and exclusive number of DE genes for the overall transcriptome of both species.

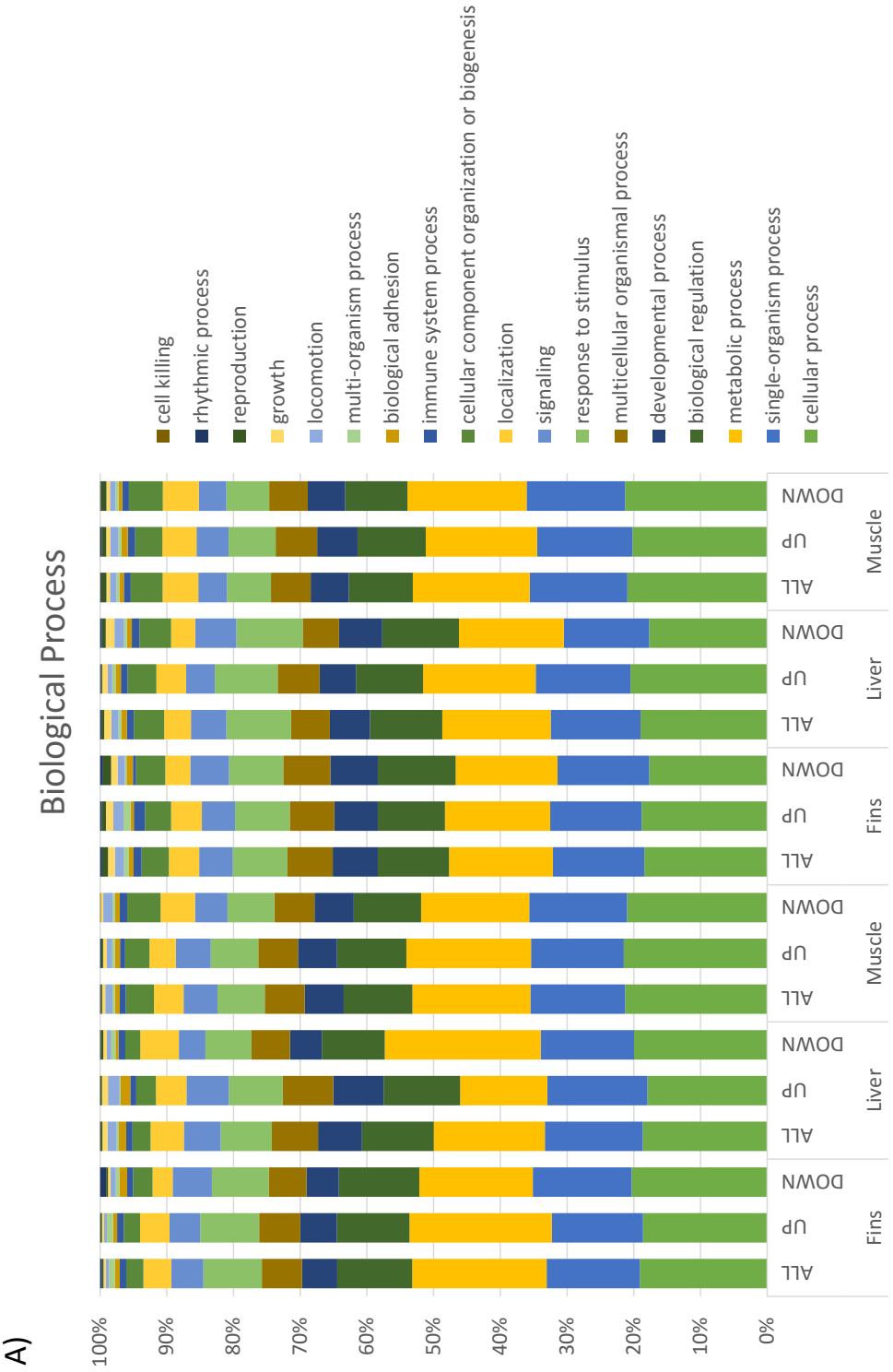


Figure 2.16: *Continues on next page.*

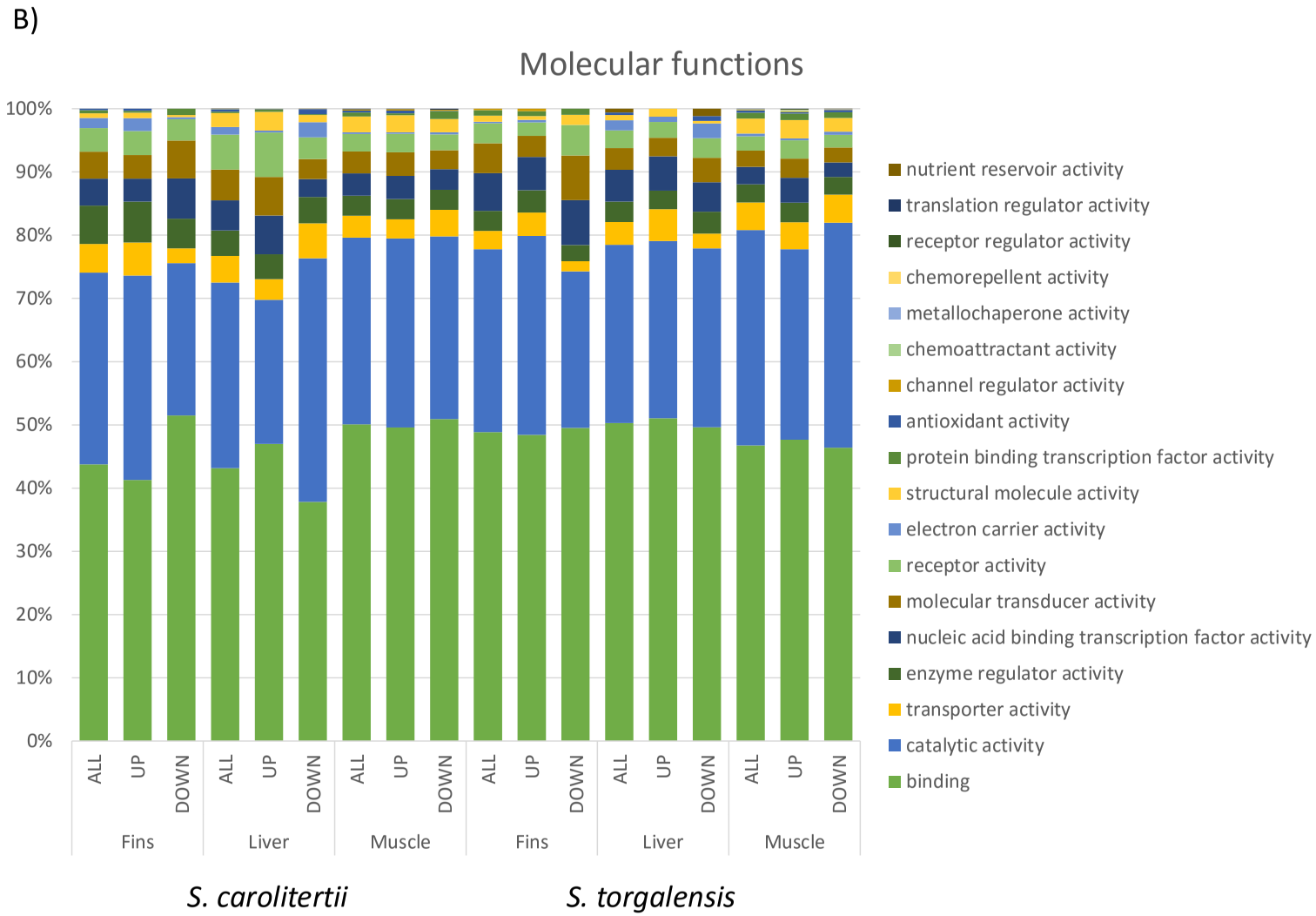


Figure 2.16: *Continues on next page.*

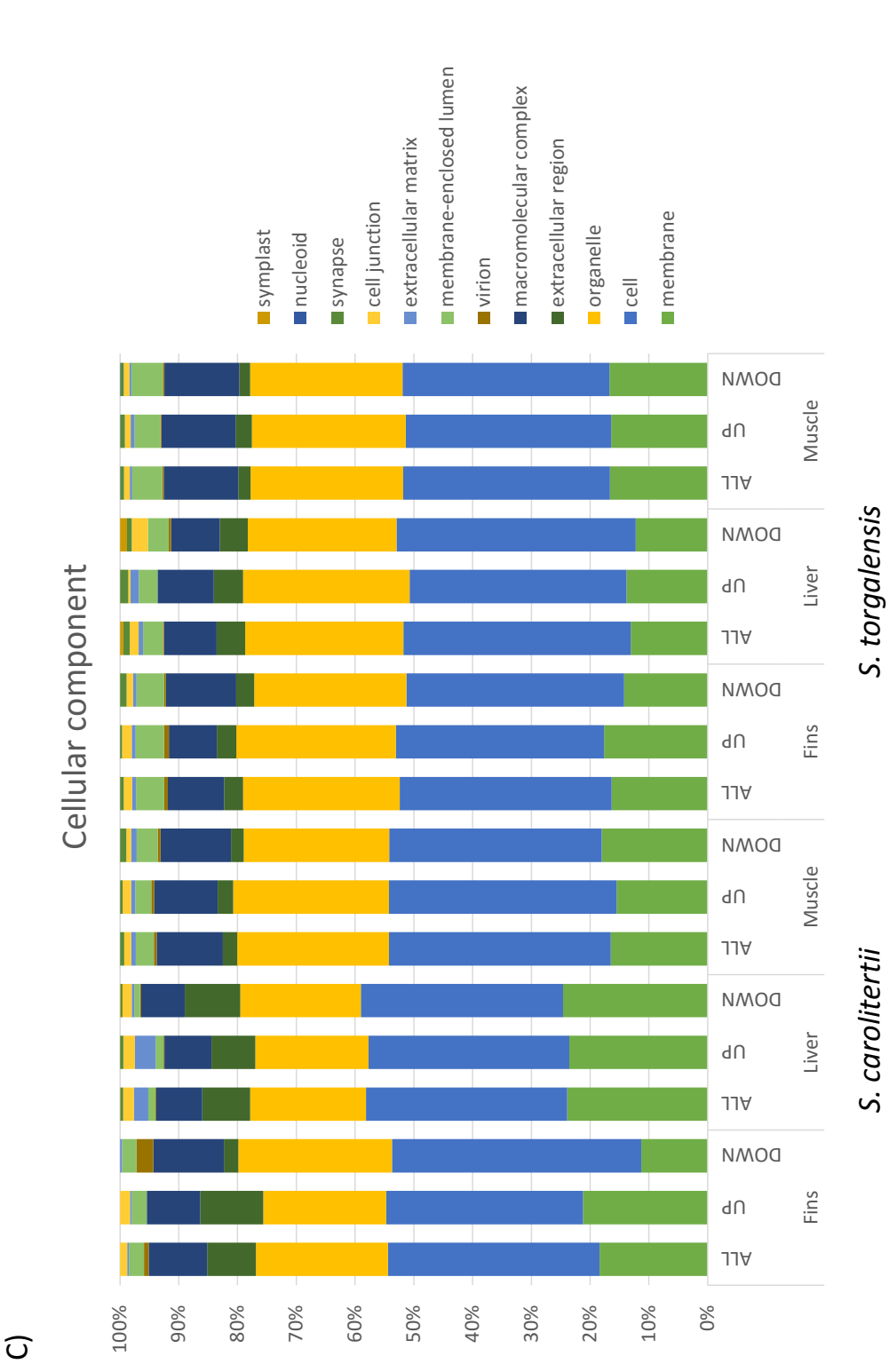


Figure 2.16: Percentage of each categories by A) Biological Process, B) Molecular Functions and C) Cellular Component for all DE genes, per tissue.

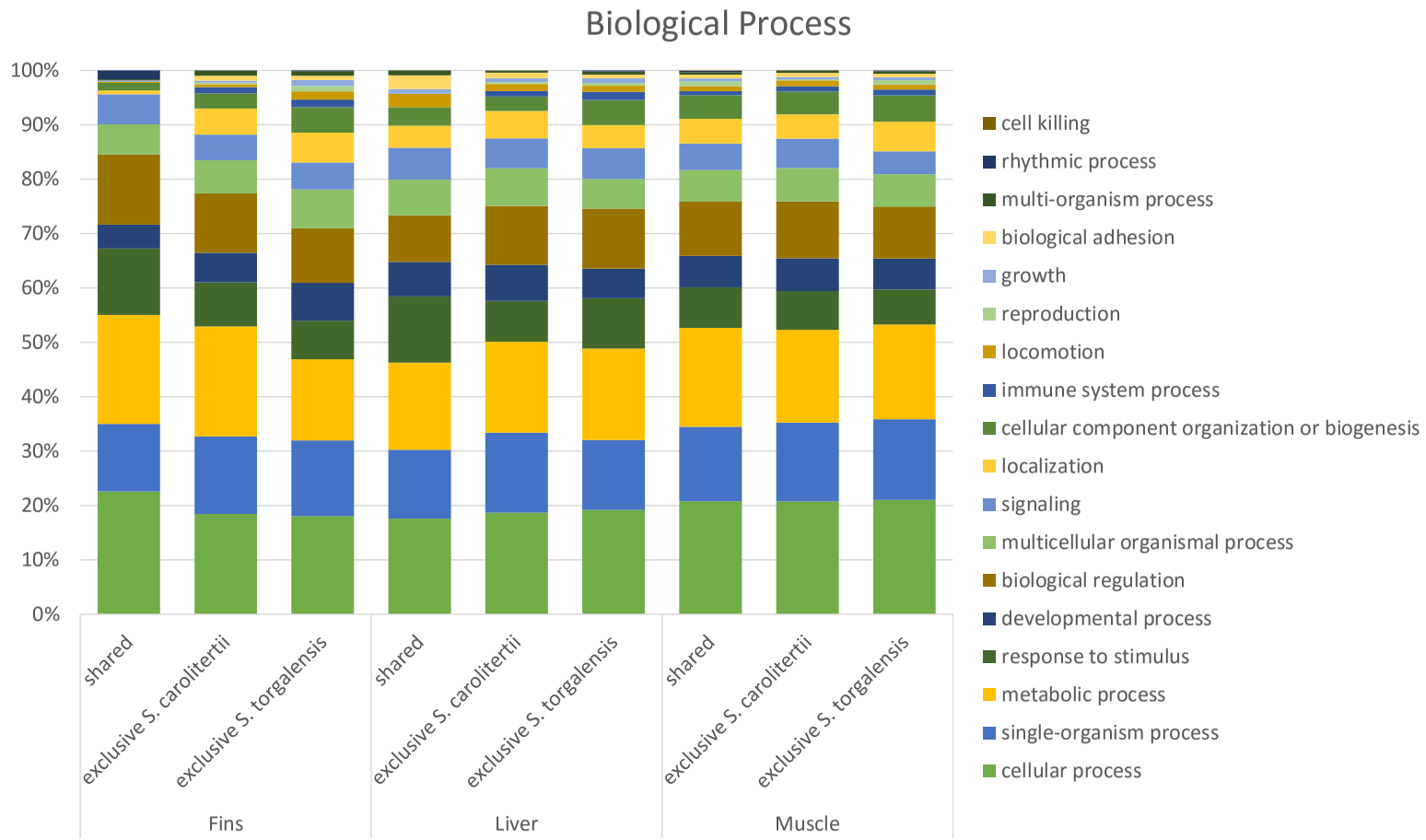


Figure 2.17: *Continues on next page.*

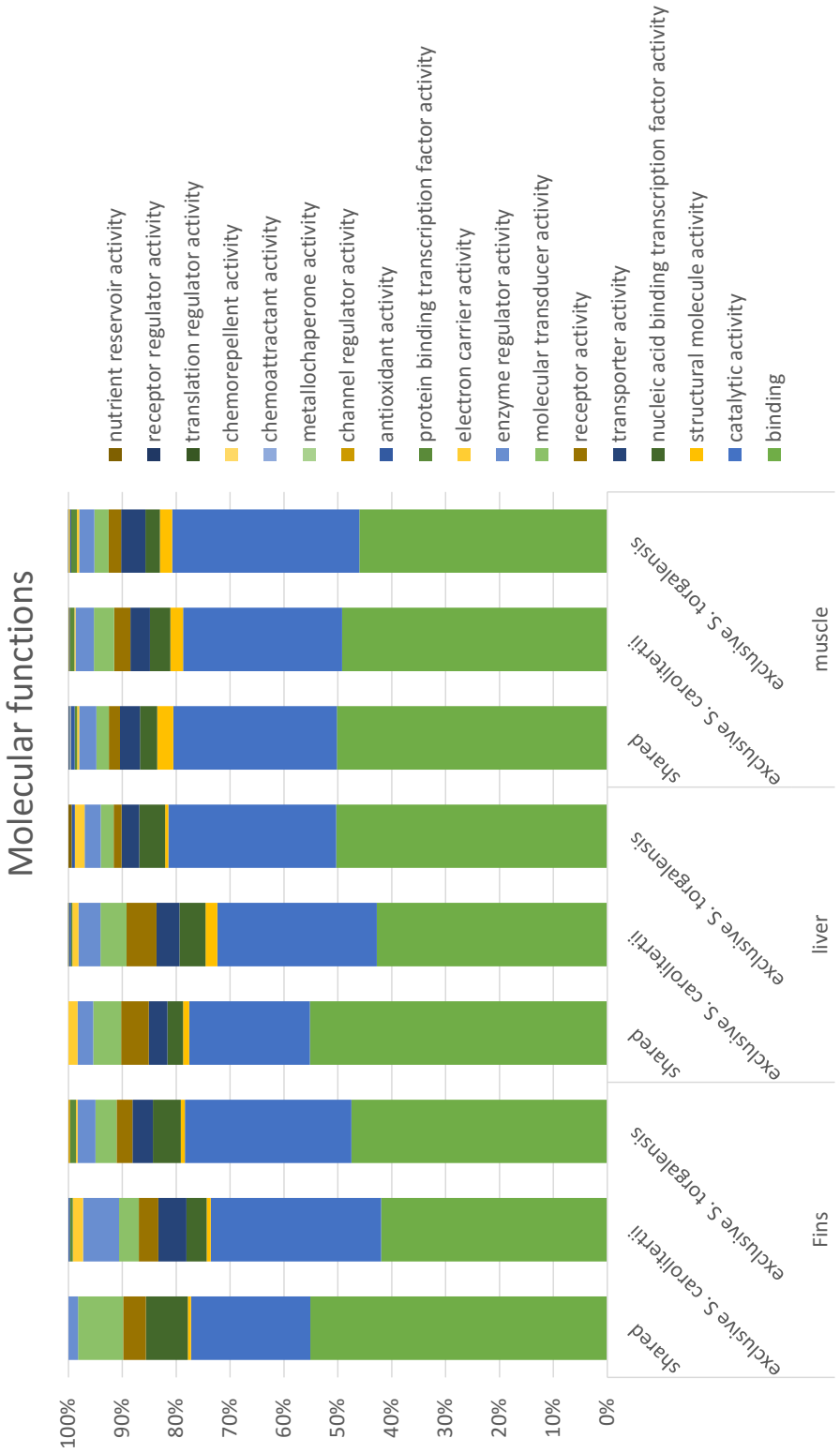


Figure 2.17: *Continues on next page.*

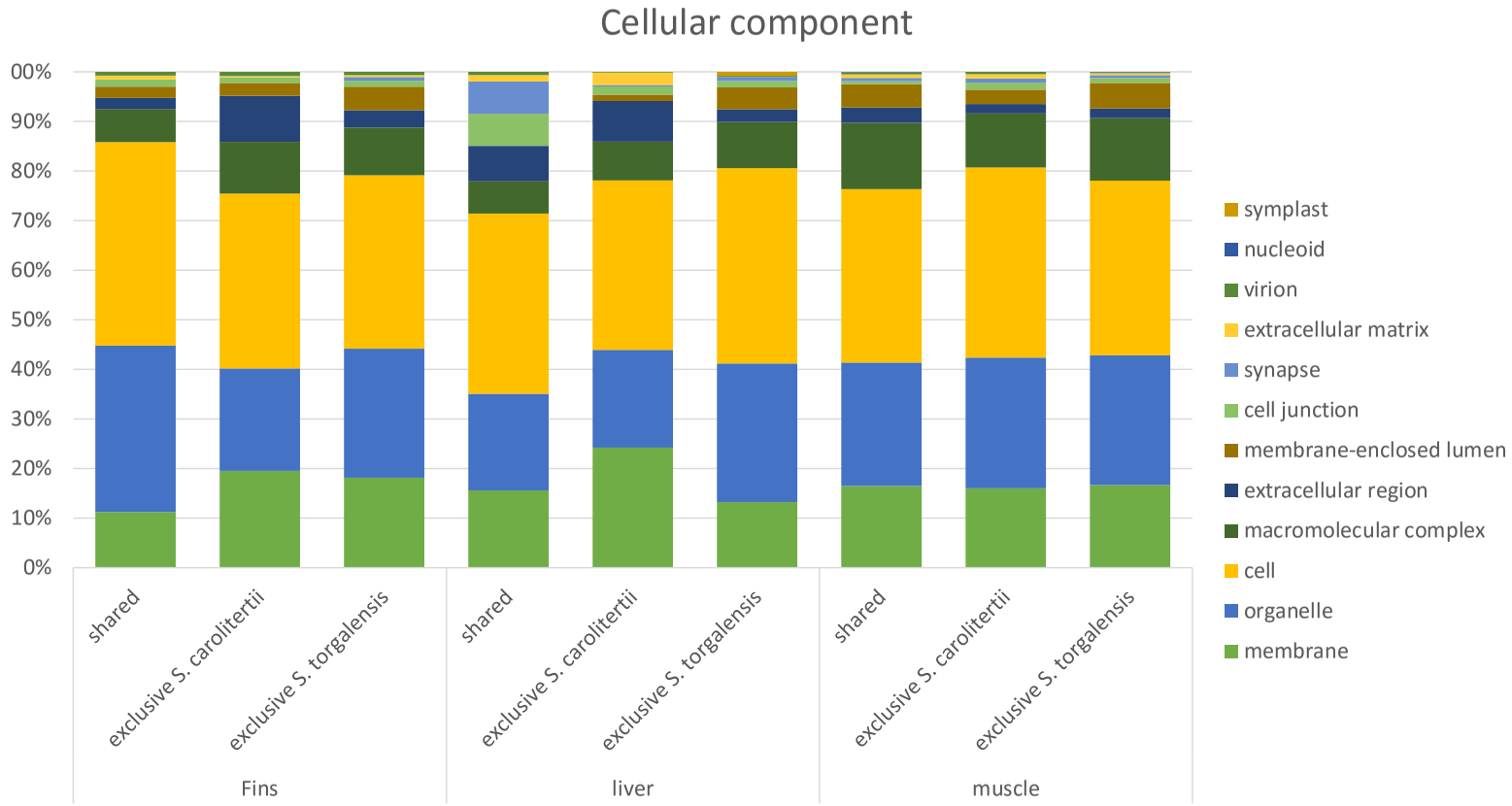
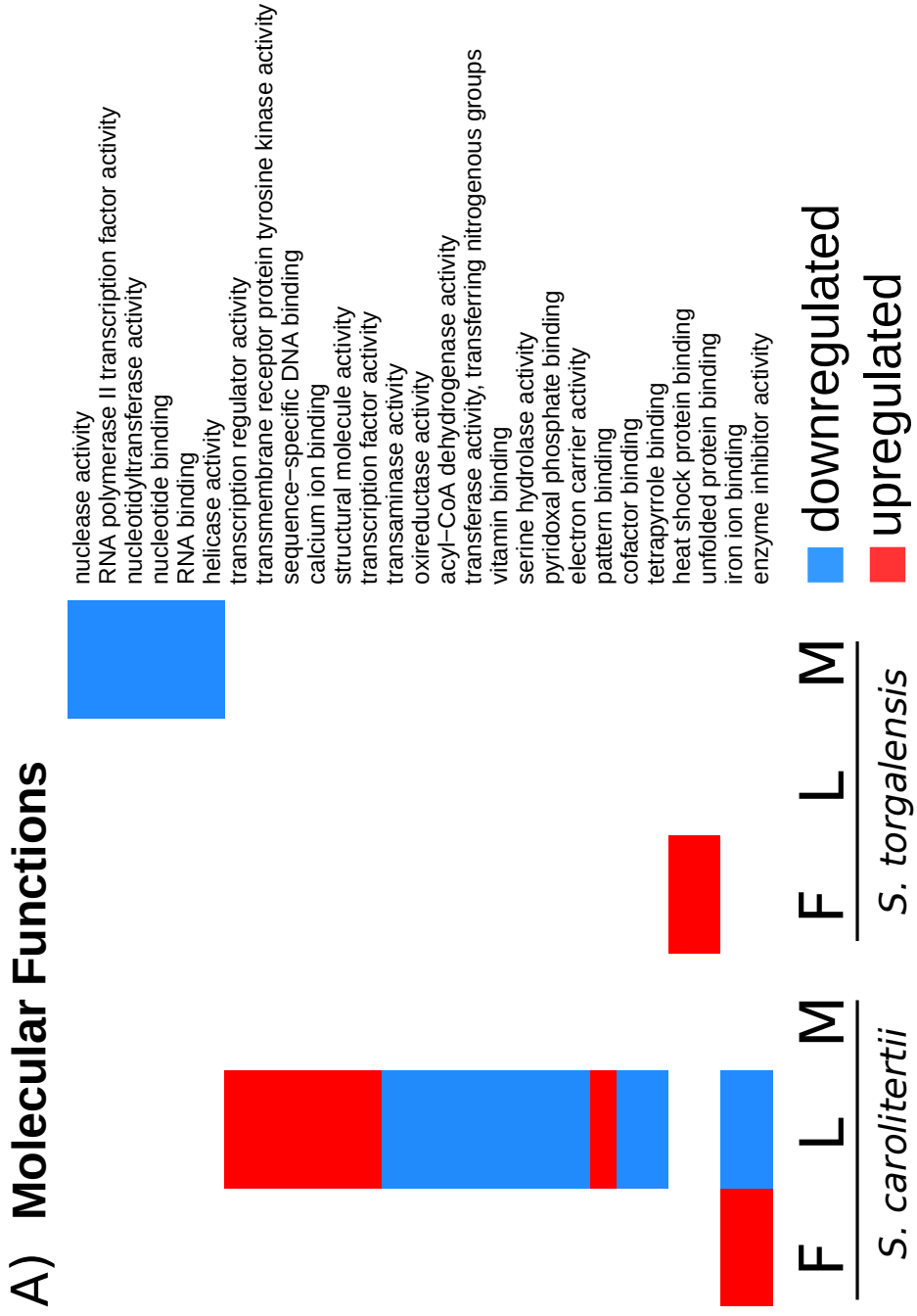


Figure 2.17: Percentage of each categories by A) Biological Process and B) Molecular Functions and C) Cellular Component, for DE genes shared between both species and exclusive to each species.



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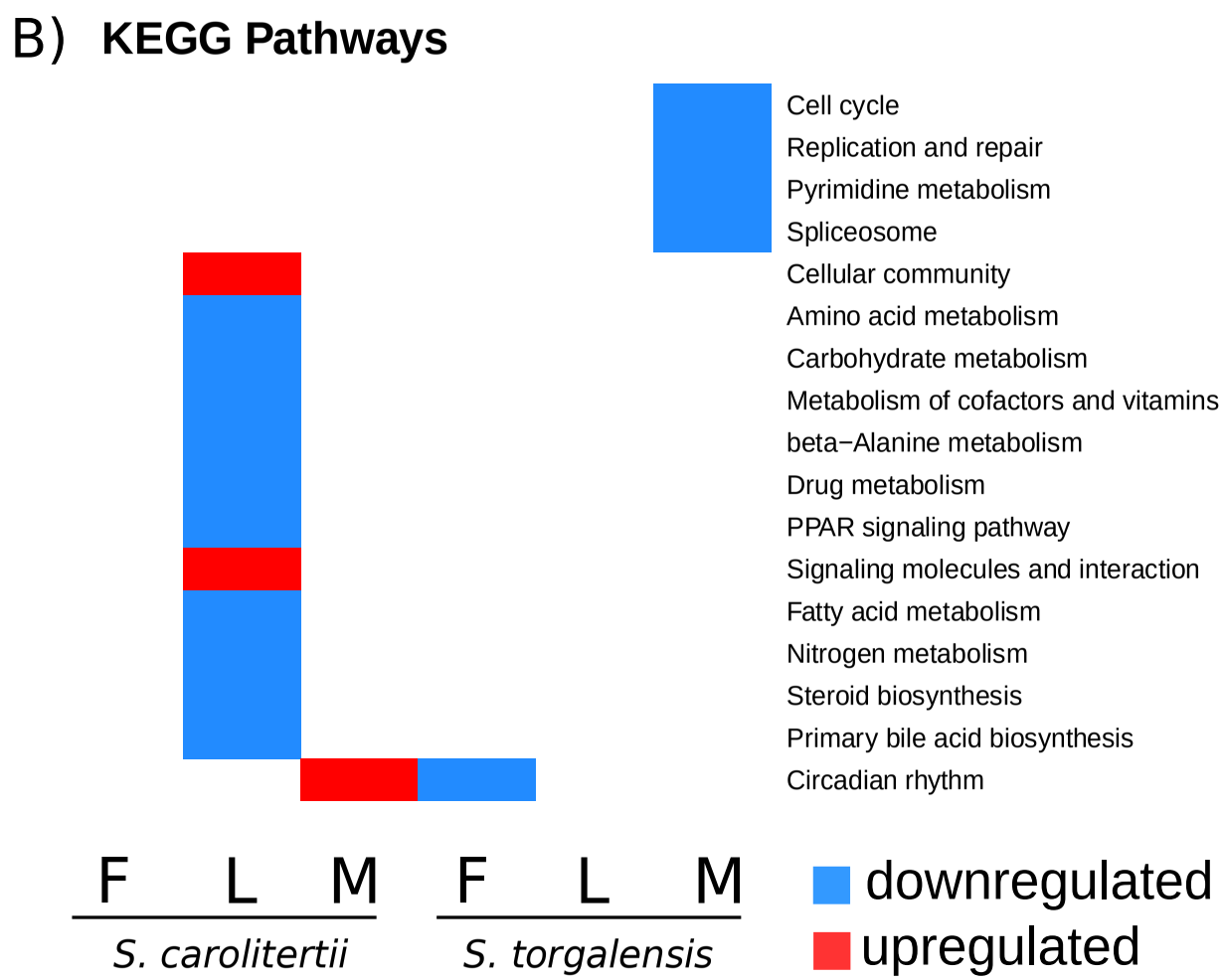

Figure 2.18: A) Enriched Molecular Functions (top heatmap) and B) Kegg Pathways of up and downregulated genes with benjamini < 0.05. F correspond to fins, L to liver and M to skeletal muscle.

Chapter 3

Acclimation and adaptation of endemic Iberian freshwater fish under climate change

3.1 Protein analysis and gene expression indicate differential vulnerability of Iberian fish species under a climate change scenario

The original work described in this subchapter is currently under revision in PLoS one.

Tiago F. Jesus[1], João M. Moreno[1], Tiago Repolho[2], Alekos Athanasiadis[3], Rui Rosa[2], Vera M.F. Almeida-Val[4] and Maria M. Coelho[1]

[1] - CE3C – Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, Edifício C2, 3º Piso, Campo Grande, 1749-016 Lisboa, Portugal

[2] - Laboratório Marítimo da Guia, MARE - Centro de Ciências do Mar e do Ambiente, Faculdade de Ciências da Universidade de Lisboa, Av. Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal

[3] - Instituto Gulbenkian de Ciência, Rua da Quinta Grande, 6, 2780-156 Oeiras, Portugal

[4] - Laboratório de Ecofisiologia e Evolução Molecular, Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM, Brasil.

Abstract

Current knowledge on the biological responses of freshwater fish under projected scenarios of climate change remains limited. Here, we examine differences in the protein configuration of two endemic Iberian freshwater fish species, *Squalius carolitertii* and the critically endangered *S. torgalensis*, that inhabit in the Atlantic-type northern and in the Mediterranean-type southwestern, respectively. We performed protein structure modeling of fourteen genes linked to protein folding, energy metabolism, circadian rhythms and immune responses. Structural differences in proteins between the two species were found for HSC70, FKBP52, HIF1α and GPB1. For *S. torgalensis*, besides structural differences, we found higher thermostability for two proteins (HSP90 and GBP1), which can be advantageous in a warmer environment. Additionally, we investigated how these species might respond to projected scenarios of 3 °C climate change warming, acidification (ΔpH=-0.4), and their combined effects. Significant changes in gene expression were observed in response to all treatments, particularly under the combined warming and acidification conditions. While *S. carolitertii* presented changes in gene expression for multiple proteins related to folding (*hsp90aa1*, *hsc70*, *fkbp4* and *stip1*), only one such gene was altered in *S. torgalensis* (*stip1*). However, *S. torgalensis* showed a greater capacity for energy production under both the acidification and combined scenarios by increasing *cs* gene expression and maintaining *ldha* gene expression in muscle. Overall, these findings suggest that *S. torgalensis* is better prepared to cope with projected climate change. Worryingly, under the simulated scenarios, disturbances to circadian rhythm and immune system genes (*cry1aa*, *per1a* and *gbp1*) raise concerns for the persistence of both species, highlighting the need to consider multi-stressor effects when evaluating climate change impacts upon fish. This work also highlights that assessments of the potential of endangered freshwater species to cope with environmental change are crucial to help decision-makers adopt future conservation strategies.

Introduction

Climate change is threatening biodiversity worldwide, with temperature and atmospheric CO_2 values rising at an unprecedented rate (Hartmann *et al.*, 2013; Field *et al.*, 2014; Pörtner *et al.*, 2014). Shifts in thermal, precipitation and flow regimes will be particularly

harmful for freshwater ecosystems (Field *et al.*, 2014). Increases in water temperature, coupled with decreased river flow and increased severity and frequency of droughts, will undoubtedly pose new challenges for freshwater fauna, particularly in the Mediterranean region (Füssel *et al.*, 2012). Such changes in natural freshwater ecosystems, will directly influence the survival, and ultimately the persistence, of extant species.

In order to cope with future climate changes, species can shift their distribution to a more suitable habitat, change their life-cycle or adapt through micro-evolution or plasticity to new environmental conditions (Bellard *et al.*, 2012). Otherwise they may become extinct (Bellard *et al.*, 2012). Fish metabolism strongly depends on the environmental temperature (Somero, 2010), and freshwater fish often have limited ability to migrate to a more suitable river, making them vulnerable to environmental changes (Hansen *et al.*, 2012). Evidence of coping mechanisms for climate change are emerging for teleost fish species such as chinook and sockeye salmon (*Oncorhynchus tshawytscha* and *O. nerka*), in which both new migration patterns and plasticity in thermal tolerance have been observed (Eliason *et al.*, 2011; Muñoz *et al.*, 2014). Also, the reef fish *Acanthochromis polyacanthus* and the rainbowfish *Melanotaenia duboulayi* have exhibited changes in gene expression in response to warming, both through plasticity mechanisms and processes that may enable them to adjust over generations (Veilleux *et al.*, 2015; Mccairns *et al.*, 2016).

European climate change reports highlight the importance of an ongoing process that has already diminished river flow and increased mean water temperature between 1 and 3 °C, over recent decades (Hartmann *et al.*, 2013; Field *et al.*, 2014; Pörtner *et al.*, 2014; Füssel *et al.*, 2012). These issues are noticeable for many European rivers during the summer season and particularly for southern European rivers where the severity and frequency of droughts has significantly increased (Füssel *et al.*, 2012).

The Iberian Peninsula is at the frontier between two contrasting climate types: the Atlantic in the northern region that is characterized by mild temperatures, and the Mediterranean in the southern region (one of 25 biodiversity hotspots (Myers *et al.*, 2000)), typified by high temperatures and droughts (Magalhães *et al.*, 2003; Carvalho *et al.*, 2010; Henriques *et al.*, 2010; Jesus *et al.*, 2013). Freshwater fish of the *Squalius* genus (Cyprinidae family) are endemic to river basins and regions in these two different climates, providing an opportunity to study closely-related species under these two climate types (Mesquita *et al.*, 2007a). *S. carolitertii* (Doadrio, 1988) inhabits the Atlantic-type northern region, whereas *S. torgalensis* (Coelho *et al.*, 1998), a critically endangered species,

has a more restricted distribution within the Mira river basin in the Mediterranean-type southwestern region (Coelho *et al.*, 1998). Hence, these two species reside under different environmental conditions, with distinct seasonal and even daily water temperature fluctuations, and demonstrate different traits that are possibly the result of adaptation to these contrasting environmental conditions (Magalhães *et al.*, 2003; Jesus *et al.*, 2013). Compared to *S. carolitertii*, *S. torgalensis* has a shorter life span, earlier spawning age, and a smaller body size, all of which are characteristics of species inhabiting more unstable environments (Magalhães *et al.*, 2003). Also, *S. torgalensis* may be better adapted to cope with higher temperatures, since it is able to induce hsps genes in response to high temperatures and acute thermal stress (Jesus *et al.*, 2013, 2016). Conversely, *S. carolitertii* was shown to be unable to cope with temperatures as high as 35 °C and either lacked or presented a weak response in terms of hsps gene expression under stress (Doadrio, 1988; Coelho *et al.*, 1998; Magalhães *et al.*, 2003; Mesquita *et al.*, 2007b; Henriques *et al.*, 2010; Jesus *et al.*, 2013, 2016). Furthermore, in a transcriptomic study, these two species presented differences in gene expression patterns between control (18 °C) and heat shock treatment (30 °C) (Jesus *et al.*, 2016). Moreover, a vast set of potential target genes involved in protein folding, energy metabolism, circadian rhythms and immune responses for use in thermal studies of these species has become available.

Climate change threatens to significantly impact the survival and persistence of fish, particularly for species living close to their thermal tolerance limits and are thus prone to be harmed by small changes in environmental temperatures (Reusch and Wood, 2007; Dahlhoff and Rank, 2007; Sorensen *et al.*, 2009; Tomanek, 2010; Hoffmann and Sgrò, 2011; Campos *et al.*, 2016). In this sense, adaptation of these species to their current environmental conditions may provide important clues as to how they might endure future environmental changes. Besides rising temperatures, acidification can also affect freshwater biota (Jiménez *et al.*, 2014). Recently, considerable attention has been given to ocean acidification and this process is widely known to affect the physiology and behavior of many marine species (e.g. Munday *et al.* (2009); Aurélio *et al.* (2013); Vinagre *et al.* (2013); Rosa *et al.* (2014); Ou *et al.* (2015); Rosa *et al.* (2016)), ranging from changes in olfactory systems (Munday *et al.*, 2009), neurotransmitter malfunctions (Nilsson *et al.*, 2012) and skeletal deformities (Bignami *et al.*, 2013; Pimentel *et al.*, 2014). Unlike ocean acidification which is caused by elevated atmospheric CO_2 concentrations, lake and river

acidification is mainly driven by acid rain (Lake *et al.*, 2000). However, freshwater acidification is also likely to be affected by future increases in CO_2 levels (Leduc, 2013). To date, few studies have examined the effects of increasing CO_2 and acidification, as mediated by climate change, on freshwater fish (Prado-Lima and Val, 2016).

Here, we aim to understand how freshwater fish might respond to projected future climate change scenarios of warming and acidification and their combined effects. We studied two Iberian endemic fish, *S. carolitertii* and *S. torgalensis*. Both species have distinct evolutionary backgrounds and experience differing environmental conditions. We simulated a climate change scenario for the year 2100, consisting of a summer average temperature increase of 3 °C and a ΔpH=-0.4. Therefore, we based our parameters on the IPCC Representative Concentration Pathways (RPC 8.5) from the fifth Assessment Report (AR5) (Field *et al.*, 2014; Settele *et al.*, 2014), since it projects an increase of air temperature ranging from 2.6 to 4.8 °C and an increase in oceanic water acidification of ΔpH=-0.42. In this context, we investigated fourteen genes linked to warming and/or water acidification responses in fish, taking advantage of their differential expression in the transcriptomes of *S. carolitertii* and *S. torgalensis* (Jesus *et al.*, 2016). Specifically, we used genes involved in protein folding, energy metabolism, circadian rhythms and immune responses in order to: i) compare the differences between the two species protein structural and functional configurations, and ii) assess alterations in gene expression between control and experimental conditions. Integration of our results allowed us to evaluate the potential capacity of the endemic freshwater fish to cope with future climate change scenarios.

Methods

Sampling

Twenty-four wild adult fish of *S. carolitertii* and *S. torgalensis* species were collected from Portuguese rivers, Mondego (40° 8'5.22"N; 8° 8'35.06"W) and Mira (37°38'1.31"N; 8°37'22.37"W), respectively, by electro-fishing (300V, 4A). Short duration pulses were used in order to avoid juvenile mortality. Sampling was performed during spring season (when average water temperatures were 17.8 ± 0.67 °C for Mondego river and 19.5 ± 0.21 °C for Mira river and average water pH were 8.08 ± 0.01 for Mondego river and 8.23 ± 0.02 for Mira river). Fish were captured under a license (263/2014/CAPT) issued by Portuguese

Table 3.1: Experimental conditions performed for both species. Control conditions defined for each species was based on summer average water temperature and pH [data obtained from snirh.pt (National Information System of Water Resources) for 4 consecutive years (2001-2005)]. Test conditions consist of an increase of 3 °C in relation to the current summer average conditions (Warming and Combined) and a decrease of 0.4 units in the current summer pH average (Acidification and Combined).

Species	Condition	Temperature	pH
S. carolitertii	control	19°C	6.9
	warming	22°C	6.9
	hypercapnia	19°C	6.5
	combined	22°C	6.5
S. torgalensis	control	23°C	7.3
	warming	26°C	7.3
	hypercapnia	23°C	6.9
	combined	26°C	6.9

authority for Conservation of endangered species (ICNF [Instituto da Conservação da Natureza e das Florestas]).

Experimental design

Upon arrival to the aquaculture facilities of Laboratório Marítimo da Guia (Faculdade de Ciências da Universidade de Lisboa, Portugal) fish were placed in tanks with conditions (temperature, pH and conductivity) similar to the ones found in nature during sampling. Then, fish were slowly acclimated to the control experimental conditions, in eight 200 L tanks (four per species), for 2 weeks, mimicking summer average values for temperature (18,68 ± 0.38 °C for *S. carolitertii* and 23.02 ± 0.77 °C for *S. torgalensis*) and pH (6.88 ± 0.33 for *S. carolitertii* and 7.31 ± 0.51 for *S. torgalensis*), under normoxic (8 mg/L) conditions (control condition, see Table 3.1).

After laboratory acclimation, four different groups (with 5 to 7 individuals) of *S. carolitertii* and *S. torgalensis* were gradually acclimated to four different conditions (Table 3.1): i) control; ii) warming; iii) acidification and iv) combined warming and acidification condition. Within these experimental conditions, we planned to simulate a moderate climate change scenario by increasing the temperature in +3 °C and applying a ΔpH=-0.4, under a 2x2 factorial design. During the acclimation and experimental periods, fish were

fed daily (ad libitum) with bloodworms (TMC Iberia, Portugal), white mosquito larvae (TMC Iberia, Portugal) and *Spirulina* spp. flake food (Ocean nutrition, Belgium). Overhead tank illumination was provided, according to prevailing natural light conditions, under a 12:12 (day: night) light regime. Ammonia, nitrite and nitrate levels were monitored daily (Salifert Profi Test, Holland) and kept always below detectable levels. Normoxic conditions were maintained and pH values were monitored and adjusted automatically by means of a computerized controlling system (Profilux 3.1N, GHL, Germany) connected to individual oxygen and pH probes (GHL, Germany), respectively. Monitoring was performed every 2 seconds and pH values were adjusted through injection of N_2/CO_2 (Air Liquide, Portugal) and upregulated by aeration with CO_2 filtered air (soda lime, Sigma-Aldrich). Conductivity was individually monitored (Profilux 3.1N, GHL, Germany) and kept between 400-500 μS/cm. Automatic dosing systems (TMC Iberia, Portugal), linked to the Profilux system, enabled inflow of freshwater (300 or 600 μS/cm), in order to lower or raise conductivity values (culture tanks), within desired interval (400-500 μS/cm). After 30 days of experimental exposure, five to seven individuals of each treatment and species were euthanized (with spinal transection followed by immediate brain removal), during early morning period. Experimental procedures used in this research were in accordance with the requirements imposed by the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (reviewed and approved by the animal ethics committee ORBEA – Animal Welfare Body of FCUL Statement 5/2016).

RNA extraction and cDNA synthesis

Liver and muscle tissue samples were immediately collected from fish and stored using RNAlater (Ambion, Austin, TX, USA), following the TRI Reagent manufacturer's instructions. For ribonucleic acid (RNA) extraction, TRI Reagent (Ambion, Austin, TX, USA) was added to liver and muscle samples. After homogenization with a Tissue Ruptor (Qiagen, Valencia, CA, USA), RNA was extracted according to the manufacturers protocol. TURBO DNase (Ambion, Austin, TX, USA) was employed to degrade any remaining genomic contaminants, followed by phenol/chloroform purification and LiCl precipitation (Cathala *et al.*, 1983). Sample quality was checked using a Nanodrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) based on the 260/280 nm and 260/230

nm absorbance ratios. Sample concentration were determined to ensure sufficient quantity of homogeneous RNA for complementary DNA (cDNA) synthesis. Synthesis of cDNA was performed, according to manufacturer's instructions, using a RevertAid H Minus First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) and stored subsequently at -20 °C.

Target genes

A total of fourteen genes of interest were chosen among the differentially expressed genes, belonging to different biological functions (protein folding, energy metabolism, circadian rhythm and immune response) (detailed in Table 3.2), in the transcriptomes of *S. carolitertii* and *S. torgalensis* (Jesus *et al.*, 2016).
For both species, the sequences of the target genes were obtained from Genomic Resources Development Consortium, Almeida-Val *et al.* (2015). All pairs of primers used were designed using PerlPrimer software v.1.1.19 (Marshall, 2004) (Table S1 and S2, supporting information). Sequences that displayed polymorphisms between both species were re-sequenced by Sanger (Table S1, supporting information). CLC Sequence Viewer v7.5 (CLC bio, Aarhus, Denmark) was employed to align nucleotide sequences. Complete sequences were obtained, except for *per1a* gene for which transcriptome information only permitted to study the partial coding sequence. The obtained sequences were deposited in GenBank (Accession numbers: KX589462-KX589485). Nucleotide sequences were translated and the resulting protein sequences were aligned using CLC Sequence Viewer v7.5 (CLC bio, Aarhus, Denmark) under default parameters (gap open cost: 10; gap extension cost: 1; end gap cost: as any other; and alignment method very accurate).

Table 3.2: List of target genes, with their official gene names, gene descriptions and functional category. *Continues on next page.*

Gene name	Gene descriptions	Function	Functional category
hsc70	heat shock cognate 70	Folding of denatured proteins; protects cells from stress.	protein folding
hsp70	heat shock protein 70	Folding of denatured proteins, protects cells from stress.	protein folding
hsp90	heat shock protein 90	Folding of denatured proteins; protects cells from stress.	protein folding
stip1	stress-induced phosphoprotein 1	links HSP70 and HSP90 together.	protein folding
fkbp4	FK506 binding protein 4	This gene is involved in immunoregulation and basic cellular processes involving protein folding and trafficking.	protein folding
hif1a	hypoxia inducible factor 1 alpha	Induces several genes involved in hypoxia response, cell proliferation, glucose and iron metabolism.	energy metabolism
ldha	lactate dehydrogenase A	Catalyzes the interconversion of pyruvate and L-lactate.	energy metabolism

Table 3.2: *Continuation of the table from previous page.*

Gene name	Gene descriptions	Function	Functional category
cs	citrate synthase	Catalyzes the first reaction of the citric acid cycle: the condensation of the acetyl-CoA and oxaloacetate to form citrate	energy metabolism.
ndufb8	mitochondrial NADH dehydrogenase (ubiquitone) 1 beta subcomplex subunit 8	Accessory subunit of the NADH dehydrogenase (ubiquitone) complex, located in the mitochondrial inner membrane, of the electron transport chain. It transfers electrons from NADH to the respiratory chain.	energy metabolism
glula	glutamate-ammonia ligase (glutamine synthase) a	Catalyzes the condensation of glutamate and ammonia to form glutamine.	energy metabolism
lox	lysil oxidase	Catalyzes the formation of aldehydes from lysine residues in collagen and elastin percursors.	energy metabolism
per1a	period circadian clock 1a	It is a member of the period gene family and is important for circadian clock maintenance.	circadian rhythm

Table 3.2: *Continuation of the table from previous page.*

Gene name	Gene descriptions	Function	Functional category
cry1a	cryptochrome 1a	It is a member of the cryptochrome gene family, which regulates the circadian clock in a light dependent fashion.	circadian rhythm
gbp1	guanylate binding protein 1	This gene is induced by interferons and presents antiviral activity by regulating the inhibition of proliferation of endothelial cells.	immune response

Protein structure prediction

In order to predict physical and chemical parameters, the ProtParam tool (Gasteiger *et al.*, 2005) was used. Protein three-dimensional structure was also predicted using the homology modelling algorithm (RaptorX Structure Prediction) offered in RaptorX webserver (Källberg *et al.*, 2012). Protein structural alignments of each species were performed following the Smith-Waterman algorithm offered in UCSF Chimera (Pettersen *et al.*, 2004), using the default parameters with a secondary structure score set to 0.70. Protein alignments were performed using the same protein of each species and differences are presented, with differing amino acid residues highlighted.

Quantitative RT-PCR

Relative expression levels of genes of interest were normalized against three reference genes [Poly(A) binding protein, cytoplasmic 1a (*pabpc1a*), ribosomal protein L35 (*rpl35*) and ribosomal protein SA (*rpsa*)] (for details on primer conditions see Table 3.4, supporting information), chosen among the most stable genes for the transcriptomes of three organs (liver, fins and skeletal muscle) of these two species exposed to different temperature conditions (18 °C and 30 °C) (Genomic Resources Development Consortium, Almeida-Val *et al.*, 2015). These reference genes were chosen from contigs with more than 1000 read counts per library, FDR > 0.05 and Fold Change < 1.5 ($\log_2$(Fold Change) < 0.58), in order to assure that they are highly expressed, but not differentially expressed. Furthermore, reference genes stability was also verified in *Squalius pyrenaicus* transcriptome (Genomic Resources Development Consortium *et al.*, 2015), to further guarantee their stability across more conditions (Table 3.5, supporting information). In order to determine the stability of these reference genes, in the qPCR analysis, we used the NormFinder software (Andersen *et al.*, 2004).

Real-time polymerase chain (PCR) reactions were performed in a Bio-Rad CFX96 system (Bio-Rad, USA), following manufacturer's instructions for Sso Advanced universal SYBR Green supermix (Bio- Rad, Hercules, CA, USA). Controls without template and without reverse transcriptase were included to check for PCR contamination and genomic deoxyribonucleic acid (DNA) contamination, respectively. Amplicons identities were confirmed through melting curve analysis. The PCR efficiency for each sample was assessed using LinRegPCR 11.1 software (Ruijter *et al.*, 2009) and ranged from 94.38% – 97.72% for all

primer pairs (Table 3.4, supporting information). Relative quantity of genes of interest was calculated, using the comparative threshold cycle (CT) method with efficiency correction, using the mean PCR efficiency for each amplicon (Ruijter *et al.*, 2009). Relative gene expression of target genes was calculated against the geometric mean of the reference genes, using the $2^{-(\Delta\Delta Ct)}$ method (Pfaffl, 2001).
Data was log transformed [$\log_{10}$(x+1)] and checked for normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test). A two-way analysis of variance (ANOVA) was performed to identify statistical differences in transcript expression patterns across the experimental conditions for all genes independently, for each tissue. Post-hoc tests for multiple comparisons (Tukey tests) were applied whenever significant differences across treatments were observed. All statistical analyses were performed using a significance level of 0.05, using a custom python script and the program STATISTICA v.12 (StatSoft Inc., USA).

Results

Protein structural and functional evolution

Four of fourteen target proteins, showed alterations in their predicted tertiary structure between the two species (Figure 3.1), and two presented different predicted physical and chemical parameters (Table 3.6, supporting information).
The physical and chemical parameters of the selected proteins were similar between species, with GBP1 and HSC70 presenting small changes in their theoretical isoelectric point (pI) and GBP1 and HSP90 having 1 unit differences in their respective aliphatic indexes (Table 3.6, supporting information).
Regarding their tertiary structures, HSC70, FKBP52 (FK506-binding protein 4, encoded by the *fkbp4* gene), HIF1α and GBP1 showed differences between species. For HSC70, there were 11 noncontiguous aminoacids (a.a.) different between the two species (Table 3.7, supporting information), but these did not coincide with the main predicted structural differences that are located in coil regions (Figure 3.1). FKBP52 had 3 non-synonymous substitutions (Table 3.7, supporting information) that also did not overlap with observed structural changes, within coil regions, but instead were located mainly at termini, as observed for HSC70 (Figure 3.1).

In addition to the above-mentioned folding proteins, two other proteins presented structural alterations. HIF1α exhibited structural changes at the helix-loop-helix (bHLH), Per-ARNT-Sim (PAS) and DNA-binding domains. The HIF1α transcription factor presented two non-synonymous substitutions between species (Table 3.7, supporting information), one of which overlaps with predicted structural changes in coil regions in the PAS domain (Figure 3.1 and Table 3.7, supporting information).
The GBP1 protein presented 11 non-synonymous substitutions in the helical and globular protein domains (Figure 3.1 and Table 3.7, supporting information). However, the locations of these altered amino acids did not coincide with the positions of structural changes observed in coil regions of the globular (GTP-binding) domain (Figure 3.1).
The remaining 10 predicted proteins presented no alterations between species (Figure 3.3, supporting information).

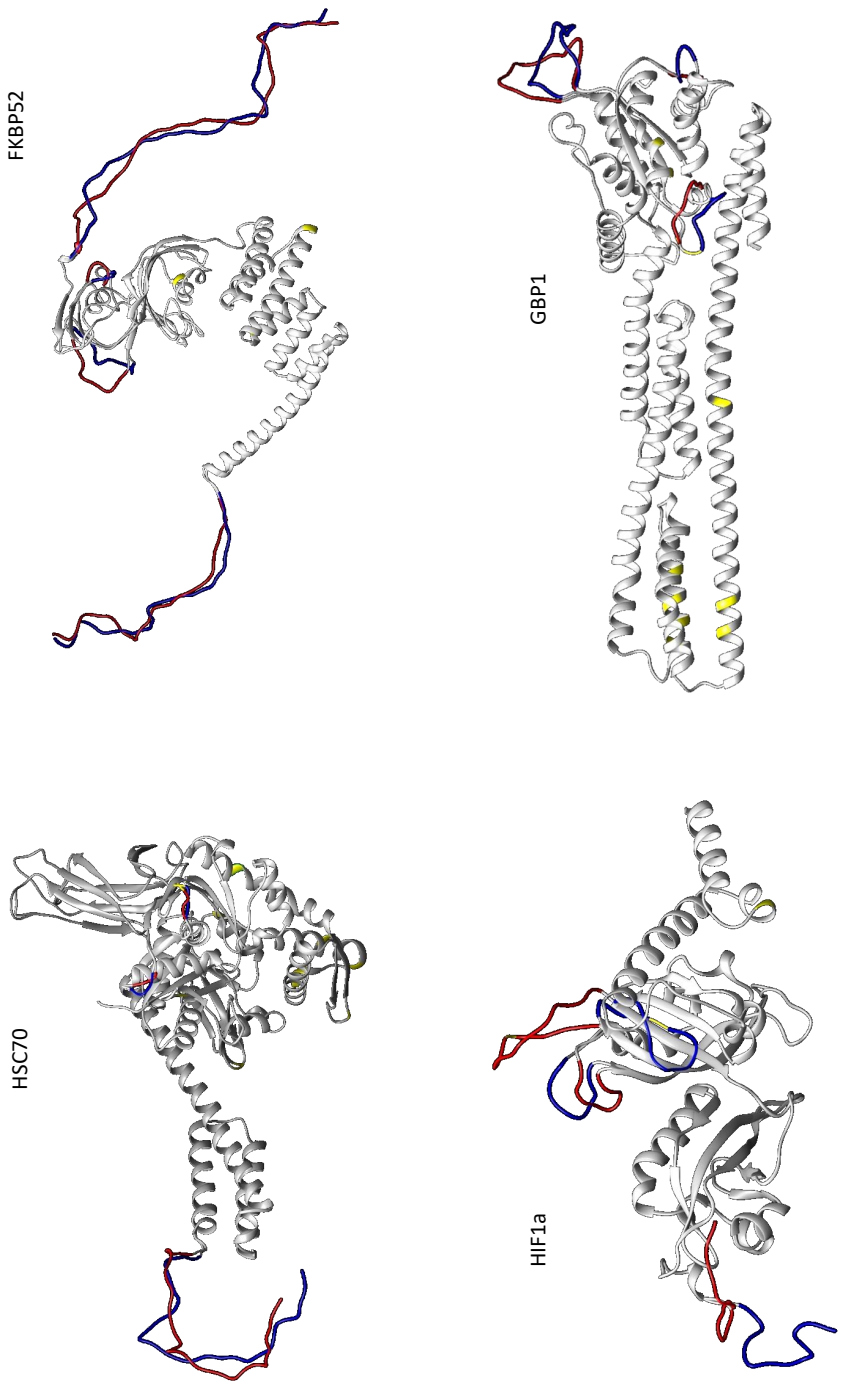

Figure 3.1: Structural differences between predicted proteins of the two species. Regions in light grey have no differences between species, blue and red indicate the conformation of *S. carolitertii* and *S. torgalensis* for that specific region and yellow represents the amino acids positions which correspond to non-synonymous substitutions.

Gene expression

Stability values of the reference genes *pabpc1a*, *rpl35* and *rpsa* were high (less than 0.06 for all tissues and temperatures tested and, on average, less than 0.045) (Figure 3.4, supporting information), with little variation (stability values of *rpsa* varied between 0.029 and 0.041, for *rpl35* between 0.017 and 0.051, and for *pabpc1a* between 0.030 and 0.059). These stability values are inferior to those observed by (Andersen *et al.*, 2004), which makes them suitable for gene expression normalization of our target genes.

Combined warming and acidification elicited the most significant changes in our genes of interest (in 11 genes for *S. carolitertii* and in 4 for *S. torgalensis*), followed by acidification (6 genes altered for *S. carolitertii* and 3 for *S. torgalensis*). Warming did not significantly alter *S. torgalensis* gene expression, but *S. carolitertii* presented significant differences in 5 genes (Figure 3.2).

Regarding differential expression of genes involved in protein folding, *S. carolitertii* presented significant changes in more genes, with differences between control and test conditions observed for *hsc70*, *hsp90aa1*, *fkbp4* and *stip1* (Figure 3.2A), while *S. torgalensis* only presented changes for *stip1* (Figure 3.2A). Most of the differences in observed gene expression were elicited by the warming and combined conditions, except for *fkbp4* for which a significant change under the acidification condition was observed in *S. carolitertii* muscle. No change was detected for the *hsp70* gene.

Regarding genes related to energy metabolism, most differences occurred under combined conditions of warming and acidification, with both species presenting several significant alterations (Figure 3.2B). The *ldha* and *cs* genes presented the greatest differences in expression, particularly for muscle tissue, in which distinct patterns were found for both species: *ldha* was downregulated in *S. carolitertii* and *cs* was upregulated in *S. torgalensis*. Both species showed a similar response in liver tissue, with both these genes being upregulated under combined conditions of warming and acidification (though for *cs* in *S. carolitertii* was only marginally significant). The *hif1a* gene was significantly upregulated in *S. torgalensis* liver under combined warming and acidification, presenting a similar pattern as that observed for the *cs* and *ldha* genes. However, only *hif1a* changes were statistically significant for *S. carolitertii* liver under the same combined conditions. Expression of the *ndufb8* and *glula* genes (Table 3.2) changed in *S. carolitertii* muscle, but that of the *lox* gene did not (Table 3.2).

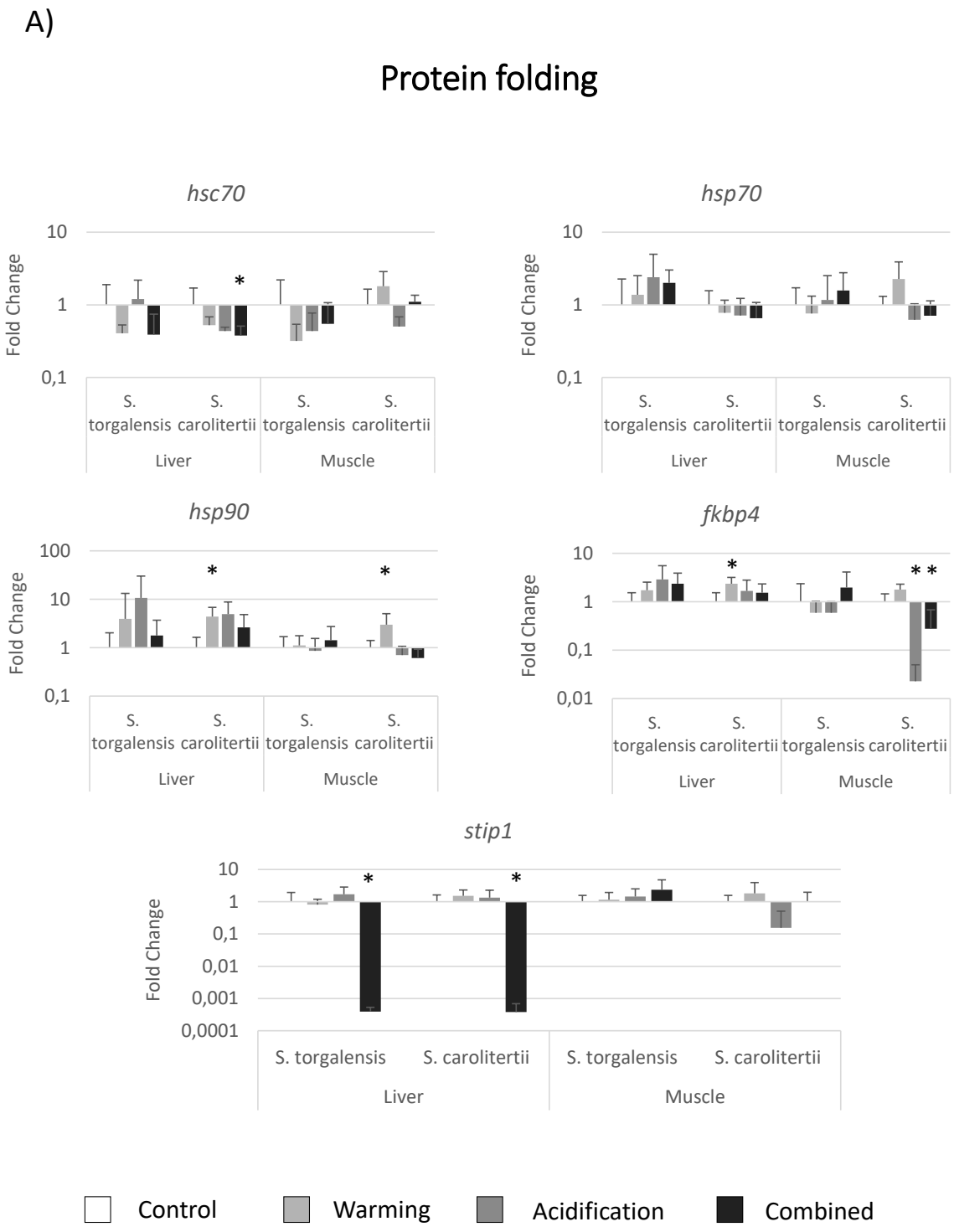

Figure 3.2: *Continues on next page.*

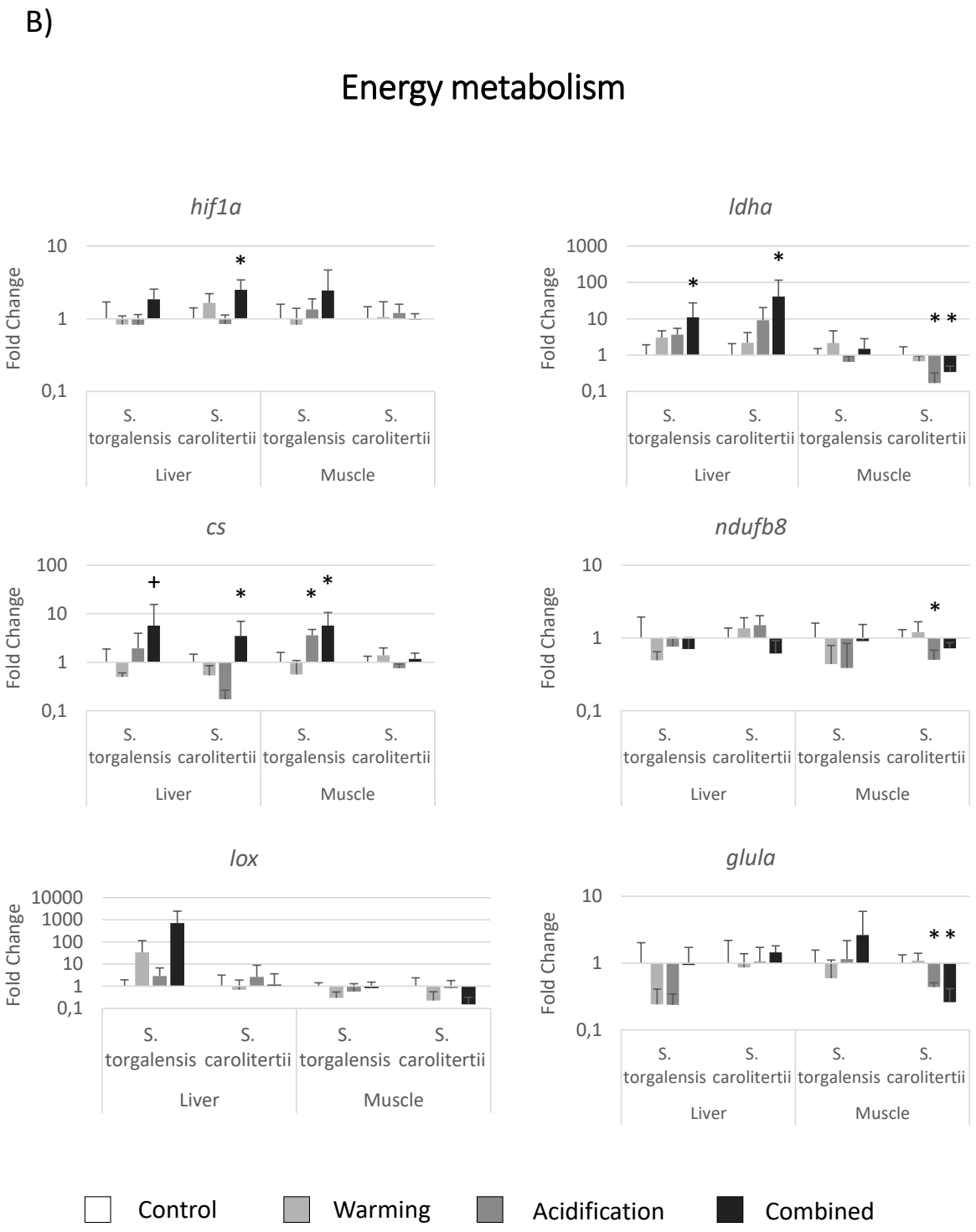

Figure 3.2: *Continues on next page.*

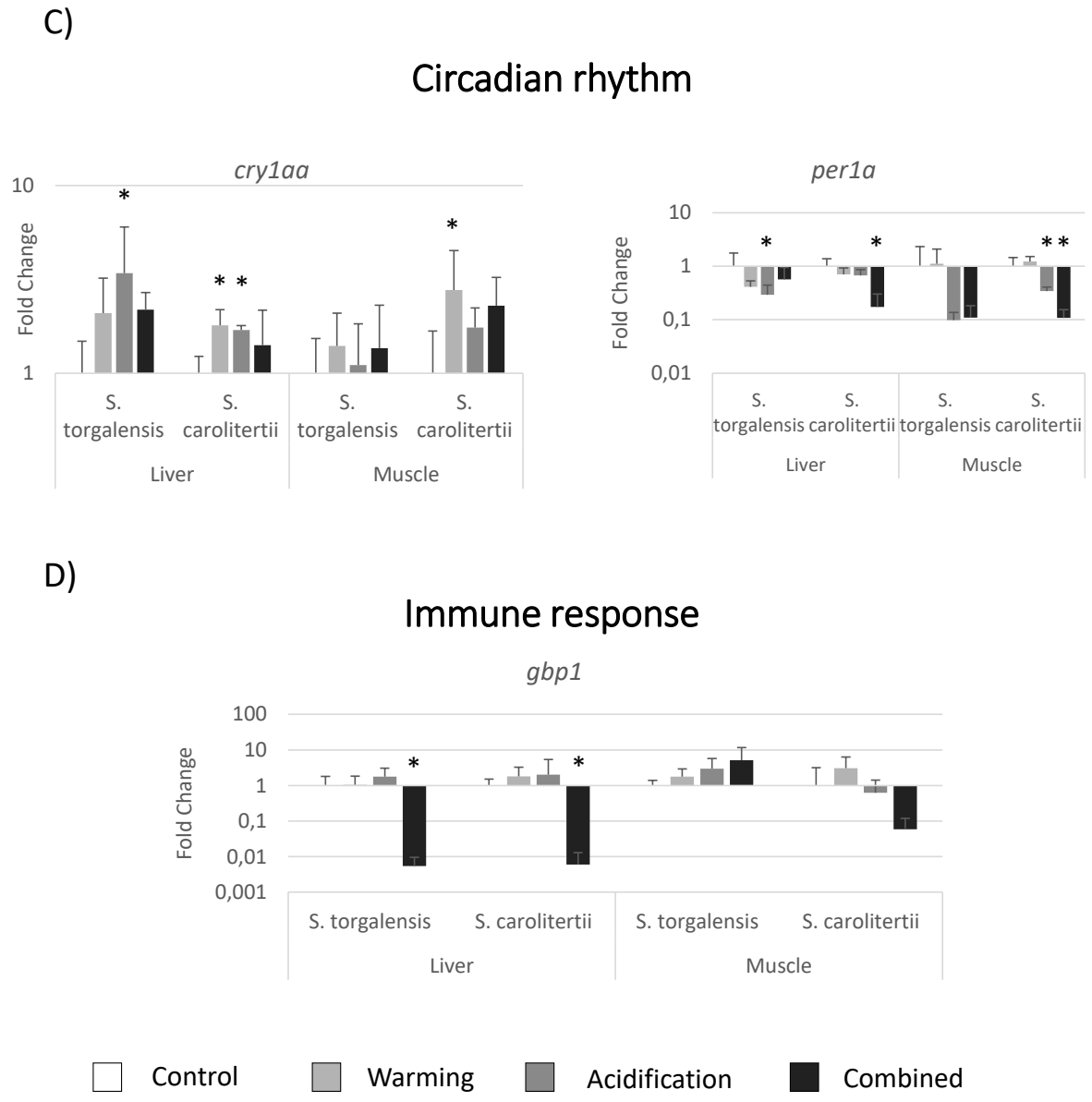

Figure 3.2: Gene expression of the genes involved in A) protein folding, B) energy metabolism, C) circadian rhythm and D) immune response. Gene expression values and significances are relative to the control condition. The * symbol represents a $p\text{-}value < 0.05$ and + symbol a $0.1 < p\text{-}value < 0.05$ (and thus marginally significant).

Circadian clock genes (*cry1aa* and *per1a*) revealed significant changes under acidification for *S. torgalensis* liver tissue, whereas *S. carolitetii* presented significantly altered expression for these genes under all three conditions for liver and muscle tissues (Figure 3.2C).

The *gbp1* gene, which is involved in the immune response, presented major changes in fish exposed to the combined warming and acidification condition, being downregulated in the livers of both species (Figure 3.2D). Significant ($p < 0.05$) synergistic effects between the combined factors of temperature and pH were observed in the liver for *S. carolitertii* (*hsp90aa1*, *fkbp4*, *stip1*, *cs*, *ndufb8* and *gbp1*) and *S. torgalensis* (*hsp90aa1*, *per1a* and *gbp1*), as well as in the muscle for *S. carolitertii* (*lox*) and *S. torgalensis* (*ndufb8*).

Discussion

It is currently assumed that climate change, namely warming and acidification, will pose serious challenges to species survival and persistence (Berg *et al.*, 2010). In general, temperate species are potentially more adapted to deal with wide ranges of temperatures and pH on a seasonal and daily basis. To date, empirical data on the biological effects of warming and acidification on freshwater biota, especially endangered fish species, is scarce or still poorly understood (Ou *et al.*, 2015; Mccairns *et al.*, 2016). To the best of our knowledge, our work represents the first comparative study integrating protein structural and functional analysis and gene expression changes in freshwater fish species exposed to experimental conditions of warming and acidification, simulating a future climate change scenario.

Protein structural and functional evolution

First, we consider at the structural and functional evolution of 14 proteins in two Iberian endemic fish species (*S. carolitertii* from the North and *S. torgalensis* from the South). Of the 14 predicted proteins we studied, 3 proteins related to protein folding presented noticeable differences between species in either their physical and chemical parameters (HSP90) or in their structure (HSC70, FKBP52). Additionally, structural differences were found for the energy metabolism-related protein, HIF1α, and both functional and structural differences were found for GBP1, which is involved in the immune response.

We found that *S. torgalensis* displays a higher thermostability for HSP90. For HSC70, several structural changes between species were found in the coil regions of functional domains, which are of uncertain importance for its protein folding function (Grishin, 2001; Trifonov and Berezovsky, 2003). The *fkbp4* gene encodes FK506-binding protein 4 (FKBP52), which possesses an N-terminal peptidylprolyl cis–trans isomerase domain (PPIase) and a C-terminal tetratricopeptide repeat domain (TPR). The PPIase domain is responsible for the cis-trans isomerization process that can limit this type of protein folding (Kang *et al.*, 2008), whereas the TPR domain mediates protein–protein interactions. For example, FKBP52 interacts with HSP90, thereby facilitating the intracellular trafficking of steroid receptors. Moreover, this protein is involved in the regulation of interferon regulatory factor-4 and plays an important role in immunoregulatory gene expression in B and T lymphocytes (Scammell *et al.*, 2003). Here, we observed alterations in both domains, suggesting that this protein has a potential role in climate change adaptation in these species. HIF1α is responsible for regulating many hypoxia-associated genes, as well as genes involved in glucose metabolism, cell proliferation and iron metabolism. Our predicted HIF1α proteins showed differences in all three functional domains, particularly in the DNA-binding domain that is crucial for the regulation of transcription (Semenza *et al.*, 1997). However, changes in bHLH and PAS domains may interfere with protein-protein dimerization (Semenza *et al.*, 1997), which may be a key element in the regulatory activity of proteins such as enzymes, ion channels, receptors and transcription factors (Marianayagam *et al.*, 2004).

We also found structural changes between both species and a higher aliphatic index (thus higher thermostability) for *S. torgalensis* in the predicted GBP1 protein, which is induced by interferons and has antiviral activity (Lu *et al.*, 2007; Itsui *et al.*, 2009). The structural differences were mostly located in the GTP-binding domain of the protein, which hydrolyzes GTP to GDP, and is crucial for the function of the protein in antiviral defense (Prakash *et al.*, 2000).

The higher thermostability of HSP90 and GBP1 and the structural differences of GBP1 may indicate an advantage for *S. torgalensis* in a warmer environment. Additionally, the structural differences found for between the two species in HSC70, and HIF1α located in coil regions between functional domains have unclear impacts on protein function (Grishin, 2001; Trifonov and Berezovsky, 2003), even though these are particularly important

regions for overall conformational flexibility (Buxbaum, 2007). These structural differences could be linked to the potential of this species to cope with warmer environments.

Gene expression under future climate change scenario

Regarding gene expression, to date, only heat shock experiments had been conducted on these species (Jesus *et al.*, 2013, 2016). In this study, we provide new clues as how these two species can acclimate to projected climate change by simulating the effects of increasing temperature and water acidity, both separately and combined. In general, the combination of both effects resulted in higher impacts on gene expression compared with the control condition. Although the resulting altered gene expression could be considered an additive effect of both conditions, for some genes, such as *stip1* and *gbp1* in the liver tissue of both species, the changes in expression were synergistic, since they were not observed in the independent temperature or pH experiments. Pimentel *et al.* (2015) observed cumulative changes in enzymatic activity under similar conditions (warming and acidification) in the flatfish *Solea senegalensis*. Despite this, to date, many studies have focused on single stressors (e.g. either temperature or pH) (Eliason *et al.*, 2011; Jesus *et al.*, 2013; Veilleux *et al.*, 2015; Jesus *et al.*, 2016). Thus, our results emphasize the necessity to consider the combined effects of these stressors when assessing the impacts of climate change scenarios on organisms, since changes are neither the simple sum of these stressors nor can they be easily predicted by considering the effects of the two factors separately.

Across all experimental conditions, genes involved in protein folding presented differential expression only for *S. carolitertii*, with the exception of *stip1* that showed changes in both species. The heat shock proteins *hsc70* and *hsp90aa1* presented changes in quantitative gene expression for *S. carolitertii*, but *hsp70* did not. The differences in gene expression found for *hsc70*, support that structural differences between the two species can be important to protein function. Long-term changes in these genes may be disadvantageous since previous studies have shown that resources are reallocated from other crucial biological processes (e.g. growth) for the folding of denatured proteins [e.g. Itsui *et al.* (2009); Veilleux *et al.* (2015)]. In previous studies, heat shock induced increased expression of both *hsp70* and *hsc70* as a response to acute thermal stress in *S. torgalensis* (Jesus *et al.*, 2013, 2016), probably to prevent protein denaturation (Lindquist and Craig,

1988; Sorensen *et al.*, 2003). However, in the present study, no change was observed for hsp genes in response to a milder temperature change for a longer period. Therefore, the fact that *S. torgalensis* have specific changes in protein structure at these genes, together with the fact that it coped with the new environment without major changes in the gene expression might indicate that this species has a higher thermal tolerance before eliciting stress responses.

HSP70 and HSP90 proteins usually form a complex of chaperones that help in the correct folding of important proteins for cell functioning. However, both proteins are capable of independent activity. While HSP70 is responsible for the folding of nascent proteins and other important cell processes (e.g. trafficking of proteins across membranes), the most common client proteins of HSP90 are regulators of transcription or protein kinases (see (Wegele *et al.*, 2004) for further details). Therefore, the observed differences in *hsp90aa1* gene expression may be related to substract interactions of HSP90 protein, with *S. carolitertii* possibly incurring altered transcriptional regulation under the warming condition. Moreover, these expression differences between the two species, in *hsp90aa1*, can be related to the higher thermostability of the corresponding coding protein observed in *S. torgalensis*. In contrast, pH per se did not affect the genes involved in protein folding, except for *fkbp4*, which possesses peptidylprolyl isomerase activity (Scammell *et al.*, 2003) and whose catalysis may depend on environmental pH (Cornish-bowden, 2013). Therefore, the lack of gene expression response in *S. torgalensis* could be related with the structural differences between the proteins of both species that encode this gene (FKBP52). Also, the observed results for *fkbp4* may be related with its immunoregulatory functions (Scammell *et al.*, 2003). Stress-Induced Phosphoprotein 1 [*stip1* or *hop* (Hsp70-Hsp90 Organizing Protein)] mediates the transfer of proteins from HSP70 to HSP90, through the formation of an "intermediate complex" composed of these three proteins and the substrate protein (mainly steroid hormone receptors) (Wegele *et al.*, 2004). The severe downregulation of *stip1* gene transcription under the combined warming and acidification condition in liver tissues of both species highlights the importance of synergistic effects in climate change studies.

The genes involved in energy metabolism presented an intricate and interconnected response (see Figure S3, supporting information for a schematic representation). The transcription factor hif1a induces many genes during hypoxia, but also participates in other pathways such as glucose metabolism, with ldha being a target gene of this transcription

factor (Semenza *et al.*, 1997; Denko, 2008). In this study, we maintained the animals under normoxic conditions and an increase in transcription of both *hif1a* and *ldha* genes was observed in liver. Thus, the induction of *hif1a* gene expression seems to be more related to glucose metabolism rather than hypoxia. Importantly, the liver is capable of catabolism and anabolism at the same time; an ability not shared by any other organ or tissue (Nelson and Cox, 2008).

However, gluconeogenesis is an expensive mechanism and we found that upregulation of the *ldha* gene was coupled with an increase in *cs* transcription, suggesting an increase in the usage of pyruvate by the citric acid cycle. Furthermore, in *S. torgalensis*, *ldha* expression in muscle was not altered between treatments, whereas *cs* was upregulated under acidic and combined conditions, suggesting a greater ability to produce ATP. However, *S. carolitertii* exhibited downregulation of *ldha* under the same conditions, with no significant change in *cs* transcription, so perhaps this species has a reduced capacity to produce ATP under the acidic and combined conditions. Also, the gene *ndufb8*, which encodes NADH dehydrogenase 1 beta subcomplex subunit 8, which is capable of independent respiratory chain activity in mitochondria (Davis *et al.*, 2010), was downregulated in *S. carolitertii* under acidified conditions. Together, these results suggest that both species prioritize aerobic metabolism for energy production in muscle, with *S. torgalensis* showing a greater capability of producing energy under our experimental conditions compared to control by increasing the expression of *cs* and by maintaining ldha expression. Also, differences found in the expression of genes related with energy metabolism can result from the higher thermostability and structural differences found in HIF1α for *S. torgalensis*, since this protein is a main regulatory agent of this function.

Glutamine ammonia ligase or glutamine synthetase (encoded by the *glula* gene) plays a key role in nitrogen metabolism, catalyzing the conversion of ammonia and glutamate to glutamine, a less toxic compound that is used in the production of several other metabolites (Liaw *et al.*, 1995). We only observed differential expression under acidification alone, with warming having little or no significant effect. Thus, the catalytic activity of this enzyme may decrease at lower pH in *S. carolitertii* muscle. Though we did not feed our experimental groups of fish differently, demand for nitrogen compounds is expected to decrease under increased temperatures and so herbivory is increased, which has been reported in omnivorous copepods and fish (Behrens and Lafferty, 2007; Boersma *et al.*,

2016). Therefore, the *glula* gene might be a suitable biomarker for the usage of nitrogen in omnivorous fish undergoing climate change.

We did not find significant differences for the *lox* gene among fish under different conditions. Lysyl oxidase (LOX) catalyzes the formation of lysine-derived cross-links in collagen and elastin and it is involved in several other biological functions (e.g. development, tumor suppression, cell motility and cellular senescence) (Csiszar, 2001). Therefore, the absence of differences may be due to the fact that the lox gene is vital during development and in atypical cell functioning (Csiszar, 2001).

Warming and acidification have impacts on many biological processes that occur in vertebrate cells. Despite limited evidence that circadian clock genes may be directly impacted by climate change, temperature may trigger the responses of such genes (Idda *et al.*, 2012; Schunter *et al.*, 2016), as observed for the species in this study (Jesus *et al.*, 2016). We found that the two circadian clock genes we studied, *cry1aa* and *per1a*, presented significant changes under both warming and acidification conditions. Expression was increased for *cry1aa* and decreased for *per1a* in both species. The *cry1aa* gene is known to be induced in fish during the early morning, whereas per1a has higher expression late at night (end of the dark period) (Amaral and Johnston, 2012). Contrary to *cry1aa*, *per1a* gene does not exhibit light-dependent expression (Amaral and Johnston, 2012). Therefore, disruption of this balance in the circadian clock of fish may have profound effects on fish metabolism and behavior (such as feeding and mating behavior), particularly given that the changes were not the result of experimental changes in photoperiod. For a more detailed mechanistic explanation on this subject, a study of all genes involved in the circadian clock would aid our understanding of the regulation of the pool of *cry* and *per* genes that are involved in clock regulation.

There is growing concern about the effects of environmental change on the immune system of vertebrates (Hansen *et al.*, 2012; Veilleux *et al.*, 2015). Some evidence that temperature may alter gene expression of immune response-related genes is already available (Smith *et al.*, 2013; Veilleux *et al.*, 2015; Jesus *et al.*, 2016). Our results, raise some concerns for medium- to long-term exposure to predicted climate change, since a drastic downregulation was observed for the *gpb1* gene for the combined warming and acidification condition. Although we analyzed only one gene related to the immune system, the combination of these two environmental factors severely decreased its expression, putatively leading to its suppression. Therefore, further attention should be paid to the effects and interactions

of the multiple environmental factors involved in climate change on genes involved in the immune response.

Conclusions

Climate change projections for freshwater ecosystems are scarce and may be worse than we simulated here, particularly for the acidification of these ecosystems, where organic matter content may be extremely variable between water bodies and seasons, contrary to what is observed in oceanic waters (Ou *et al.*, 2015; Settele *et al.*, 2014). In this study, we examined differences in protein configuration and in gene expression between two endemic Iberian freshwater fish species that inhabit different climatic regions, *S. carolitertii* in the Atlantic-type northern region and *S. torgalensis* in the Mediterranean-type southwestern region. We observed protein structural differences between the two species for HSC70, FKBP52 and HIF1α and higher thermostability for HSP90 and GBP1 in *S. torgalensis*. Most of the changes in gene expression were observed for *S. carolitertii*, whereas *S. torgalensis* showed no major changes in the heat shock response or in respiratory capacity. Taken together, these results suggest that *S. torgalensis*, which lives in a warmer environment, is less impacted by temperature increases and acidification. Consequently, our results suggest that *S. torgalensis* could be capable of dealing with the IPCC projections of warming and/or acidification at the end of this century. Our study highlights the importance of assessing the potential of endangered freshwater species to cope with projected climate change conditions for the proper implementation of conservation strategies.

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Supporting information

Tables

Table 3.3: *Continues on next page.* (part 1/3)

Genes	Primer names	Primer Sequence
ldha (1)	ldha_f1	Forward: 5' - GCTGAAAGGAGAGGTTATGG - 3'
	ldha_r1	Reverse: 5' - AATGGTTAGAGGCAGTGAGG - 3'
ldha (2)	ldha(2)_fw	Forward: 5' - GACCTGTTAGCCAATAGACC -3'
	ldha(2)_rv	Reverse: 5' - TCCAGCTGATACACAAAGTG - 3'
cry1a (1)	cry1a_fw	Forward: 5' - TCTTCCAGCAGTTCTTCCAC - 3'
	cry1a_rv	Reverse: 5' - TGTGCAGATTACAGAGCCAG - 3'
cry1a (2)	cry1a(2)_fw	Forward: 5' - CACGGCAGGATGGTTTAC - 3'
	cry1a(2)_rv	Reverse: 5' - TGTGCAGATTACAGAGCC - 3'
gbp1	gbp1_fw	Forward: 5' - GAAGTCCTACCTTATGAACC -3'
	gbp1_rv	Reverse: 5' - ATGCTTACAGCTTCCTCCAG -3'
hif1a	hif1a_fw	Forward: 5' - GAGTCCGAGGTGTTCTACGAG - 3'
	hif1a_rv	Reverse: 5' - GCTCTGTCATGGTCTGCTGC - 3
hsc70	hsc70_fw	Forward: 5' - GACCTTCACCACTTACTCAG - 3'
	hsc70_rv	Reverse: 5' - CACTTCCTCAATGGTAGGAC - 3'
fkbp4	fkbp4_fw	Forward: 5' - CGCAGGATCATCACTAAGG - 3'
	fkbp4_rv	Reverse: 5' - CATGCCATTATGCTGCAGTT - 3'
hsp90	hsp90_fw	Forward: 5' - GCTTTCCCTCAAGGACTACG -3'
	hsp90_rv	Reverse: 5' - GGTTGAGTAATGTCCTCCACAG - 3'

Table 3.3 (part 2/3)

Genes	Initial Denaturation		Denaturation		Annealing		Extension		Cycles	Final Extension	
	Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)		Temp (°C)	Time (s)
ldha (1)	95	300	95	60	60	60	72	60	35	72	600
ldha (2)	95	300	95	60	54	60	72	60	35	72	600
cry1a (1)	95	300	95	60	56	60	72	60	35	72	600
cry1a (2)	95	300	95	60	56	60	72	60	35	72	600
gbp1	94	300	95	45	56	60	72	60	35	72	600
hif1a	95	300	95	60	60	60	72	60	35	72	600
hsc70	95	300	95	60	56	60	72	60	35	72	600
fkbp4	95	300	95	60	58	60	72	60	35	72	600
hsp90	95	300	95	60	52	60	72	60	35	72	600

Table 3.3: Primer pairs used to re-sequence genes in Sanger with their PCR amplification conditions. (part 3/3)

Genes	Taq Buffer (5x)	MgCl2 (10 mM)	dNTP's (10 mM) (2 mM each dNTP)	Primers (10 μM)	Taq (5U/μL)
ldha (1)	5	2	2.5	0.75	0.12
ldha (2)	5	2	2.5	0.75	0.1
cry1a (1)	5	2	2.5	0.75	0.12
cry1a (2)	5	2	2.5	0.75	0.12
gbp1	5	2	2.5	0.75	0.15
hif1a	5	2	2.5	0.75	0.12
hsc70	5	1.5	2.5	0.75	0.12
fkbp4	5	2	2.5	0.75	0.15
hsp90	5	2	2.5	0.75	0.12

Table 3.4: *Continues on next page.*

Gene name	Primers		Efficiency (%)	References
	forward	reverse		
pabpc1a	5' - GCAAAGTGT	5' - CTCGTCATCC	97.06	N/A
	TCGTCGGTC - 3'	ATATCCTCTCC - 3'		
rpl35	5' - CAAGCCTTT	5' - GGTTCTCCTC	96.56	N/A
	GGACCTGAGG - 3'	GTGTTTGGTCA - 3'		
rpsa	5' - CATCCCAAC	5' - TCCACCACAT	96.54	N/A
	CATTGCCCT - 3'	CAGACCCA - 3'		
cry1a	5' - CCTTCTTCCA	5' - GTATGTAGTC	97.22	N/A
	GCAGTTCTTC - 3'	TCCGTTGGG - 3'		
cs	5' - CTGTTGCCCA	5' - GCCCACTCCT	94.38	N/A
	AAGCTTCCG - 3'	TAGACAACCA - 3'		
fkbp4	5' - AATCCCACCC	5' - CACACTTCCA	97.21	N/A
	AACGCTACC - 3'	CAGATGCACC - 3'		
gpb1	5' - GAAGTCCTAC	5' - CCAGCCGTCA	96.96	N/A
	CTTATGAACCGC - 3'	TTCTTAGAGTC - 3'		
glula	5' - CCAGTCAGTC	5' - GCCACACTAA	97.38	N/A
	TACGAGCA - 3'	CTTTAGCACC - 3'		
hif1a	5' - CCTCATCCCTC	5' - GGCTCATATCC	97.24	N/A
	AAACATCG - 3'	CATCAGC - 3'		
hsc70	5' - TTTGCTGTTGG	5' - GTGGGAATGG	96.92	Jesus *et al.* 2013
	ATGTCACTC - 3'	TGGTGTTC - 3'		
hsp70	5' - AATTCCACCTG	5' - TCTCCTCTTTG	97.47	Jesus *et al.* 2013
	CACCACG - 3'	CTCAGTCTG - 3'		
hsp90	5' - CTGTTTATTCCC	5' - TGTCCATGATA	96.65	N/A
	AGAAGAGCCTCC - 3'	AAGACCCTGCG - 3'		

Table 3.4: Real-time RT-PCR primer pairs for reference and target genes and their efficiency values calculated in LinRegPCR (Ruijter *et al.*, 2009). Real-time PCRs were done in a final volume of 10 μL, containing 5 μL of Sso Advanced universal SYBR Green supermix (2x) (Bio-Rad. Hercules. CA. USA) and 0.4 μL of each primer (with a concentration of 0.4 μM). The assay conditions included an initial denaturation step at 95 °C for 30 s, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s.

Gene name	Primers		Efficiency (%)	References
	forward	reverse		
ldha	5' - TCTGACTGACG AACTCGCC - 3'	5' - TCCAGCAGTC ACAACCACC - 3'	96.04	N/A
lox	5' - ACCAGATACTT CCAGAACGGT - 3'	5' - GAACCTCAGC AGAACCCT - 3'	96.32	N/A
nkx3.2	5' - CCGTTCTCCAT TCAAGCCA - 3'	5' - TGTCGTTGTC CTCGCTCAG - 3'	97.65	N/A
nudb8	5' - GAAGATTACCA GCCCTTTCC - 3'	5' - CGTGTCAACC CTATTCCTG - 3'	96.65	N/A
per1a	5' - GAGTTAACGCA GGTCCAC - 3'	5' - GGAGGAGTCA AGAAATCTGG - 3'	97.41	N/A
stip1	5' - GCCTTAGACCC TTCCAATCAC - 3'	5' - AGTCGCCCAA GAAACTCC - 3'	97.01	N/A

Table 3.5: *Continues on next page.*

gene name	*S. carolitertii*			*S. torgalensis*			*S. pyrenaicus*	GO description	Functional category
	Fins	Liver	Muscle	Fins	Liver	Muscle	Brain*		
rpsa	-0.24	-0.29	-0.21	0.34	-0.11	0.36	-0.27	N/A	N/A
rpl35	-0.14	-0.02	-0.58	0.1	0	0.28	-0.26	N/A	N/A
pabpc1a	-0.01	-0.1	-0.03	-0.09	-0.47	-0.45	-0.24	N/A	N/A
per1a	non DE	non DE	non DE	-3.39	non DE	-7.47	N/A	response to oxidative stress	circadian rhythm
cry1a	non DE	non DE	non DE	-10.7	non DE	-9.08	N/A	response to oxidative stress	circadian rhythm
hsc70	non DE	4.33	-0.77	non DE	non DE	-9.72	N/A	protein folding	protein folding
hsp70	9.41	7.4	18.49	16.54	18.31	20.07	N/A	protein folding	protein folding
hsp90	8.21	8.18	5.59	8.87	4.24	4.69	N/A	protein folding	protein folding
stip1	non DE	non DE	non DE	3.84	5.75	9.35	N/A	protein folding	protein folding
fkbp4	non DE	non DE	non DE	non DE	3.4	12.09	N/A	protein folding	protein folding
hif1a	non DE	non DE	-0.73	non DE	-1.38	0.99	N/A	response to oxidative stress	energy metabolism
ldha	non DE	non DE	non DE	non DE	non DE	-6.38	N/A	response to oxidative stress	energy metabolism
cs	non DE	non DE	non DE	0.93	-0.7	non DE	N/A	response to oxidative stress	energy metabolism

Table 3.5: Gene expression values of reference and target genes in the transcriptomes of both species described in Jesus *et al.* (2016). Reference genes have a column for the differential gene expression value between *S. pyrenaicus* males and females from (Genomic Resources Development Consortium *et al.*, 2015)). Non-DE and N/A stands for genes that are not significantly differentially expression and not applicable, respectively.

gene name	*S. carolitertii*			*S. torgalensis*			*S. pyrenaicus*	GO description	Functional category
	Fins	Liver	Muscle	Fins	Liver	Muscle	Brain*		
ndub8	non DE	non DE	-4.26	non DE	non DE	13.1	N/A	response to oxidative stress	energy metabolism
glula	non DE	non DE	2.23	non DE	non DE	-6.61	N/A	response to oxidative stress	energy metabolism
lox	non DE	non DE	7.3	non DE	non DE	-8.65	N/A	skeletal system development	energy metabolism
gbp1	non DE	non DE	7.06	non DE	non DE	-4.36	N/A	immune response	immune response

Table 3.6 (part 1/5)

Physical and chemical parameters	LDHA		HIF1		CRY1AA	
	S. torgalensis	*S. carolitertii*	*S. torgalensis*	*S. carolitertii*	*S. torgalensis*	*S. carolitertii*
Molecular Weight (kDa)	30.13	29.59	85.29	85.22	70.88	70.90
Theorethical pI	7.81	7.81	5.20	5.24	8.15	8.15
Instability index	31.7	32.7	56.28	56.22	48.02	48.07
Aliphatic index	100.15	100.93	81.52	82.03	76.18	76.34
GRAVY	-0.051	-0.045	-0.394	-0.390	-0.412	-0.409
Size (aa)	274	269	770	770	626	626

Table 3.6 (part 2/5)

Physical and chemical parameters	HSC70		HSP90		HSP70	
	S. torgalensis	*S. carolitertii*	*S. torgalensis*	*S. carolitertii*	*S. torgalensis*	*S. carolitertii*
Molecular Weight (kDa)	70.55	70.53	88.11	88.58	70.92	70.92
Theorethical pI	5.32	5.26	5.25	5.25	5.55	5.55
Instability index	33.64	34.42	39.04	39.11	34.83	34.83
Aliphatic index	81.32	81.77	85.77	84.71	82.32	82.32
GRAVY	-0.444	-0.45	-0.638	-0.648	-0.450	-0.450
Size (aa)	644	644	694	694	647	647

Table 3.6 (part 3/5)

Physical and chemical parameters	STIP1		FKBP52		LOX	
	S. torgalensis	*S. carolitertii*	*S. torgalensis*	*S. carolitertii*	*S. torgalensis*	*S. carolitertii*
Molecular Weight (kDa)	47.37	47.37	52.43	52.41	42.45	42.45
Theorethical pI	6.72	6.72	5.55	5.55	8.56	8.56
Instability index	38.35	37.92	34.35	34.92	58.32	58.32
Aliphatic index	65.95	65.95	69.94	70.77	61.82	61.82
GRAVY	-0.928	-0.929	-0.647	-0.645	-0.574	-0.574
Size (aa)	415	415	469	469	374	374

Table 3.6 (part 4/5)

Physical and chemical parameters	CS		NDUFB8		PER1A	
	S. torgalensis	*S. carolitertii*	*S. torgalensis*	*S. carolitertii*	*S. torgalensis*	*S. carolitertii*
Molecular Weight (kDa)	52.80	52.80	21.68	21.68	15.58	15.54
Theorethical pI	8.44	8.44	6.58	6.58	4.68	4.68
Instability index	27.31	27.31	53.68	53.68	42.84	42.84
Aliphatic index	89.33	89.33	57.30	57.30	74.09	74.09
GRAVY	-0.163	-0.163	-0.795	-0.795	-0.546	-0.526
Size (aa)	476	476	189	189	137	137

Table 3.6: Predicted proteins physical and chemical parameters. (part 5/5)

Physical and chemical parameters	GLULA		GBP1	
	S. torgalensis	*S. carolitertii*	*S. torgalensis*	*S. carolitertii*
Molecular Weight (kDa)	43.04	43.04	61.25	61.27
Theorethical pI	5.75	5.75	5.03	5.11
Instability index	44.04	43.32	46.03	45.57
Aliphatic index	66.48	66.48	80.96	79.85
GRAVY	-0.501	-0.501	-0.623	-0.613
Size (aa)	383	383	531	531

Table 3.7: Non-synonymous substitutions for the translated predicted protein structures (in a.a.).

Functional category	gene	non synonymous residues	raptor model coverage	raptor template id
protein folding	*hsp70*	N/A	100%/100%	5E84
	hsc70	20; 47; 81; 191; 243; 249; 254; 255; 288; 291; 315	100%/100%	5E84
	hsp90	741; 744; 745	100%/93%	2CG9
	fkbp4	222; 323; 351	100%/100%	1kt1
	stip1	244; 248	100%/100%	1elw
energy metabolism	*hif1a*	44; 200	46%/46%	4zp4
	ldha	N/A	100%/100%	1v6a
	cs	N/A	100%/100%	2cts
	ndufb8	N/A	100%/100%	1t7n
	glula	4; 10	100%/100%	4wa0
	lox	N/A	43%/43%	3ob8
circadian rythm	*cry1a*	97	80%/80%	4ct0
	per1a		59%	4ct0
immune system	*gbp1*	54; 57; 65; 343; 345; 348; 352; 399; 403; 407; 430	100%/100%	1dg3

Figures

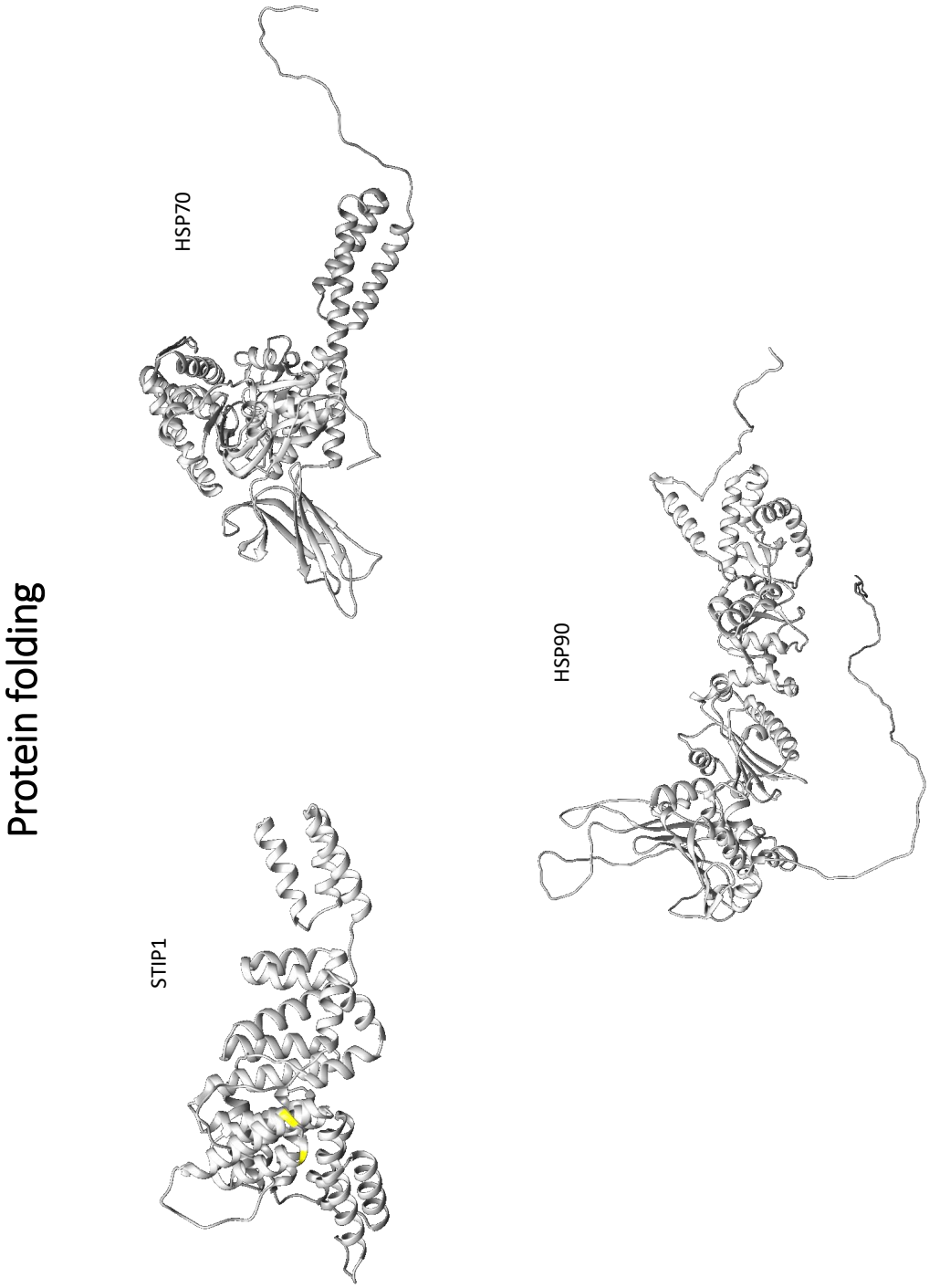

Figure 3.3: *Continues on next page.*

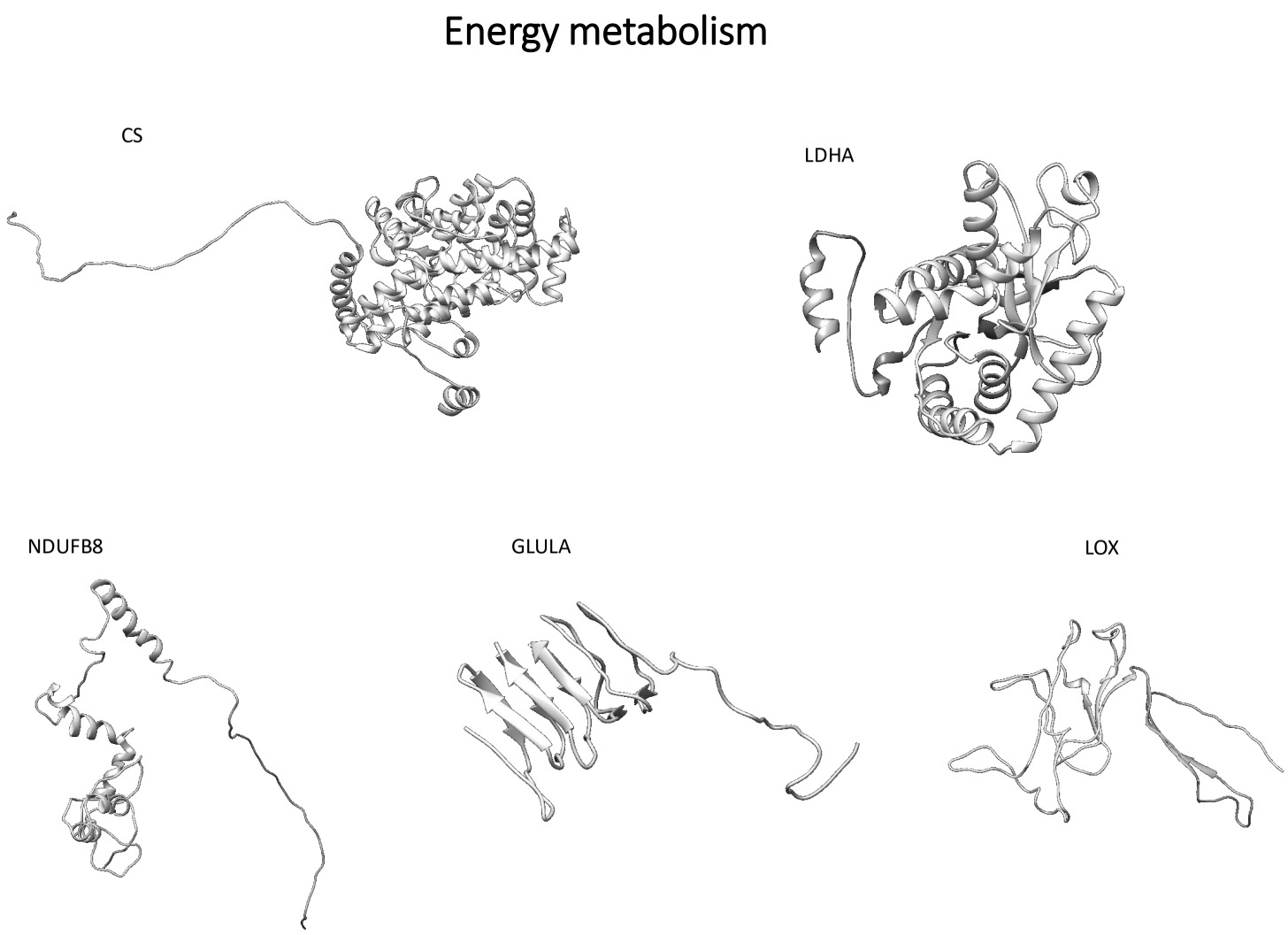

Figure 3.3: *Continues on next page.*

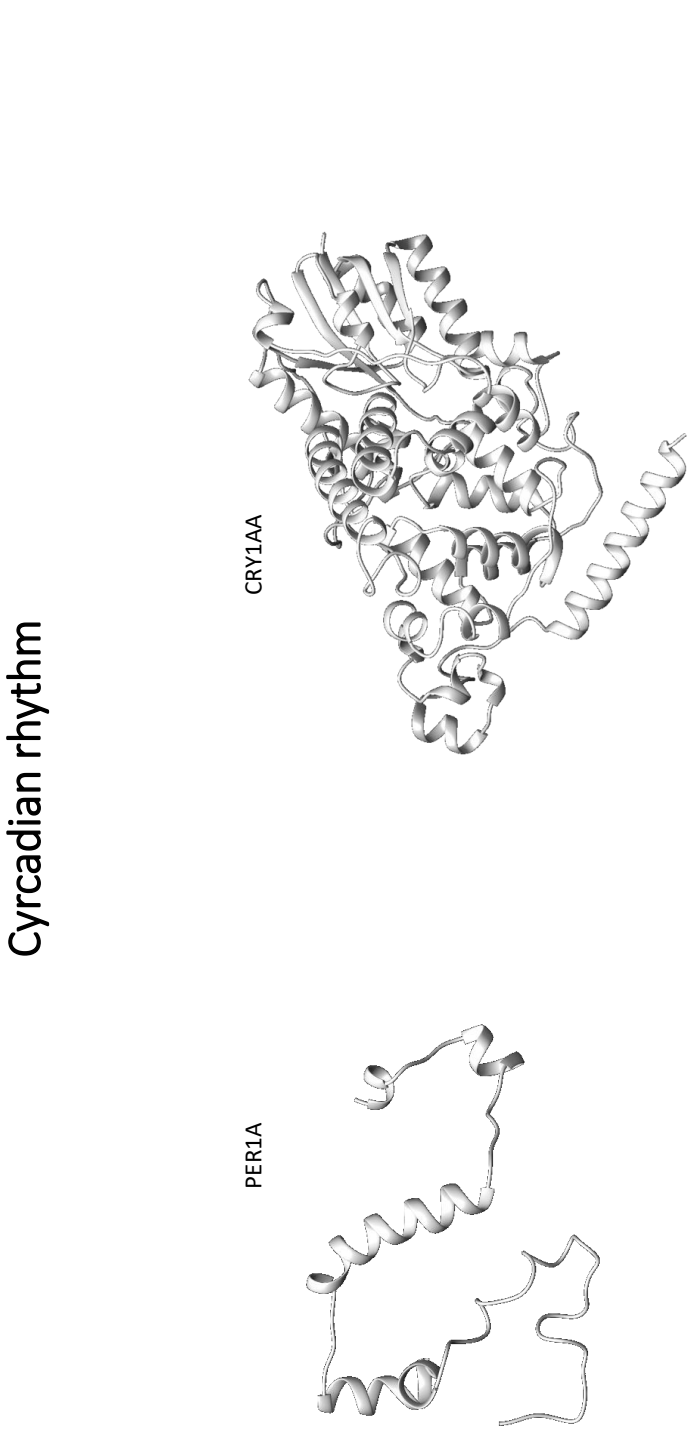

Figure 3.3: Protein structure predictions for proteins with minor or no differences between species. Regions in light grey have no differences between species, blue and red indicate the conformation of *S. carolitertii* and *S. torgalensis* for that specific region and yellow represents the amino acids which correspond to non-synonymous substitutions.

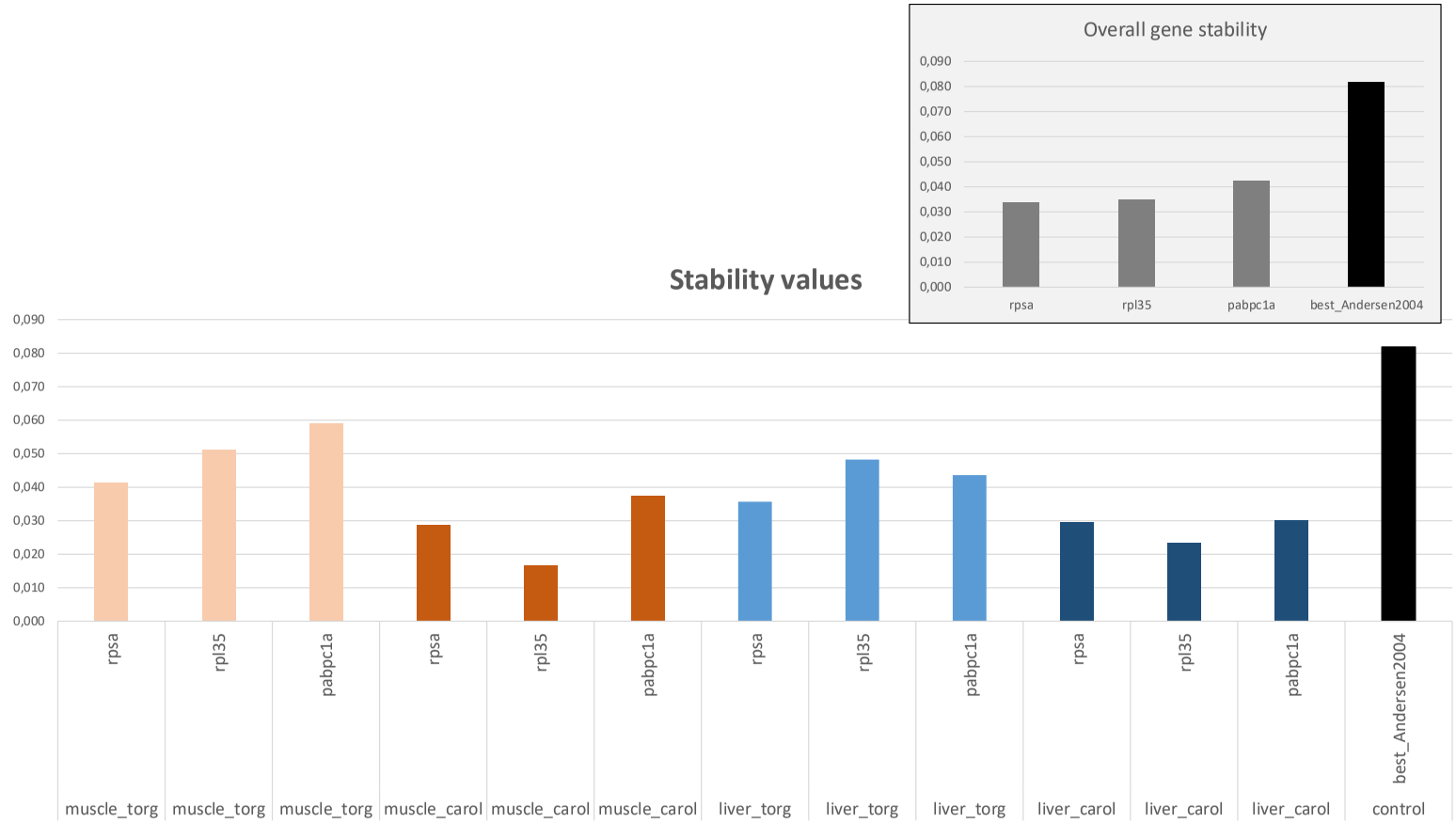

Figure 3.4: Stability values calculated for the reference genes (*rpsa*, *rpl35* and *pabpc1a*), showing their overall stability and for each organ and condition analyzed. The lower the stability value the better the reference gene and thus less variable across the experimental conditions.

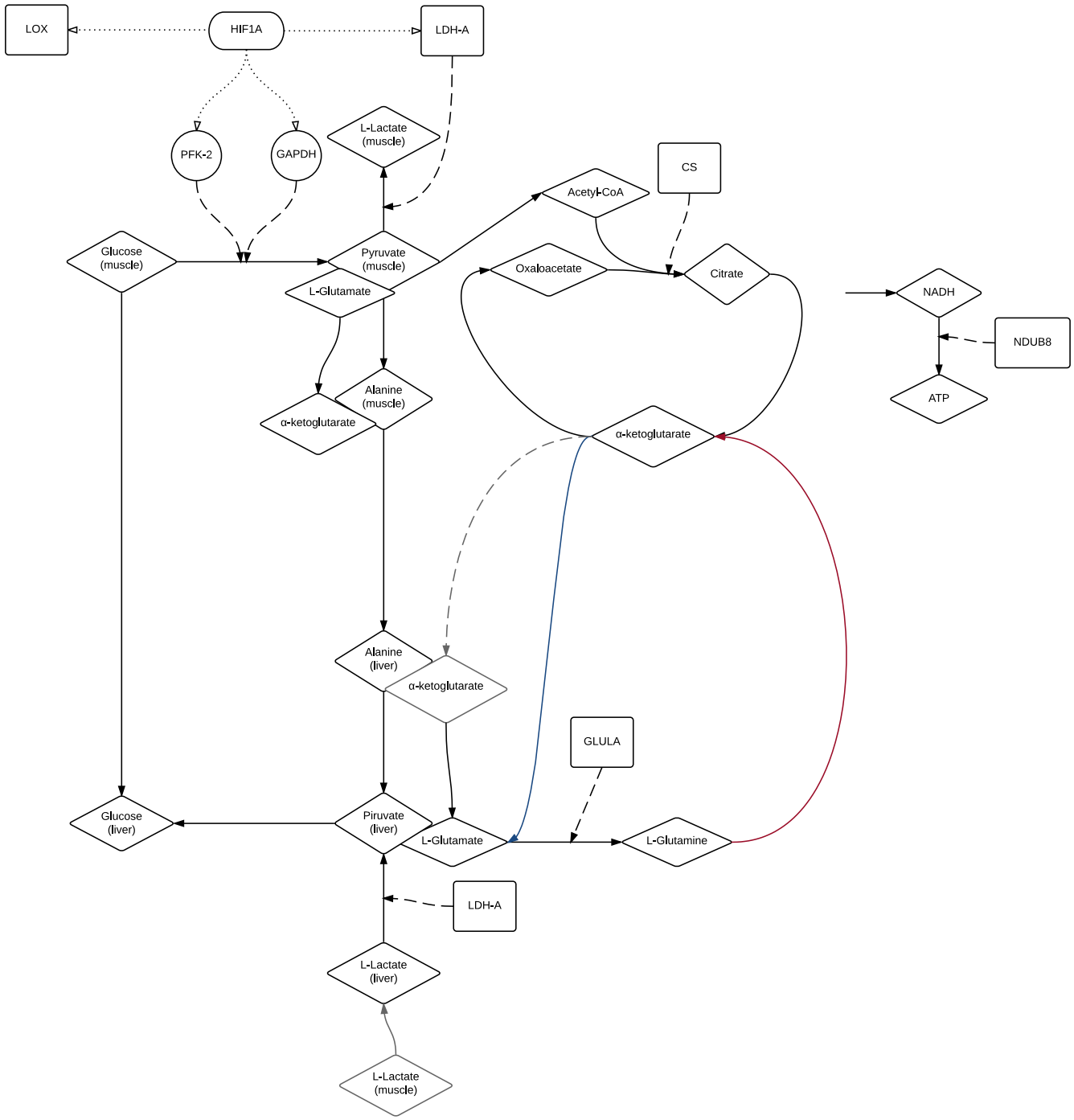

Figure 3.5: *Continues on next page.*

Figure 3.5: Schematic representation of the pathways discussed in this research for the genes involved in energy metabolism. Doted arrows indicate gene expression regulation from the source to the sink gene; dashed arrows represent a source gene that encodes a protein is responsible for substrate conversion; and full arrows indicate a direct conversion. Target genes are represented with squares, except for *hif1a* (represented with a rectangle with two curved sides), which is a key gene in the regulation of many gene involved in these pathways. Circles indicate genes which regulate relevant pathways but that are not target genes and polygons symbolize the substrates.

3.2 Different ecophysiological responses of freshwater fish to warming and acidification

The original work described in this subchapter is currently in preparation and awaiting publication of the results reported in 3.1 Protein analysis and gene expression indicate differential vulnerability of Iberian fish species under a climate change scenario.

Tiago F. Jesus[1], Inês C. Rosa [2], Tiago Repolho[2], Ana R. Lopes[2], Marta S. Pimentel[2], Vera M.F. Almeida-Val[3], Maria M. Coelho[1], Rui Rosa[2]

[1] - CE3C – Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, Edifício C2, 3º Piso, Campo Grande, 1749-016 Lisboa, Portugal

[2] - MARE - Marine and Environmental Sciences Centre, Laboratório Marítimo da Guia, Faculdade de Ciências da Universidade de Lisboa, Avenida Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal

[3] - Laboratório de Ecofisiologia e Evolução Molecular, Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM, Brasil.

Abstract

Predictions based upon future climate change scenarios elicit threatening outcomes to the biodiversity worldwide. Available empirical data concerning biological response of freshwater fish to climate change remains scarce. In the present study, we investigated the physiological and biochemical responses of two Iberian freshwater fish species (the northern *Squalius carolitertii* and the southern endangered *S. torgalensis*), inhabiting different climatic conditions, to projected future scenarios of warming (+3 °C) and acidification (ΔpH = -0.4 units). Herein, the metabolic enzyme activities of glycolytic (citrate synthase - CS, lactate dehydrogenase - LDH) and antioxidant (glutathione S-transferase, catalase and superoxide dismutase) pathways, as well as the heat shock response (HSR) and lipid peroxidation were determined. Our results show that, under current water pH, warming causes differential interspecific changes on LDH activity, increasing and decreasing its activity in *S. carolitertii* and in *S. torgalensis*, respectively. Furthermore, the synergistic effect of warming and acidification caused a significant increase in LDH activity of *S. torgalensis*, comparing with the warming condition. As for citrate synthase (CS) activity, water acidification significantly decreased its activity in *S. carolitertii* whereas in *S. torgalensis* no significant effect was observed. These results suggest that *S. carolitertii* is more vulnerable to climate change, possibly as the result of its evolutionary acclimatization to milder climatic conditions in its environment, while *S. torgalensis* evolved in a warmer Mediterranean climate. Regarding the oxidative stress responses, there is a general lack of changes in antioxidant enzymatic activities on both species. Nevertheless, significant increases in HSR were observed under the combined warming and acidification (*S. carolitertii*) or only under acidification (*S. torgalensis*). Our results underlie the importance of conducting experimental studies and address species endpoint responses under projected climate change scenarios in order to improve conservation and mitigation strategies, and to safeguard endangered freshwater fish species, in a changing environment.

Introduction

Earth's climate is changing at an unparalleled pace, threatening biodiversity worldwide (Hartmann *et al.*, 2013; Field *et al.*, 2014; Pörtner *et al.*, 2014). In fact, air temperature is projected to increase between 2.6 and 4.8 °C (Collins *et al.*, 2013) and atmospheric CO_2

concentration can reach values between 420 and 940 ppm by 2100 (Collins *et al.*, 2013; Pörtner *et al.*, 2014). Freshwater ecosystems are particularly at risk due to alterations in thermal and precipitation regimes which, in turn, will drastically change the dynamics between floods and droughts, decrease of river flow and increase of the risk of extreme events (e.g. heat waves) (Füssel *et al.*, 2012; Field *et al.*, 2014). Also, the increase in acid rainfall, resulting from emissions of sulfur dioxides and nitrogen oxides to the atmosphere, will contribute to the acidification of lakes and rivers (Van De Waal *et al.*, 2010; Leduc, 2013). All of this will unquestionably pose further challenges for fauna living in these habitats (Leduc, 2013).

Freshwater fish, as ectotherms, strongly rely on environmental temperature in order to regulate their metabolism and may have a reduced migration ability, making them prone to warming conditions (Angilletta, 2002; Berg *et al.*, 2010). Increasing temperature is even more alarming for those species living closer to their thermal tolerance limits (Reusch and Wood, 2007; Somero, 2010; Tomanek, 2010; Hoffmann and Sgrò, 2011). Even though many studies have approached the subject of thermal stress in freshwater fish (e.g. Podrabsky and Somero (2004); Yamashita *et al.* (2004); Fangue *et al.* (2006); Jesus *et al.* (2013, 2016); Campos *et al.* (2016), only a few have attempted to study the effects under the context of climate change (e.g. de Oliveira and Val (2016); Mccairns *et al.* (2016); Prado-Lima and Val (2016); Jesus *et al.* (2017). Furthermore, the acidification of freshwater ecosystems have been poorly studied, despite the predictable effects that freshwater biota will suffer as a result of it (Leduc, 2013; Ou *et al.*, 2015a). In fact, major focus has been given to ocean acidification and this process is widely known to affect many marine species physiology and behavior (e.g. Munday *et al.* (2009); Aurélio *et al.* (2013); Vinagre *et al.* (2013); Rosa *et al.* (2014); Pimentel *et al.* (2015); Rosa *et al.* (2016).

The Iberian chubs, *Squalius carolitertii* (Doadrio, 1988) and *Squalius torgalensis* (Coelho, Bogutskaya, Rodrigues and Collares-Pereira, 1998), are two closely related endemic freshwater fish species, which inhabit two distinct regions with different climatic conditions (Carvalho *et al.*, 2010): *S. carolitertii* inhabits the northern region of Iberian Peninsula, whereas *S. torgalensis* has a restricted distribution to the Mira river basin, in the south-western region of Portugal (Coelho *et al.*, 1998). These two distinct climates, expose these species to different seasonal and even daily water temperature fluctuations, which in turn result in different life history traits such as different life span, spawning age and body size (Magalhães *et al.*, 2003). Additionally, previous works on gene regulation of both species

suggest that *S. torgalensis* seems to be better adapted to higher temperatures, presenting higher survival rates and stronger responses in gene expression under high temperatures when compared to *S. carolitertii* (Jesus *et al.*, 2013, 2016).

When facing stressful conditions, organisms may display several physiological responses to survive under the adversities. Adjustments in metabolic performance are amongst the most common responses and may lead to shifts in energy production (Mwangangi and Mutungi, 1994; Campos *et al.*, 2016). The activities of citrate synthase (CS) and lactate dehydrogenase (LDH) can reflect these modifications in aerobic and anaerobic potential, respectively, and thus represent good biomarkers for these metabolic pathways (McClelland *et al.*, 2006). Another highly common response to stressful conditions is the heat shock response (HSR) (Wegele *et al.*, 2004; Morris *et al.*, 2013), which consists in the synthesis of a specific group of proteins (heat shock proteins (HSP)) that are responsible for the stabilization and refold of denatured proteins as a response to increasing temperatures (Yamashita *et al.*, 2004; Fangue *et al.*, 2006; Dong *et al.*, 2008; Tomanek, 2010). In addition, the production of molecules that derive from oxygen, i.e. reactive oxygen species (ROS), (e.g. superoxide anion and hydrogen peroxide) (Sun *et al.*, 2007; Sevcikova *et al.*, 2011) is also a good indicator of stress (Storey and Storey, 2005; Sun *et al.*, 2007; Sevcikova *et al.*, 2011). ROS trigger the individual's antioxidant defense system by producing antioxidant enzymes, trying to reestablish the oxidant balance. However, in excess ROS situations, several biological features of the organisms may be damaged, including cellular health and integrity due to lipid peroxidation (Sevcikova *et al.*, 2011).

The present study aims to understand the effects of warming plus acidification on the physiology of the Iberian chubs, *S. carolitertii* and *S. torgalensis*, inhabiting different climatic regions, by using conventional stress-related biomarkers (metabolic and antioxidant responses). Particularly, we investigated the combined effects of warming (+3 °C) and acidification (ΔpH = -0.4), in relation to summer average parameters, on the metabolic potential (CS and LDH activities), heat shock response, antioxidant enzymatic machinery [glutathione S-transferase (GST), superoxide dismutase activity (SOD) and catalase (CAT)] and peroxidative damage [malondialdehyde (MDA)] of these two species.

This study provides important insights on the threats of climate change, a scenario presently considered irreversible to freshwater species (Collins *et al.*, 2013). Moreover, since *S. torgalensis* is a critically endangered species (Coelho *et al.*, 1998), this work is of

utmost importance for surveying the threats that this species may face in future, in order to adopt proper conservation measures.

Methods

Sampling

S. carolitertii and *S. torgalensis* specimens were field collected in two river basins (Mondego: 40°8'5.22"N - 8°8'35.06"W; Mira: 37°38'1.31"N - 8°37'22.37"W), located in the west coast of Portugal. An electro-fishing device (300V, 4A; Hans Grassl, Model EL 62) was used to perform fish collection, and the avoidance of juvenile mortality was accomplished by applying short duration pulses (3-6 milliseconds). Organism sampling was performed during spring (May to June 2014), where water temperature and pH varied between 17.80 ± 0.67 °C and 8.08 ± 0.01 for Mondego river, and 19.50 ± 0.21 °C and 8.23 ± 0.02 for Mira river (measured with a YSI-85 handheld system). Capture procedures were performed under ICNF license (nº 263/2014/CAPT, Instituto da Conservação da Natureza e Florestas).

Experimental design

After collection, fish were transported in isothermal cases, under constant aeration conditions, to the Laboratório Marítimo da Guia (Cascais, Portugal). Subsequently, fish were progressively acclimated (2 weeks) to laboratory conditions, mimicking summer average values at collection sites (national information system of water resources, snirh.pt) for temperature and pH under normoxic (8 $mg.L^{-1}$) conditions (control condition, see 3.1). After this acclimation period, each fish species (*S. carolitertii* and *S. torgalensis*) was exposed (30 days) to four different experimental conditions (3.1), under a 2×2 factorial design: i) control (19 and 23 °C, respectively, pH 6.9 and 7.3 for both species); ii) warming (22 and 26 °C, respectively, pH 6.9 and 7.3 for both species); iii) acidification (19 and 23 °C, respectively, pH 6.5 and 6.9 for both species); iv) combined warming and acidification scenario (22 and 26 °C, respectively, pH 6.5 and 6.9 for both species). Warming and acidification conditions were accomplished in order to experimentally assess the responses of each fish species to the tested climate change scenarios (temperature increase = +3 °C;

ΔpH = -0.4), based on the IPCC's RCP 6.0 scenario (Field *et al.*, 2014). During laboratory acclimation and experimental exposure, a mixture of bloodworms/white mosquito larvae (TMC Iberia, Portugal) and *Spirulina* spp. (flake food, Ocean nutrition, Belgium) was provided ad libitum to fish, on a daily basis. Light regime was set to 12h:12h (light/dark cycle), in accordance to prevailing natural light conditions. Monitoring of nitrate, nitrite and ammonia levels was performed daily, using colorimetric tests (Profi Test, Salifert, Holland), with abiotic parameters being kept below detectable levels, during the entire experimental procedure. Monitoring of dissolved oxygen and pH was performed through an automatic control device (Profilux 3.1N, GHL, Germany), with set point values being adjusted and monitored automatically. Individual oxygen (PL-0368, GHL, Germany) and pH (PL-0071, GHL, Germany) sensors were used. Conductivity levels were continuously (Profilux 3.1N, GHL, Germany) and individually (PL-0055, GHL, Germany) monitored, while being kept at 400 to 500 μS.cm^{-1}. Additional daily conductivity checks were performed, using handheld monitoring equipment (CO30, VWR, Portugal). Programmable dosing systems (Easy Dose 3, TMC Iberia, Portugal) connected to indoors-freshwater tanks (300 or 600 μS.cm^{-1}), allowed inflow of freshwater to experimental tanks, in order to maintain conductivity levels within desired range (400-500 μS.cm^{-1}). Maintenance of dissolved oxygen/pH values was accomplished, as follows: injection of certified N_2/CO_2 (Air Liquide, Portugal) to down regulate values and aeration with atmospheric filtered air (soda lime, Sigma-Aldrich) to up regulate. All water parameters for the different experimental treatments are shown in 3.1.

After experimental exposure, a set number of fish (n = 6), derived from each treatment and species, was euthanized and the collected samples were immediately frozen in liquid nitrogen and stored at -80 °C for biochemical analyses. All experimental procedures were performed under EU compulsory requirements/guidelines (Directive 2010/63/EU, 22nd September 2010) for animal's protection for scientific purposes (ORBEA – Animal Welfare Body of FCUL Statement 5/2016).

Metabolic enzyme activity

Maximum activity levels of citrate synthase (CS) and lactate dehydrogenase (LDH) were estimated in muscle of both species (n = 6 specimens per treatment). CS and LDH determinations were performed based on an adaptation of Driedzic and Almeida-Val (1996);

Rosa *et al.* (2009). Samples were first homogenized in a buffer containing 150 mM imidazole, 1 mM EDTA at pH 7.4 in a glass/PTFE potter–Elvehjem tissue grinder (Kartell, Italy) kept on ice. Homogenates were then centrifuged at 10,000 g for 10 min at 4 °C. LDH activity was assayed using 1 mM pyruvate as substrate in a buffer containing 0.15 mM NADH, 50 mM imidazole and 1 mM EDTA at pH 7.4. CS activity was assayed in a buffer containing 0.25 mM DTNB, 75 mM Trisbase, and 0.4 mM acetyl CoA at pH 8.0, and the reactions were initiated by adding 0.5 mM oxaloacetate. LDH activity was measured following the oxidation of NADH (extinction coefficient of 6220 M^{-1} cm^{-1}) at 340 nm while CS activity was determined based on the reaction of coenzyme A with DTNB (5,5 V dithio-bis (2-nitrobenzoic acid)) at 412 nm (extinction coefficient of 13,600 M^{-1} cm^{-1}). Changes in absorbance were measured at 20 °C during 1 min, using a Shimadzu UV-1800 spectrophotometer (Shimadzu Scientific Instruments, Japan). For both enzymes, each sample was run in triplicate (technical replicates). The enzyme results were normalized by measuring the total protein content of the samples according to the Bradford method (Bradford, 1976).

Heat shock response, antioxidant enzymes activities and peroxidative damage

Preparation of tissue extracts

Muscle samples (n = 6 per treatment) were homogenized (Ultra-Turrax, Ika, Staufen, Germany) in accordance to body mass of each sample in homogenization buffer [300 mg tissue per 1 mL phosphate-buffered saline solution (PBS, pH 7.4): 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na2HP04, 1.47 mM KH2P04)]. All homogenates were then centrifuged (20 min at 14,000 g at 4 °C) and the HSR, antioxidant enzyme activities and lipid peroxidation were quantified in the supernatant fraction as described below. All enzyme assays were tested with commercial enzymes obtained from Sigma (Missouri, USA), and each sample was run in triplicate (technical replicates). The enzyme results were normalized by measuring the total protein content of the samples according to the Bradford method (Bradford, 1976).

Heat shock response

HSP content (HSC70/HSP70) was assessed by ELISA (Enzyme-Linked Immunoabsorbent Assay) as previously described by Rosa *et al.* (2014). Briefly, a total of 10 μL of homogenate supernatant was diluted in 990 μL of PBS, and 50 μL of the diluted sample was added to 96-well microplates MICROLON600 (Greiner Bio-One GmbH, Germany) and incubated overnight at 4 °C. Microplates were washed on the next day in 0.05% PBS-Tween-20. Subsequently, 100 μL of blocking solution (1% Bovine Serum Albumin, BSA) was added to each well. The microplates were then incubated for 2 h at room temperature in darkness. Then, 50 μL of a solution of 5 μg mL^{-1} primary antibody anti-HSP70/HSC70 (that detects both 72 and 73 kDa proteins, which corresponds to the molecular mass of inducible HSP70 and constitutive HSC70, respectively) was added to each well. Plates were then incubated overnight at 4 °C. The non-linked antibodies were removed by repeating the abovementioned washing method, microplates were then incubated for 90 min at 37 °C with 50 μL of the secondary antibody [anti-mouse IgG Fab specific, ALP conjugate (1 μg mL^{-1}) from Sigma-Aldrich (Germany)]. After another wash, 100 μL of substrate p-nitrophenyl phosphate tablets (Sigma-Aldrich, Germany) were added to each well and the microplates were incubated at room temperature (10 to 30 min). Finally, 50 μL of stop solution (3M NaOH) was added to each well and the absorbance was read at 405 nm in a 96-well microplate reader (UVM 340, Biochrom, USA). The amount of HSP70/HSC70 in the samples was calculated from a standard curve of absorbance based on serial dilutions (from 0 to 2000 μg mL^{-1}) of purified HSP70 active protein (Acris, USA). HSP70/HSC70 concentrations are presented as μg mg^{-1} total protein.

Glutathione S-transferase (GST) activity

GST total activity (EC 2.5.1.18) was determined according to Habig *et al.* (1974) and optimized for 96-well microplate (Sigma Technical Bulletin, CS0410). This procedure measure the conjugation of the thiol group of glutathione to the 1-chloro-2,4-dinitrobenzene (CDNB) substrate. To perform the assay, aliquots (20 μL) from the supernatant fraction of each sample and 180 μL of substrate solution (Dulbecco's Phosphate Buffered Saline with 200 mM L-glutathione reduced and 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution, all from Sigma-Aldrich, Germany), were added to 96-well microplates (Nunc-Roskilde, Denmark) and the enzymatic activity determined

spectrophotometrically every minute for 6 min at 340 nm, using a microplate reader (UVM 340, Biochrom, USA). Thereby, the increase in absorbance is directly proportional to GST activity. GST activity was calculated using a molar extinction coefficient for CDNB of 5.3 εmM (Sigma Technical Bulletin, CS0410). The results are expressed as nmol min^{-1} mg^{-1} total protein.

Catalase (CAT) activity

Catalase activity was assessed through an adaptation to the method described by Johansson and Borg (1988). In this assay, 20 μl of each sample, 100 μl of 100 mM Potassium phosphate and 30 μl of methanol were added to a 96-well microplate, which was promptly shaken and incubated for 20 minutes. Afterwards, 30 μl of potassium hydroxide (10 M KOH) and 30 μl of purpald (34.2 mM in 0.5 M HCl) were added to each well, and the plate shaken and incubated for another 10 minutes. Subsequently, 10 μl of potassium periodate (65.2 mM in 0.5 M KOH) was added to each well and a final incubation was performed, for 5 minutes. Using a microplate reader (UVM 340, Biochrom, USA), enzymatic activity was determined spectrophotometrically at 540 nm. Formaldehyde concentration of the samples was calculated based on a calibration curve (from 0 to 75 μM formaldehyde), followed by the calculation of the CAT activity of each sample, were one unit of catalase is defined as the amount that causes the formation of 1.0 nmol of formaldehyde per minute at 25 °C. The results are expressed in relation to total protein content (nmol min^{-1} mg^{-1} protein).

Superoxide dismutase (SOD) activity

The SOD assay follows the nitrobluetetrazolium (NBT) method adapted from Sun *et al.* (1988). In this assay, 10 μL of SOD standard or sample were added to a 96-well microplate (Nunc Roskilde, Denmark), followed by the addition of 200 μL of 50 mM phosphate buffer (pH 8.0) (Sigma-Aldrich, Germany), 10 μL of 3 mM EDTA (Riedel-de Haën, Germany), 10 μL of 3 mM xanthine (Sigma-Aldrich, Germany) and10 μL of 0.75 mM NBT (Sigma-Aldrich, Germany) to each well. The reaction was started with the addition of 100 mU XOD (Sigma-Aldrich, Germany), and the absorbance recorded every 5 min for 25 min, at 550 nm, using a microplate reader (UVM340, Biochrom,

USA). Negative control included all components except SOD or sample and produced a maximal increase in absorbance at 560 nm. This allowed determining the inhibition percentage per minute. SOD from bovine erythrocytes (Sigma-Aldrich, Germany) was used as standard and positive control. The total SOD activity was expressed in % of inhibition mg^{-1} total protein.

Lipid peroxide assay (malondialdehyde concentration)

Lipid peroxide assay was determined according to the thiobarbituric acid reactive substances (TBARS) protocol, adapted from /Uchiyama and Mihara (1978), through the quantification of a specific end-product of the oxidative degradation process of lipids, malondialdehyde (MDA). Aliquots (5 μL) of each sample were added to 45 μL of 50 mM monobasic sodium phosphate buffer in a microtube. Following this, 12.5 μL of sodium dodecyl sulfate (8.1 %), 93.5 μL of trichloroacetic acid (20 %, pH 3.5) and 93.5 μL of thiobarbituric acid (1 %) were added to each microtube. Afterwards, 50.5 μL of ultrapure water was added to this mixture and vortexed for 30 s and the microtube lids punctured just before the incubation in boiling water, for 10 min, after which they were allowed to cool on ice. Subsequently, 62.5 μL of ultrapure water and 312.5 μL of n-butanol pyridine (15:1, v/v) (Sigma-Aldrich, Germany) were added and microtubes were centrifuged (5000 $\times$ g; 5 min.). Duplicates of 150 μL of the supernatant of each reaction were put into a 96-well microplate (Nunc Roskilde, Denmark) and absorbance was measured at 532 nm. To quantify the lipid peroxides, an eight-point calibration curve (0 – 0.3 μM TBARS) was calculated using malondialdehyde (dimethylacetal; MDA; Merck, Switzerland) standards. MDA values are expressed as nmol mg^{-1} total protein.

Statistical analyses

In order to infer the statistical significance of warming and acidification in underlying metabolic (i.e. CS and LDH) and antioxidant stress responses (i.e. HSP, GST, SOD, CAT and MDA), a two-way MANOVA was performed for each group. As MANOVA revealed significant differences, two-way ANOVA followed by Tukey post-hoc tests were performed whenever the interaction between temperature and pH was significant, to understand the effect of explaining variables on each enzyme. In these analyses, the Dunn-Sidak correction

was applied in order to adjust associated significance level of the family-wise type-I error (Quinn and Keough, 2002). A total of 4 comparisons were applied (2 temperature levels combined with 2 pH values), resulting in a significance level of 0.013. Prior to all performed analyses, data was checked for normality and homocedascity using Shapiro-Wilk's and Levene's tests, respectively. Unless stated otherwise, a significant level of 0.05 was used. All statistical analyses were performed, using Statistica 7.0 software (StatSoft Inc., USA).

Results

Metabolic enzymes

Acidification caused significant changes in the metabolic enzyme activities of *S. carolitertii* and *S. torgalensis*, with a significant interaction with temperature (two-way MANOVA: $p < 0.05$; Table 3.8). Regarding the former species, LDH activity was significantly affected by both variables, together with a significant interaction between temperature and pH (two-way ANOVA: $p < 0.013$; Table 3.9). In fact, increasing temperature prompted an increase in the activity of LDH but only under normocapnia (Tukey post-hoc test: $p < 0.013$; Figure 3.6 A). On the other hand, CS activity was only significantly affected by pH with a significant decrease of its activity in organisms exposed to water acidification (two-way ANOVA: $p < 0.013$; Figure 3.6 B; Table 3.9). Regarding *S. torgalensis*, LDH activity was significantly affected by pH and by its interaction with temperature (two-way ANOVA: $p < 0.013$; Table 3.9). In more detail, individuals exposed to the combined warming and acidification showed significantly higher LDH activity (Tukey post-hoc test: $p < 0.013$; Figure 3.6 A). Moreover, under normocapnia, LDH activity was significantly decreased by increasing temperature (Tukey post-hoc test: $p < 0.013$; Figure 3.6 A). As for CS activity, neither temperature nor pH exerted a significant effect (two-way ANOVA: $p > 0.013$; Figure 3.6 B; Table 3.9).

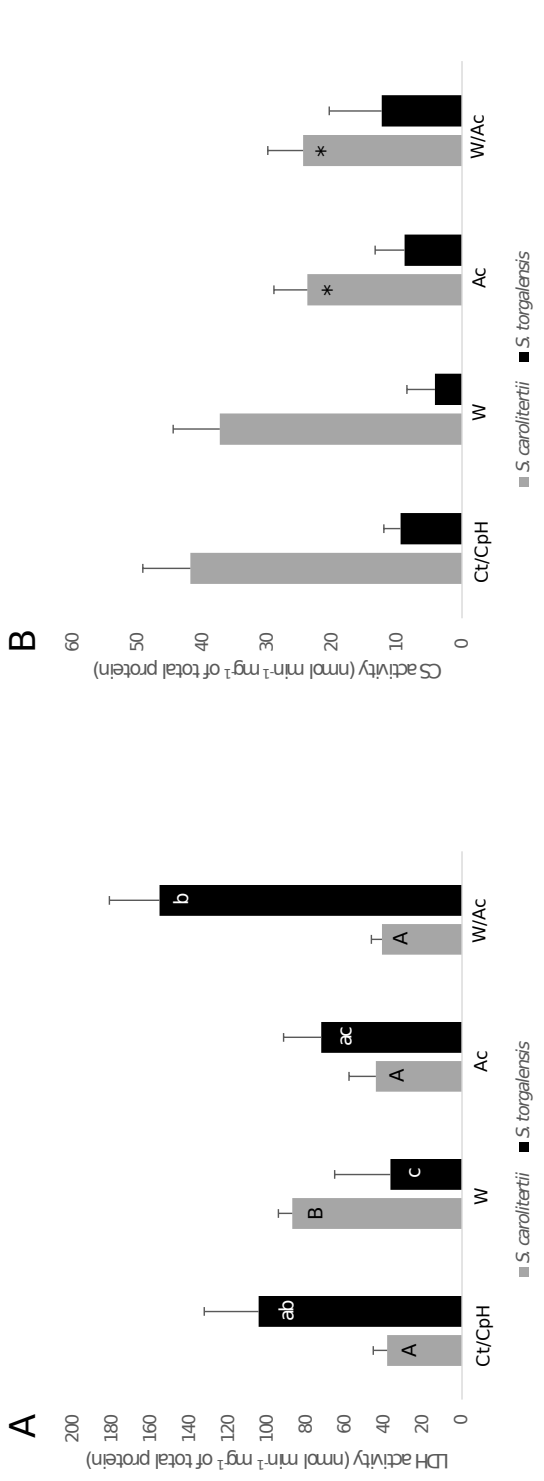

Figure 3.6: Activity of metabolic enzymes: A) lactate dehydrogenase (LDH, nmol min^{-1} mg^{-1} of total protein), and B) citrate synthase (CS, nmol min^{-1} mg^{-1} of total protein) in the muscle of *Squalius carolitertii* and *S. torgalensis* exposed for 30 days to control temperature (Ct) and pH (CpH), warming (W; +3 °C) and acidification (Ac; ΔpH = -0.4). Values represent mean $\pm$ SD (n = 6). Different letters represent significant differences between treatments ($p < 0.013$). Asterisks represent significant differences between pH within the same temperature ($p < 0.013$).

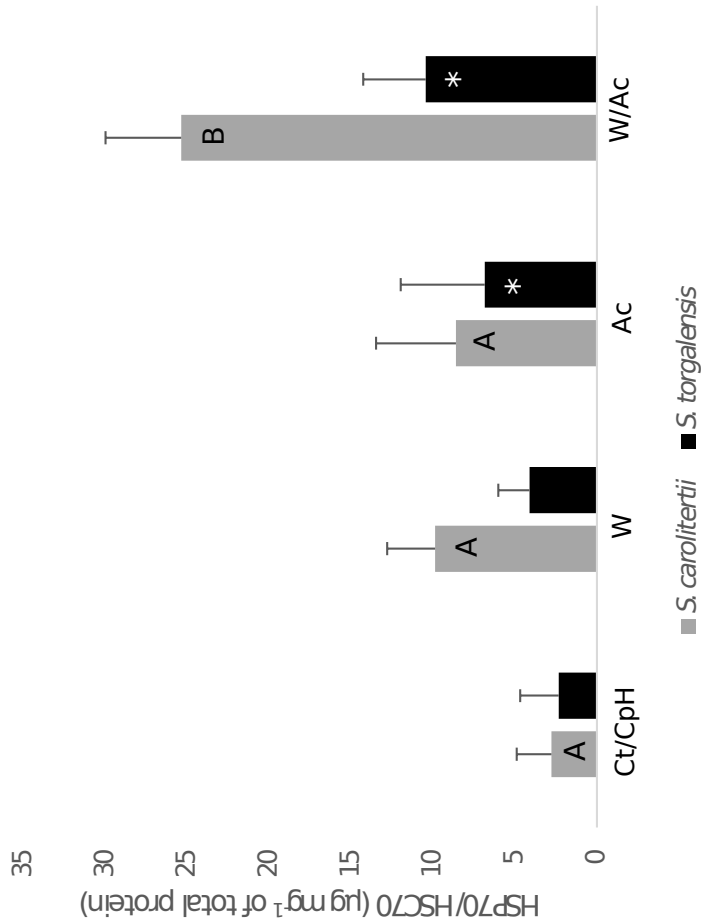

Figure 3.7: Concentration of heat shock proteins (HSP, μg mg^{-1} of total protein) in the muscle of *Squalius carolitertii* and *S. torgalensis* exposed for 30 days to control temperature (Ct) and pH (CpH), warming (W; +3 °C) and acidification (Ac; ΔpH = -0.4). Values represent mean $\pm$ SD (n = 6). Different letters represent significant differences between treatments ($p < 0.013$). Asterisks represent significant differences between pH within the same temperature ($p < 0.013$).

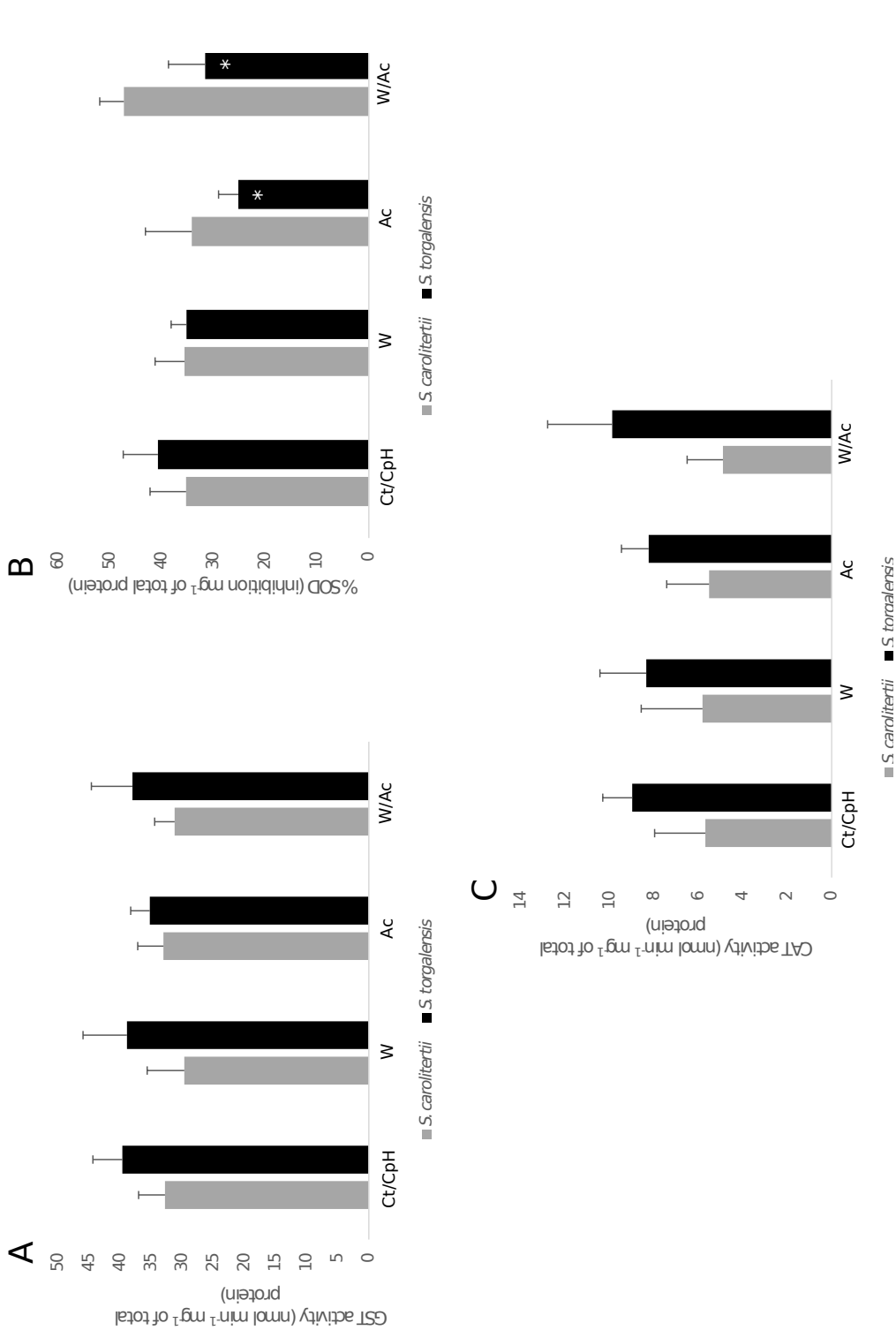

Figure 3.8: Activity of antioxidant enzymes: A) Glutathione s-transferase (GST, nmol min^{-1} mg^{-1} of total protein), B) percentage inhibition of superoxide dismutase (SOD, % inhibition mg^{-1} of total protein) and C) catalase (CAT, nmol min^{-1} mg-1 of total protein) in the muscle of *Squalius carolitertii* and *S. torgalensis* exposed for 30 days to control temperature (Ct) and pH (CpH), warming (W; +3 °C) and acidification (Ac; ΔpH = -0.4). Values represent mean ± SD (n = 6). Asterisks represent significant differences between pH within the same temperature ($p < 0.013$).

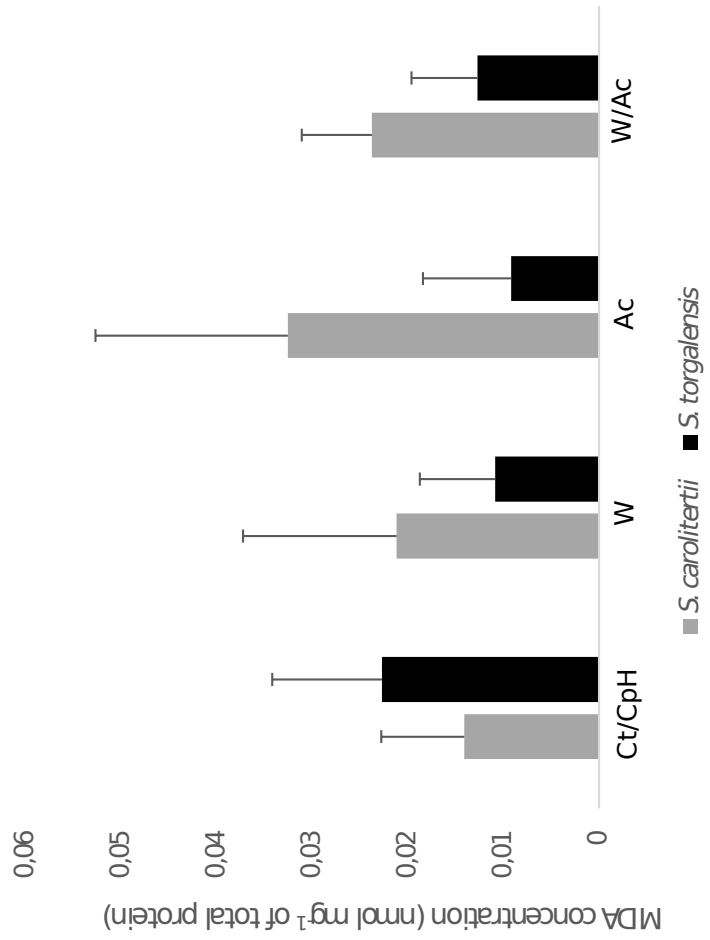

Figure 3.9: Concentration of malondialdehyde (MDA, nmol mg^{-1} of total protein) in the muscle of *Squalius carolitertii* and *S. torgalensis* exposed for 30 days to control temperature (Ct) and pH (CpH), warming (W; +3 °C) and acidification (Ac; ΔpH = -0.4). Values represent mean ± SD (n = 6).

Heat shock response, antioxidant enzymes activities and peroxidative damage

The heat shock and antioxidant (i.e. GST, CAT and SOD) responses, as well as cellular damage assessed in *S. carolitertii* were significantly influenced by both warming and acidification together with a significant interaction between the explaining variables (two-way MANOVA: $p < 0.05$; Table 3.8). However, only HSP content showed significant differences between the experimental conditions (Figure 3.7 and Table 3.10). In more detail, individuals exposed to conditions simulating a future climate change scenario (i.e. warming and acidification tested together) showed a significant increase in HSP content when compared with the ones exposed to the other conditions (Tukey post-hoc test: $p < 0.013$; Figure 3.7). Regarding *S. torgalensis*, pH and the interaction of this factor with temperature significantly affected heat shock, antioxidant, and peroxidative damage responses (two-way MANOVA: $p < 0.05$; Table 3.8), with no significant effect of temperature (two-way MANOVA: $p > 0.05$; Table 3.8). Looking into more detail to each response, HSP concentration significantly increased under the acidification condition (two-way ANOVA: $p < 0.013$; Table 3.10) whereas SOD inhibition decreased significantly in the acidification condition (two-way ANOVA: $p < 0.013$; Table 3.10). As for the other endpoints (i.e. GST, CAT and MDA), neither warming nor acidification significantly affected their activity/concentration (two-way ANOVA: $p > 0.013$; Figure 3.8 and 3.9; Table 3.10).

Discussion

Even though freshwater ecosystems are considered to be extremely vulnerable to environmental changes (Field *et al.*, 2014), the physiology of freshwater fishes under the context of climate change is poorly known (Ou *et al.*, 2015b; Mccairns *et al.*, 2016). This study reports the first results on the impact of climate change related variables in the metabolism and oxidative stress enzymatic machinery of two species of the genus Squalius inhabiting different climatic regions.

Our results showed that conditions simulating a climate change scenario affected the enzymatic activity of citrate synthase (CS) and lactate dehydrogenase (LDH) on *S. carolitertii* and *S. torgalensis*. For both species, increasing temperature under normocapnia, elicited changes on LDH activity, causing an increase in its activity in *S. carolitertii* with an opposite trend in *S. torgalensis*. It is important to note that, under the combined effects of

warming and acidification, *S. torgalensis* showed the higher LDH activity suggesting the existence of a synergistic effect between these two climate change related variables. On the other hand, acidification caused a significant decrease in CS activity of *S. carolitertii*, contrary to *S. torgalensis* which was not affected by conditions simulating climate change. Altogether, these results suggest that the anaerobic potential was required by *S. carolitertii* to compensate for the increase in the metabolic rate due to the higher temperature, whereas CS decreased as the result of CO_2 increase, which may have affected the oxidative metabolism. The increase in anaerobic and decrease in aerobic potentials, albeit in different conditions, may not be a viable long-term response, given that the anaerobic metabolism requires finite fermentable subtracts and leads to cytotoxicity (Rosa and Seibel, 2008; Rosa *et al.*, 2016). On the other hand, *S. torgalensis* sustained its metabolic rate under higher temperatures, requiring no increase in both aerobic and anaerobic metabolism. Thus, it seems that *S. torgalensis* is able to maintain its metabolic homeostasis, being able to cope with climate changes. Moreover, when exposed to increased temperature (under control pH), the anaerobic potential is reduced, which may be explained by the adaptation of this species to higher temperatures as it is usually exposed to high temperatures (e.g. 38 °C) during summer (Jesus *et al.*, 2013). These results are in agreement with previous transcriptomic studies on both species, which suggest that *S. torgalensis* may be better suited to deal adverse environmental conditions (Jesus *et al.*, 2016, and Chapter 3 Section 3.1). The performance of this species under stressful conditions may be the result of the adaptation to a harsher environment giving it tools to survive to a wider range of environmental conditions.

In general, the responses related with the HSR and oxidative stress of both *S. carolitertii* and *S. torgalensis* were not extremely affected by the variables related with climate change. Yet, in a long-term perspective certain results may raise future concerns, which make them still interesting and worth to discuss. With regard to HSR, conditions simulating a future scenario of climate change significantly affected both species, although in different ways. While HSP concentration of *S. carolitertii* significantly increased under the combined warming and acidification condition, suggesting a synergistic effect of temperature and pH, HSP concentration of *S. torgalensis* was only affected by pH, regardless of the temperature to which fish were exposed. These results raise concerns regarding the long-term persistence of both species, with particular emphasis for *S. carolitertii*, which showed the greatest increase in HSP concentration in the more realistic scenario simulating the future

conditions of their habitats. In fact, maintaining high levels of HSP for extended periods of time may be disadvantageous since the resources may be relocated from other key biological processes (e.g. growth, energy production) to the refolding of denatured proteins (Sorensen *et al.*, 2003; Veilleux *et al.*, 2015). Once again, our results are in line with previous gene expression studies on these species, which demonstrated that *S. torgalensis* presents a better fine-tuned HSR than *S. carolitertii*, both during short (Jesus *et al.*, 2013, 2016) and long-term exposure periods (Jesus *et al.*, 2017).

Interestingly, none of the antioxidant enzymes was significantly affected by any tested condition, except for SOD activity of *S. torgalensis* that significantly decreased with acidification regardless of the temperature to which fish were exposed. These results, along with the absence of changes in lipid peroxidation, indicate that both species were not under oxidative stress in the projected climate change conditions.

Overall, our results, suggest that *S. carolitertii* may struggle to cope with future climate change, particularly due to the effects of warming and acidification in metabolic activity and heat shock response. On the other hand, *S. torgalensis* seem to be better suited to these changes. Nevertheless, this species may still be at risk mainly due to the inability to maintain the trade-off between the upregulation of HSP and its costs. This study is of utmost importance to better comprehend how freshwater fishes will cope with future climate change and for the adoption of proper conservation strategies, which is particularly relevant for species such as *S. torgalensis*, considered a critically endangered species. Although our results do not raise a serious concern on the future of these species, further studies should focus on the combined effects of warming and acidification (Rosa *et al.*, 2014; Pimentel *et al.*, 2015, and Chapter 3 Section 3.1) together with other climate change related variables (Crozier *et al.*, 2008; Prado-Lima and Val, 2016).

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Supplementary material

Table 3.8: Results of two-way MANOVA performed in order to assess the effects of temperature (Temp) and pH on the activity of metabolic enzymes and heat shock proteins, antioxidant enzymes and malondialdehyde of *Squalius carolitertii* and *S. torgalensis* following an exposure of 30 days to conditions simulating present day and future climate change scenarios. Significant values ($p < 0.05$) are highlighted in bold.

Metabolic enzymes				
		Pillai's test	F	*p-value*
S. carolitertii	Temp	0.243	0.772	0.588
	pH	0.782	8.594	**0.001**
	Temp*pH	0.712	5.933	**0.005**
S. torgalensis	Temp	0.022	0.179	0.838
	pH	0.432	6.073	**0.011**
	Temp*pH	0.695	18.196	**<0.001**
Heat shock proteins, antioxidant enzymes and malondialdehyde				
		Pillai's test	F	*p-value*
S. carolitertii	Temp	0.945	17.560	**0.003**
	pH	0.842	5.320	**0.045**
	Temp*pH	0.855	5.885	**0.037**
S. torgalensis	Temp	0.243	0.772	0.588
	pH	0.782	8.594	**0.001**
	Temp*pH	0.712	5.933	**0.005**

Table 3.9: Results of two-way ANOVA performed in order to assess the effects of temperature (Temp) and pH on the activity of each metabolic enzymes (lactate dehydrogenase (LDH) and citrate synthase (CS)) of *Squalius carolitertii* and *S. torgalensis*, following an exposure of 30 days to conditions simulating present day and future climate change scenarios. Significant values ($p < 0.013$) are highlighted in bold.

S. carolitertii			*S. torgalensis*		
	F	*p-value*		F	*p-value*
LDH					
Temp	15.764	**0.001**	Temp	0.503	0.487
pH	12.289	**0.004**	pH	15.832	**0.001**
Temp*pH	20.245	**<0.001**	Temp*pH	48.279	**<0.001**
CS					
Temp	0.542	0.478	Temp	0.176	0.680
pH	33.461	**<0.001**	pH	3.060	0.096
Temp*pH	0.947	0.343	Temp*pH	4.103	0.057

Table 3.10: Results of two-way ANOVA performed in order to assess the effects of temperature (Temp) and pH on the activity of heat shock proteins (HSP), each antioxidant enzymes (glutathione S-transferase (GST), superoxide dismutase activity (SOD) and catalase (CAT)) and malondialdehyde (MDA) of *Squalius carolitertii* and *S. torgalensis* following an exposure of 30 days to conditions simulating present day and future climate change scenarios. Significant values ($p < 0.013$) are highlighted in bold.

S. carolitertii			*S. torgalensis*		
	F	*p-value*		F	*p-value*
HSP					
Temp	50.705	**<0.001**	Temp	3.839	0.063
pH	40.531	**<0.001**	pH	15.554	**0.001**
Temp*pH	8.339	**0.011**	Temp*pH	0.436	0.516
GST					
Temp	1.428	0.248	Temp	0.224	0.641
pH	0.185	0.672	pH	1.490	0.235
Temp*pH	0.105	0.750	Temp*pH	0.683	0.417
CAT					
Temp	0.061	0.808	Temp	0.406	0.531
pH	0.284	0.601	pH	0.241	0.629
Temp*pH	0.139	0.714	Temp*pH	2.054	0.167
SOD					
Temp	4.333	0.054	Temp	0.039	0.846
pH	2.717	0.119	pH	18.804	**<0.001**
Temp*pH	3.956	0.064	Temp*pH	7.258	0.014
MDA					
Temp	0.019	0.891	Temp	1.375	0.846
pH	2.835	0.109	pH	2.703	0.114
Temp*pH	1.620	0.219	Temp*pH	4.714	0.041

Chapter 4

Discussion and final remarks

4.1 Acclimatization and Acclimation of freshwater fish

In this thesis a major focus was given to the effects of climate changes, particularly temperature increases, in two congeneric fish species acclimatized to different environmental conditions. *S. carolitertii* is acclimatized to Atlantic climate, with mild environmental conditions. On the other hand, *S. torgalensis* is acclimatized to Mediterranean climate, being exposed to a marked interchange between floods and droughts (Magalhães *et al.*, 2003; Carvalho *et al.*, 2010; Henriques *et al.*, 2010), which subjects individuals of this species to higher daily and seasonal variations of temperatures and to higher maximum temperatures.

Temperature is a serious constraint for living beings, however it is certainly more significant for ectotherms, which rely on environmental temperature for their metabolism. Global warming is increasing water temperature in both marine and freshwater ecosystems (Field *et al.*, 2014). As a result, freshwater fish species, with a distribution confined to river basins, must be able to cope with changing environmental conditions in order to survive and persist along generations, otherwise they may become extinct. In this regard, the study of the responses of extant species to high temperatures may provide important hints on the thermal tolerance of species.

4.1.1 Acute thermal stress responses

Chapter 2 of this thesis focused on short-term responses of *S. carolitertii* and *S. torgalensis* to acute thermal stress. In Section 2.1 (Jesus *et al.*, 2013), it was observed that *S. torgalensis* induced the mRNA levels of *hsp70* and *hsc70*, suggesting that this species has a strong heat shock response (HSR). On the other hand, no increments in *hsp70* and *hsc70* were observed in *S. carolitertii*. In nature, *S. torgalensis* may be naturally exposed to temperatures as high as 38 °C, while *S. carolitertii* is usually exposed to temperatures below 31 °C (SNIRH, 2010). However, two, out of the seven individuals of *S. carolitertii*, did not survive to the 35 °C treatment, suggesting that its upper thermal tolerance limit may have been reached. It is important to emphasize, though, that those individuals were not previously acclimated to temperatures higher than their natural habitat, which ranged from 18 °C to 22 °C during sampling. Therefore, the absence of heat shock response in this species does not seem to be the result of acclimation, leading to a new homeostatic

state, but rather an inability to cope with such short time exposure to high temperatures. Thermal tolerance studies (e.g. thermal tolerance polygons or critical thermal limits) would have helped to better understand the physiological constraints of these species. However, the number of individuals to perform such studies, with statistical significance, is not easily attained for endangered species.

Using a next generation sequencing approach (presented in Section 2.2) (Genomic Resources Development Consortium, Almeida-Val *et al.*, 2015), we compared the differences in the transcriptomes of *S. carolitertii* and *S. torgalensis* between fish kept and acclimated for 15 days at a control temperature (18 °C) and the test condition (30 °C). For that, we pooled samples from seven individuals for each tissue (skeletal muscle, liver and fins). Sample pooling was a commonly used strategy at the beginning of this work, since it considerably reduced the costs of sequencing and increased the representativeness of the transcriptome or genome of a given species (Ekblom and Galindo, 2010; Yek *et al.*, 2013; Rajkumar *et al.*, 2015), compared with single individual sequencing. Although this approach has limitations for the statistical analyses, there are programs that can deal with the absence of actual replicates by adjusting the distribution of read count data to a given statistical distribution (e.g. negative binomial or Poisson) (Rajkumar *et al.*, 2015). Nevertheless, we did a conservative approach in order to reduce the number of false positives, by lowering the FDR cutoff value to 5×10^{-4} (Jesus *et al.*, 2016).

The analysis of differential gene expression obtained from the transcriptomes of both species also showed changes in *hsp70* and *hsc70* genes. *Hsp70* was upregulated in all three analyzed tissues (skeletal muscle, liver and fins), particularly in the skeletal muscle of *S. carolitertii* and in all tissues of *S. torgalensis*. These results corroborate our findings for *S. torgalensis* from the previous work (Jesus *et al.*, 2013) in which this species showed a significant increase in *hsp70* and *hsc70* gene expression. However, in this transcriptome-wide study (Jesus *et al.*, 2016), *hsp70* was significantly upregulated and *hsc70* was significantly downregulated in skeletal muscle tissue of *S. carolitertii*, suggesting that, contrary to *S. torgalensis*, it is unable to induce the *hsc70* gene under thermal stress conditions. These differences observed between the two experiments, suggest that for *S. carolitertii* the *hsc70* is a constitutively expressed gene rather than a stress induced gene, as in the case of *S. torgalensis*. On the other hand, the *hsp70* gene is a stress induced gene in both species, though, *S. torgalensis* present a stronger induction of this gene in both experimental conditions. Besides *hsp70* and *hsc70*, many other *hsps* involved in the HSR were

upregulated in both species (e.g *hsp90* and *hsp40*, also known as *dnajs*). These results suggest that the HSR is, in fact, an important defense mechanism against acute thermal stress for both species since it helps to adjust metabolic disorders caused by protein degradations (Lindquist and Craig, 1988; Sorensen *et al.*, 2003).

However, there are costs in triggering the HSR, and thus the survival of individuals, as well as the persistence of species, lies in the trade-off between the costs and benefits of the HSR (Sorensen *et al.*, 2003; Dahlhoff and Rank, 2007; López-Maury *et al.*, 2008). While the HSR helps the organism to deal with protein degradation and denaturation during periods of thermal stress, it may have deleterious effects on organisms' fitness (Sorensen *et al.*, 2003; López-Maury *et al.*, 2008). Up regulation of hsps may have impacts on the organisms' energy consumption, development, growth and even fertility and fecundity, since it redirects energy from normal cell functions to the HSR (Sorensen *et al.*, 2003). Coupled with the HSR, *S. carolitertii* showed increased activity of genes involved in transcription and in RNA metabolic process, suggesting that this species responds by increasing the mRNA levels of genes (e.g. HSR involved genes), in order to maintain homeostasis. However, *S. torgalensis* displays a stronger increase in HSR related genes in all tissues, and a downregulation of many biological processes involved in cellular growth (e.g. nuclear division, cell cycle, chromosome organization). This process is widely known as a mechanism to save energy during stressful conditions, re-directing energy towards the repair of damaged molecules (such as denatured proteins) (Sorensen *et al.*, 2003; Buckley *et al.*, 2006; López-Maury *et al.*, 2008). Therefore, *S. torgalensis* seems to be better suited to cope with stressful conditions for short periods of time, since it is able to conserve energy by downregulating molecular pathways involved in growth, and to survive at higher temperatures than *S. carolitertii*. However, long-term exposure to high temperatures, such as those predicted by climate change scenarios, might hinder the survival chances of fish (Reusch and Wood, 2007; López-Maury *et al.*, 2008; Tomanek, 2010). Additionally, species which are commonly exposed to higher temperatures are usually closer to their upper thermal tolerance, and thus future warming might still threaten them (Reusch and Wood, 2007; Sorensen *et al.*, 2009; Somero, 2010; Tomanek, 2010; Hoffmann and Sgrò, 2011).

Interestingly, among the differentially expressed genes of the transcriptomes of both species are genes involved in the circadian rhythm. This is surprising because individuals were maintained in a constant day:night cycle (12h:12h), however zebra fish circadian

clock is influenced by environmental temperature, inducing changes in the transcription of clock involved genes (Lahiri *et al.*, 2005; Vatine *et al.*, 2011).

4.1.2 Projected warming and acidification and their synergistic effects

Individuals of both species were exposed, for 30 days, to warming and acidification, individually and combined, simulating an increase in temperature of 3 °C and a decrease in pH of 0.4 units in relation to summer average conditions (Chapter 3). Despite the absence of projections of acidification for freshwater systems from the Intergovernmental Panel on Climate Change's (IPCC) fifth assessment report, we based upon these parameters following the IPCC Representative Concentration Pathways (RPC 8.5) (Field *et al.*, 2014). This experimental setting aimed to find whether species would acclimate to the new conditions [i.e. reach a new steady-state (non-stressed) condition] (López-Maury *et al.*, 2008; de Nadal *et al.*, 2011) after a period of one month, thus simulating long-term responses of these species. Conte (2004) considered 15 days, after a change in water parameters, enough time for a species to acclimate, thus our experimental setting may be seen as long-term exposure in which acclimation effects are absent.

As previously stated in Chapter 1, to date, only three studies were published regarding the effects of multiple climate change stressors (i.e. synergistic scenarios) in freshwater fish. Prado-Lima and Val (2016) studied *Colossoma macropomum* responses to three climate change scenarios (B1, A1B, A2) (simulating the forecasted atmospheric temperature, CO_2, humidity and O_2 concentrations), for 5 and 15 days. As previously stated, physiological recovery after a change in water parameters may take up to 15 days (Conte, 2004), which suggests that the experimental period used by Prado-Lima and Val (2016) may have been insufficient for the proper acclimation of fish to the simulated climate change conditions. In fact, many genes involved in binding molecular functions (including a large percentage of protein binding GO terms) were found to be differentially expressed in *Colossoma macropomum* in response to these conditions, which is more characteristic of acute stress responses than of acclimation responses (Kassahn *et al.*, 2007; Lewis *et al.*, 2010; Long *et al.*, 2012; Smith *et al.*, 2013; Jesus *et al.*, 2016). Also studying *Colossoma macropomum* de Oliveira and Val (2016) showed increased food intake, growth, as well as haematocrit,

after 30 days of exposure to the same climate change scenarios, which suggest that this species can adjust its physiology to these new environmental conditions. Furthermore, Mccairns *et al.* (2016) exposed the rainbow fish *Melanotaenia duboulayi* during 80 days to the foreseen 2070 summer average temperatures. In these latter two studies, the effects of acclimation were removed, since the fish were acclimated to the projected climatic conditions for a period exceeding 15 days. Here, we acclimated fish for 15 days to aquaria conditions, after being captured, and only after that period, we exposed fish for 30 days to the projected climate change scenarios as described above.

4.1.2.1 Gene expression responses to climate change and their relationship with evolution of protein function and structure

Differences between species in protein structure and function, as well as in gene expression, result from the different adaptations of each species and may confer them advantages in their environmental setting (Stapley *et al.*, 2010; Hoffmann and Sgrò, 2011). These different adaptations between species may help us understand which species are more threatened by climate change. In Section 3.1 we searched for gene expression changes and functional and structural differences in fourteen genes selected from the transcriptomes of *S. carolitertii* and *S. torgalensis* (Table 3.2).

Gene expression results showed striking differences between both species, with *S. carolitertii* having more genes with changes in expression than *S. torgalensis* for the tested conditions (warming, acidification and combined warming and acidification).

Regarding warming, *S. torgalensis* properly acclimated to an increase of 3 °C in average summer water temperature, with no significant changes in gene expression of *hsps*. On the other hand, after one month, *S. carolitertii* presented many changes in gene expression under the 3 °C warming condition. It presented changes in protein folding (*hsp90aa1.1*, *fkbp4*), circadian rhythm (*cry1a*) and immune response (*gbp1*) related genes. These results suggest that *S. torgalensis* has a higher thermal tolerance before eliciting the stress response, when compared with *S. carolitertii*, and hence might be better adapted to cope with future climate change. Therefore, *S. torgalensis* individuals seem to have acclimated to the experimental conditions after the period of one month or the conditions were not stressful enough to induce protein degradation or denaturation. On the other hand, *S. carolitertii* individuals presented a stress response and were unable to re-adjust

gene expression to levels similar to the control condition during the time of the experiment. Furthermore, comparative biochemical and structural analysis of the fourteen encoded proteins between *S. carolitertii* and *S. torgalensis* showed differences in physical and chemical parameters of HSP90 and GBP1. These two proteins presented higher thermostability in *S. torgalensis* than in *S. carolitertii*, thus reinforcing that *S. torgalensis* may be better suited to tolerate a wider range of temperatures.

In turn, acidification elicited gene expression changes in both species. Six genes had significant changes in expression for *S. carolitertii* (*fkbp4*, *ldha*, *ndufb8*, *glula*, *cry1a*, *per1a*), while *S. torgalensis* presented changes in three genes (*cs*, *cry1a* and *per1a*). The combination of warming and acidification triggered a larger number of gene expression differences (eleven in *S. carolitertii* and four in *S. torgalensis*) in relation to the control condition. This observation raises awareness towards the study of multiple climate change stressors rather than focusing on warming alone. Even for *S. torgalensis*, which presented a better acclimation, with less changes in gene expression in the majority of protein folding genes (including several genes involved in the HSR) and higher energy production performance (increasing *cs* and maintaining *ldha* mRNA levels), there were severe downregulations under the synergistic scenario (for the protein folding *stip1* gene and the immune-related *gbp1* gene). Furthermore, several significant changes in gene expression were observed in the two circadian rhythm genes (*cry1a* and *per1a*) for both species, suggesting that both warming and acidification, as well as their synergy, might disrupt the biological clock of these fish. Disturbance of the circadian clock may have profound effects on fish's metabolism and behavior, such as changes in mating season and feeding (Idda *et al.*, 2012; Brudler *et al.*, 2003; Amaral and Johnston, 2012).

Mccairns *et al.* (2016) demonstrated differences in gene expression in the freshwater fish *Melanotaenia duboulayi*, exposed to future climate change conditions. However, the authors studied the effects of warming, while herein we studied both warming and acidification plus their synergy. In that study, they increased 10 °C in relation to summer season conditions, which is an extreme temperature increase compared with the 3 °C increase used in Chapter 3, as foreseen by IPCC for the year 2100 (Field *et al.*, 2014). They also used a target gene approach, retrieving 12 genes from other transcriptomic study (Smith *et al.*, 2013), but none of them are common with our 14 target genes. Mccairns *et al.* (2016) suggested that transcriptional changes may enhance the odds of species to cope

with future climate change, however whether there is a link between transcriptional variation and the fitness is still unknown. On the other hand, in Chapter 3 we argue that in order to cope with long-term changes in environmental conditions, species cannot rely solely in the stress response, but instead they need to have a re-adjustment that allows them to reach a new homeostatic state (Sorensen *et al.*, 2003; López-Maury *et al.*, 2008; de Nadal *et al.*, 2011).

The observed differences in gene expression between both species might be explained by some of the differences between the proteins' structure that we have described, particularly for HSC70, FKBP52 and HIF1α. For these three proteins, structural differences were found, however their encoding genes did not present any change in gene expression for *S. torgalensis*, but presented for *S. carolitertii*. This might suggest that these structural differences are advantageous for *S. torgalensis*, making it unnecessary to upregulate these genes in the projected climate change conditions.

Although it is expected that proteins with different physical and chemical parameters present distinct tertiary structure, 7% of *S. torgalensis*' HSP90 structure could not be predicted based on existing database templates (see Chapter 3, section 3.1). In fact, three non-synonymous substitutions were found in *hsp90*, though they were not in the modeled region of HSP90 protein of *S. torgalensis*. Therefore these differences between *S. carolitertii* and *S. torgalensis* are absent from the modeled tertiary structure, although they can be important for the final protein function, since non-synonymous substitutions result in different amino acids that can change the conformation of the protein. Other genes presented non-synonymous substitutions between species: *hsc70*, *fkbp4*, *stip*, *hif1a*, *glula*, *per1a* and *gbp1*. These non-synonymous substitutions resulted in differences between the two species in protein structure of HSC70, FKBP52, HIF1α and GPB1. FKBP52 and GBP1 presented structural changes in important protein domains, while HSC70 and HIF1α presented all changes in coil regions with unclear function for the protein. However, even these coil regions might have a relevant function, adding flexibility that allow for conformational changes in proteins (Buxbaum, 2007). While model coverage of Glutamine Synthetase (GLULA) and Period 1A (PER1A) were less than 60% of the protein, resulting in the absence of structural differences from the model of the two species (similarly to HSP90), model coverage of STIP1 was 100%, but with no impact in the modeled protein structure.

4.1.2.2 Physiological responses

In order to access the physiological impacts of the simulated conditions of warming and acidification in *S. carolitertii* and *S. torgalensis*, we used a set of state of the art markers (Vinagre *et al.*, 2012; Pimentel *et al.*, 2015; Rosa *et al.*, 2016). Regarding the metabolic enzymatic activity, *S. carolitertii* was the most affected species, with an increase in lactate dehydrogenase (LDH) activity under warming condition, and a decrease of citrate synthase (CS) activity under hypercapnia. On the other hand, *S. torgalensis* presented a diminished LDH activity under warming condition in relation to control condition (current summer average temperature). These differences might be the result of the adaptation of *S. torgalensis* to warmer conditions during summer, resulting in the development of mechanisms to keep aerobic metabolism at higher temperatures. Therefore, *S. torgalensis* seems to be better suited than *S. carolitertii* to deal with future warming and acidification, favoring the aerobic (CS activity) instead of the anaerobic (LDH activity) metabolism, which is more efective in producing energy (ATP). On the other hand, *S. carolitertii* activated anaerobic metabolism to better cope with higher ATP demands, once higher temperatures increases general metabolism, causing higher ventilation, higher osmoregulation, and other pathways responsive to heat (Storey and Storey, 2005; Campos *et al.*, 2016).

Except for superoxide dismutase (SOD), which presented a decreased activity under the acidic condition in *S. torgalensis*, no other changes were observed for antioxidant enzymes for both species. SOD catalyzes the dismutation of superoxide ($\cdot O_2^-$) radical into oxygen (O_2) or hydrogen peroxide (H_2O_2), thus reducing the production of most damaging reactive oxygen species (ROS) present in cells (e.g. $\cdot OH$) (Madeira *et al.*, 2013; Rosa *et al.*, 2016). The absence of significant increases in CAT, GST, SOD activities, for both species, suggest that these conditions were not stressful enough to induce the formation of ROS. Moreover, no significant changes were observed in the peroxidative damage marker [malondialdehyde (MDA)], for both species, suggesting that cell membrane was not damaged, keeping the cell integrity (Lushchak, 2011; Patil and David, 2013). These results indicate that these species were not under oxidative stress and, thus, at least for these species, ROS are not a major threat under the projected climate change conditions.

In fact, as already mentioned, the HSR was also impacted by the projected climate changes. Despite both species presented changes in HSP70, *S. carolitertii* presented a

higher increment than *S. torgalensis* under the combined warming and acidification condition, suggesting that its HSR was more responsive to climate change conditions. Although acute stress responses are important to keep cellular homeostasis, increased HSP70 expression might not be a viable long-term strategy since organisms cannot indefinitely pause normal cell function, relocating resources from other key biological processes, such as cell growth and energy production, towards the folding of denatured proteins (Sorensen *et al.*, 2003; Veilleux *et al.*, 2015). Further studies may clarify the role of HSP70 increases once no mortality was observed during the experiments.

4.2 Final remarks

Climate change is threatening biodiversity worldwide and each species must be able to deal with future changes, otherwise they may perish. The difficulty in predicting the impacts of climate change in a given species is linked with the uniqueness of each species' response. In this thesis, by studying two congeneric species of the *Squalius* genus living in different environmental conditions, it was observed that both species present different responses to warming and acidification.

In all experimental settings, *S. torgalensis* (acclimatized to the warmer Iberian climate) consistently showed higher performance than *S. carolitertii* (acclimatized to the Atlantic temperate climate) when exposed to both acute heat shock and to projected climate changes. Under acute thermal stress, *S. torgalensis* seemed to outperform *S. carolitertii*, since it greatly induced the stress machinery, which may be an adaptive trait to deal with periods of extreme temperatures in which this species periodically lives. On the other hand, *S. carolitertii* is not usually exposed to temperatures as high as the ones tested in Chapter 2, or to such sudden temperature variations. However, long-term exposure to changing temperature requires an acclimation to the new environmental conditions rather than a stress response (López-Maury *et al.*, 2008; de Nadal *et al.*, 2011). So, the responses to long-term exposure were more complex.

The observed differences in gene expression and protein structure between species may be considered adaptive as well as the result of the evolutionary adaptation (acclimatization) of each species to their current environmental conditions (Ouborg *et al.*, 2010). However, whether species will be able to evolve improved responses to future climate changes at

the pace that they are occurring is an answer only achieved through experiments that involve several generations of fish exposed to these future changes. For instance, Veilleux *et al.* (2015), studying the reef fish *Acanthochromis polyacanthus*, discovered that molecular processes, such as gene expression changes, can be adjusted along generations in order to improve the response of fish to future climate change conditions. Hence, to better predict the adaptive ability of *S. carolitertii* and *S. torgalensis*, it would be interesting to perform a trans-generational approach, tracking the progress of the responses of further generations to the same projected climate change conditions. Additionally, common garden experiences, in which laboratory bred individuals of both species would be subject to the same control conditions, might help to better understand the environmental and genetic components of the plastic responses found in these species throughout this thesis. However, such experiments are limited by the long generation time of these species (2-3 years) (Magalhães *et al.*, 2003; Maia, 2006), the high mortality and the difficulty to reproduce these species in captivity, particularly if we intend to analyze more than one generation.

Although *S. torgalensis* has a reduced genetic diversity and reduced population effective size (Henriques *et al.*, 2010), this can in fact be the result of its adaptation to the harsh environmental conditions in which this species live, particularly during summer season. Corroborating this hypothesis, we found that *S. torgalensis* is better adapted to deal with acute thermal stress conditions (e.g. a heat wave) and to the projected warming and acidification conditions.

However, future conditions predicted by IPCC's models are far more complex than those tested here. For example, in aquatic environments the increase in water temperature is coupled with an increase in dissolved CO_2 and a decrease in dissolved O_2, which in some places may lead to hypoxic conditions, particularly where droughts are harsher, as occurs in *S. torgalensis* habitats. Also, climate change is boosting other already harmful threats, both biotic and abiotic (e.g. invasive species and pollutants, respectively) (Field *et al.*, 2014). Hence, and given the synergistic effects of temperature and pH found in the results of this thesis, future research should pay attention to the combined effects of climate change stressors.

The protection of the critically endangered *S. torgalensis* is more important than ever. With the decrease in availability of suitable habitats for this species, particularly during the dry season if the severity of droughts is intensified, the conservation and monitoring of

these watercourses will be paramount. In this regard, and given the high anthropogenic pressure on this species (e.g. construction of dams and introduction of invasive species) (Cabral *et al.*, 2006), the recovery and maintenance of the riparian vegetation and deepening of the ponds in which these fishes stay during the dry season might help them to cope with future threats. Moreover, although *S. carolitertii* population is currently larger and despite its environment undergoes lower temperature variations (daily and along the year), both acute heat stress and future climate change projections have elicited changes in its physiological responses, suggesting that this species might also struggle with future environmental changes. Therefore, the constant monitoring of environmental conditions should necessarily be part of conservation plans for both species, in order to detect if future climate will corroborate the projections assumed in this dissertation.

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