UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

HEAT TOLERANCE LIMITS AND PHYSIOLOGICAL RESPONSES TO CLIMATE WARMING IN SHRIMPS FROM DIFFERENT TIDAL HABITATS AND LATITUDINAL REGIONS

Ana Rita José Lopes

DISSERTAÇÃO
MESTRADO EM ECOLOGIA MARINHA

2012

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

HEAT TOLERANCE LIMITS AND PHYSIOLOGICAL RESPONSES TO CLIMATE WARMING IN SHRIMPS FROM DIFFERENT TIDAL HABITATS AND LATITUDINAL REGIONS

Ana Rita José Lopes

DISSERTAÇÃO

MESTRADO EM ECOLOGIA MARINHA

Dissertação orientada pelo Doutor Rui Rosa (CO/LMG)
E co-orientada pelo Doutor Mário Diniz (FCT-UNL)

2012

Acknowledgements

I would like to express my gratitude to everyone that helped, guided and inspired me along this work, namely to:

Professor Doutor Rui Rosa for kindly accepting me as his student and for all the support, motivation and trust. I want to thank for all his supervision along this work, friendship and enthusiasm, no doubt that all his knowledge and expertise helped me grow as a scientist and to get even more passionate about this profession. He's a role model.

Doutor Mário Diniz for receiving me so well into his guard at Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa and for all his teachings. Also I wish to thank all the trust deposited in me and his unconditional supervision, support friendship and companionship.

Professor Doutor Luís Narciso for his help during field work. His experience and knowledge really helped me through this work.

To all of my dearest friends, to longtime friends from my childhood and new ones I made during my stay in FCUL and FCT, in short all my new friends in Lisbon, mainly to Manuel Neves for all of his support and company along this work, I would like to thank him for all his help, it was really useful to have another friend and biologist's eye on these matters, no doubt that his companionship made this journey much more fun.

All the team from the Laboratório Marítimo da Guia, in particular Filipa Faleiro, Marta Pimentel, Tatiana Teixeira and Tiago Repolho for all their companionship, encouragement, help and friendship. Their advices throughout this work really help me.

My family, mainly my parents for believing in me, there is no doubt that they made me the woman I am today, because they taught me to be strong, hard working, to fight for what I want, but most important to believe in me. Without them I couldn’t achieve my biologist dream. I would like to thank to my grandparents for all of their love and pride. I feel very fulfilled and happy in my family they not only love me as they make it a point to demonstrate each passing day.

To all those who received me in their home, as if I were part of the family, mainly to my cousins Cristina, for being my “backup mother”, Armando and their girls (Maria and Constança) for their kindness and love and to João Gonçalves, Aldina Gonçalves and João Miguel for being so wonderful.

At last, but certainly not the least to João Pedro, who gave me his unconditional love, support and care. I would like to thank him for being such an amazing boyfriend who gave me motivation and encouraged my work. Thank you to cheering me up on bad moments and to be present in the good ones.

Resumo

Atualmente, a temática das alterações climáticas na biosfera marinha reveste-se de importância fulcral com especial ênfase nos possíveis efeitos nefastos que poderão provocar ao nível de ecossistemas, populações e espécies marinhas. Como principais efeitos, podemos salientar alterações na distribuição geográfica de espécies, extinções locais, migrações em grande escala, alterações fenológicas e a própria estrutura das cadeias tróficas. Um dos principais problemas associados às alterações globais, é o aquecimento médio dos oceanos (entre +3°C e +6°C segundo as previsões do IPCC 2007), o qual influenciará a bio-ecologia (mortalidade, reprodução, crescimento, comportamento) e ecofisiologia dos organismos marinhos.

Esta tese teve como principais objetivos a determinação da tolerância térmica (LT_{50} e LT_{100}), os padrões de expressão de proteínas de choque térmico (HSP's) e mecanismos de defesa face ao stress oxidativo (i.e. à produção de ROS), de quatro espécies de camarões (*Lysmata seticaudata*, *Lysmata amboinensis*, *Palaemon elegans* e *Palaemon serratus*), oriundos de diferentes habitats com o objetivo de avaliar o impacto do aumento da temperatura na biologia destas espécies.

Mais concretamente, e numa primeira abordagem, pretendeu-se inferir a suscetibilidade biológica de duas espécies congéneres (*Palaemon elegans e Palaemon serratus*), oriundas da mesma localização geográfica mas de diferentes habitats costeiros (intertidal e subtidal), face a um aumento de temperatura; e numa segunda abordagem, pretendeu-se compreender a resposta fisiológica de congéneres do género *Lysmata* de diferentes latitudes, nomeadamente de uma região tropical (*Lysmata amboinensis*) e de uma região temperada (*Lysmata seticaudata*).

O estudo da tolerância térmica constituiu a primeira abordagem para compreender a vulnerabilidade/resiliência das espécies estudadas face a um aumento da temperatura. Deste modo, foi determinado o limite de tolerância térmica máxima (LT's) para as quatro espécies estudadas, que consistiu em submeter os organismos a um aumento crescente de temperatura (1°C/30 minutos) até o seu limite térmico biológico ser atingido. Desta forma, concluiu-se que a espécie *P. elegans* possui um limite de tolerância mais elevado do que *P. serratus*. No que diz respeito às espécies congéneres oriundas de zonas temperada/subtropical e tropical, observou-se que a espécie mais vulnerável ao stress térmico foi *L. amboinensis* (espécie tropical), provavelmente devido a uma baixa amplitude térmica ao nível do seu *habitat* e ao facto do seu limite de tolerância máximo se encontrar próximo da temperatura máxima do seu habitat natural, tornando-a deste modo mais vulnerável que *L. seticaudata*. De igual modo constatou-se que num cenário de aquecimento extremo (+ 6°C acima da média do seu habitat) *L. amboinensis* exibiu um processo de supressão no seu metabolismo ($Q_{10} < 1.5$).

Posteriormente estudou-se os mecanismos de defesa celular face a stress térmico (e ao aumento de produção de ROS). As proteínas de choque térmico, são um grupo de proteínas específico, que são induzidas quando ocorrem fatores de stress, de forma a protegerem as células dos impactos negativos dos mesmos. Outro mecanismo de defesa celular baseia-se na produção de enzimas antioxidantes que atuam sobre espécies reativas responsáveis pelos efeitos tóxicos do oxigénio (ROS).

Assim sendo, determinaram-se os padrões de expressão das HSP de peso molecular 70 DKa (HSP70), através do método ELISA (Enzyme Linked Immunosorbent Assay), bem como a ocorrência de peroxidação lipídica (através da determinação do

MDA, Malonaldeído), e as actividades da catalase (CAT), glutationa s-transferase (GST) e superóxido dismutase (SOD).

Para ambas as espécies do género *Palaemon* houve um aumento da peroxidação lipídica (considerado o mecanismo mais frequente de lesão celular) e da atividade dos restantes mecanismos enzimáticos de defesa celular, com especial ênfase para a espécie *P. serratus*. No que respeita ao género *Lysmata*, verificou-se que tanto a expressão de HSP´s assim como dos restantes mecanismos de defesa celular foi superior para *L. seticaudata*.

Em conclusão, os resultados deste estudo demostram que para as espécies estudadas, as que demonstraram maior vulnerabilidade face ao aquecimento global foram aquelas que habitam ambientes mais estáveis, nomeadamente subtidais (*P. serratus)* e tropicais *(L. amboinensis)*, em oposição a espécies características de ambientes intertidais (*P. elegans*) e temperadas/subtropicais (*L.seticaudata)*.

Abstract

This study was aimed to determine thermal tolerance limits (LT_{50}, LT_{100}), heat shock protein (HSP) expression, activity of antioxidative enzymes (CAT, GST and SOD) and lipid peroxidation (MDA buildup) in four species of shrimps (*Lysmata seticaudata*, *Lysmata amboinensis*, *Palaemon elegans* e *Palaemon serratus*), in order to understand how climate change will influence their vulnerability.

The present work was designed and performed taking into consideration two different approaches. In the first instance, our study focused on congener's shrimps of genus *Palaemon*, since these organisms can be found in different tidal habitats and thus determine which species would be more susceptible to an increasing temperature, if an intertidal species, such as *Palaemon elegans*, or an subtidal one, such as *Palaemon serratus*; a second approach was to compare species from different latitudinal habitats, namely *Lysmata amboinensis*, a cleaner shrimp of coral reefs inhabiting the tropical region and *Lysmata seticaudata*, also called the Monaco's shrimp, which inhabits temperate/subtropical region.

Through the method of maximum thermal tolerance limit's (LT's), species were ranked in terms of their vulnerability. Results showed that species inhabiting unstable tidal zones and temperate/subtropical environments, had a higher tolerance than their congener's, inhabiting subtidal and tropical habitats. Additionally and under an extreme warming scenario (+ 6° C above habitat temperature), the tropical species suppressed its metabolism ($Q_{10} < 1.5$), which showed that in response to increased temperature, *L. amboinensis* does not prevent a decrease in their metabolic performance.

Secondly, cellular defense mechanisms against stress were analyzed. Higher mass-specific metabolic rate of intertidal and temperate shrimps was compensated by physiological mechanisms in order to minimize negative effects of thermal stress on fitness, like HSP70 expression, lipid peroxidation, SOD, GST and CAT activities.

In conclusion, the results derived from our study show which species may be more vulnerable to climate change and how molecular mechanisms may account for thermotolerance. Additionally, evidence that species have adapted their tolerance limits to coincide with microhabitat conditions is presented, which to some extent can corroborate assumptions that ocean warming is expected to drive more profound biological impacts on species that evolved in relatively stable and aseasonal environments (*P. serratus* and *L. amboinenis*) - thermal specialists - in comparison to species inhabiting seasonal and unstable habitats (*P. elegans* and *L. seticaudata*).

Table of contents

ACKNOWLEDGEMENTS .... **I**

RESUMO .... **III**

ABSTRACT .... **VII**

GENERAL INTRODUCTION **1**

1. CLIMATE CHANGE 1

1.1 Climate change and tidal differences: Genus Palaemon case *3*

1.2 Climate change and latitudinal differences: Genus Lysmata case *4*

2. MOLECULAR MECHANISMS BEHIND THERMAL TOLERANCE AND ADAPTATION 5

2.1 Heat shock proteins *5*

2.2 Oxidative stress enzymes and cellular damage (Lipid peroxidation) *8*

REFERENCES 11

CHAPTER 1 .... **19**

HEAT TOLERANCE LIMITS AND PHYSIOLOGICAL RESPONSES FROM GENUS *PALAEMON* SHRIMPS TO A WARMING OCEAN **19**

ABSTRACT 19

INTRODUCTION 20

MATERIALS AND METHODS 22

Specimen Collection and Maintenance *22*

Thermal Tolerance Limits *22*

Oxygen Consumption Rates and Thermal Sensitivity *23*

Preparation of tissues extracts *24*

Heat shock response (HSP70/HSC70) *24*

Lipid peroxidase (determination of malondialdehyde, MDA) *25*

Enzymatic Analyses *26*

Statistical Analyses *28*

RESULTS 29

DISCUSSION 35

REFERENCES 37

CHAPTER 2 .... **43**

CLIMATE WARMING AND PHYSIOLOGICAL RESPONSES IN SHRIMPS OF GENUS *LYSMATA*: A LATITUDINAL APPROACH **44**

ABSTRACT 42

INTRODUCTION 43
MATERIALS AND METHODS 45
Specimen Collection and Maintenance *45*
Thermal Tolerance Limits *46*
Oxygen Consumption Rates and Thermal Sensitivity *46*
Preparation of tissues extracts *47*
Heat shock response (HSP70/HSC70) *48*
Lipid peroxide assay (determination of malondialdehyde, MDA) *49*
Enzymatic Analyses *50*
Statistical Analyses *52*
RESULTS 52
DISCUSSION 62
REFERENCES 64

FINAL CONSIDERATIONS 69

REFERENCES 72

General Introduction

1. Climate change

Over the past century, the earth's climate has increased by approximately 0.6°C and oceans net heat uptake has approximately been 20 times greater than that by the atmosphere (Levitus et al., 2005).

Expected alterations to physical environment under climate change scenarios include potential sea level rise, increased warming of water temperatures, oceanic circulation disruption and increased frequency and severity of storm occurrences (Petes et al., 2007).

Considering marine ectotherms, the effects can be very pronounced, once temperature is one of the most important factors affecting molecules kinetic energy as well as biochemical reactions (Somero, 1969; Mora and Ospina, 2001). For instance and at a molecular level, temperature affects the biochemical reactions leading to physiological and behavioral changes (Mora and Ospina, 2001), which have pronounced consequences on individual fitness and performance. Considering higher levels of organization, ecosystem changes in response to climate warming include poleward or altitudinal shifts in geographical distribution (Perry et al., 2005; Brander et al., 2003; Grebmeier et al., 2006), population collapse or local extinction (Pörtner and Knust, 2007), large scale migration failure (Farrell et al., 2008), changes in the seasonal timing of biological events (Wiltshire et al., 2004), or even changes in food availability and food web structure.

Dynamic fluctuations of these abiotic variables (e.g. temperature) can interfere and dominate life history, demographics and competition between species (Christian et al., 1983; Huey, 1991; Huey and Berrigan, 2001; Munday et al., 2009; Porter, 1989) explaining to some extent the adaptation diversity among organisms (Lutterschmidt and Hutchison, 1997). Therefore and according to climate change scenarios, it is reasonable to expect inter and intraspecific competition to occur if thermal microhabitat boundaries are narrow. These changes might involve stock redistribution, invasion by exotic species (Bennett et al., 1997; Kimball et al., 2004) and resilience of native or threatened species (Walsh et al., 1998).

In the face of climate change, these issues are particularly important because in order to protect ecosystems there is the need to understand the causal-effect relationship between climatic changes and ecosystem changes. Additionally and in order to address the impacts of climate forcing on marine organisms, thus making realistic predictions for the future, it is essential to know the species current vulnerability status. This vulnerability is dependent not only on thermal limits but also on factors like fishing pressure (once it alters the genetic structure of the population and leads to a fragmentation in the food web - Perry et al., 2010), duplication time, adaptation capacity, regional rate of temperature increase and predicted changes in food availability due to climate forcing (Pörtner and Knust, 2007).

Facing current concerns about climate change scenarios, the knowledge of thermal tolerance is the first step to understand how vulnerable species are (Rivadenera and Fernández, 2005). The tolerance window for each species is described as a favorable range of temperature or performance breadth. It includes an optimal zone and a suboptimal zone. Above or below this range, performance is negatively affected and the species cannot survive unless it occurs for a limited period of time.

To some extent, several studies already show and predict the effects of ongoing changes. At the physiological level, oxygen availability can limit aerobic metabolism and consequently thermal tolerance (Frederich and Pörtner, 2000; Pörtner et al., 2004; Pörtner and Knust, 2007; Melzner et al., 2007; Rosa and Seibel, 2008). As such, ocean warming may decrease oxygen availability and alter organism metabolism, thus leading to aerobic performance decline, with a progressive transition towards an anaerobic mode of energy production (the ‘‘oxygen limitation of thermal tolerance’’ concept (Pörtner and Knust, 2007; Pörtner et al., 2004)). The reduction in aerobic scope is not caused by lower levels of ambient oxygen but through limited capacity of oxygen supply mechanisms (ventilatory and circulatory systems), in order to meet an animal’s temperature-dependent oxygen demand (Pörtner and Knust, 2007). If these previously mentioned parameters are altered by thermal regime, both population survival as well as reproduction, recruitment and structure (Mora and Ospina, 2001) will be affected once temperature has an direct effect over time and frequency of spawning and survival of eggs, larvae and juveniles (IPCC, 2001) but also on temperature-dependent sex determination (Ospina-Alvaréz and Piferrer, 2008). At the behavioral level, changes in reproductive strategies (Angilletta et al., 2006) and life history patterns might also occur.

1.1 Climate change and tidal environments

Coastal marine ecosystems are among the most ecologically important habitats. and they are warming at a much faster rate than many other ecosystems (MacKenzie and Schiedek, 2007). Because many coastal organisms already live close to their thermal tolerance limits (Stillman and Somero, 2000, Helmuth et al., 2006), ocean warming will have a profound and negative impact on their performance and survival.

Shrimps of the genus *Palaemon* (Decapoda; Caridea; Palaemonidae) have a broad distribution. While some are found in the intertidal zone, within seagrass beds or rocky tidal pools (*Palaemon elegans* (Rathke, 1837)), others inhabit subtidal zones, in rocky substrates and submerged caves, up to a depth of 40 m (*Palaemon serratus* (Pennant, 1777)) (Udekem d'Acoz, 1999, Morais et al., 2002). Due to vertical distribution variation, these congener species may experience different patterns of emersion during each tidal cycle.

Studies focused on abiotic stress (e.g. temperature) have shown that species living higher in the intertidal zone generally possess a greater resistance to abiotic factors in comparison to species living in the subtidal zone (Stillman and Somero, 1996). It has also been suggested that warm-adapted species of the intertidal/supratidal zone may be particularly at risk since they live closer to their upper thermal limit (Hopkin et al., 2006; Somero, 2010) with limited scope to tolerate further warming (Pörtner et al., 2004; Somero, 2010; Stillman, 2002). Despite the fact that they are more thermally tolerant, there is a high probability that maximum habitat temperatures surpass their upper thermal limit (Somero, 2010) because they live in a hot and unstable environment with daily and seasonal broad thermal amplitudes.

1.2 Climate change and latitudinal differences

Organisms inhabiting different latitudinal habitats are expected to suffer differently according to temperature. In this manner, studies of congener's species are important as a method to determine which one is more vulnerable to ocean warming.

An example are shrimps of the genus *Lysmata,* once they have a large geographic distribution. While some species occur in dense aggregations at rocky intertidal and/or subtidal temperate and subtropical zones, e.g. *Lysmata seticaudata* (Risso, 1816), others live as socially monogamous pairs in tropical zones, e.g. *Lysmata amboinensis* (De Man, 1888) (Fieldler, 1998).

Because tropical organisms experience far more warm temperatures throughout the year than temperate organisms do, it might be expected that tropical animals have a greater heat tolerance. Surprisingly, that is often not the case - heat tolerance typically varies very little across latitude in ectotherms (Ghalambor et al., 2006; Addo-Bediako et al., 2000; Deutsch et al., 2008).

Most literature has focused on tropical regions, not only because models suggest that impacts will be more severe in the tropics (Tewksbury et al., 2008) but also because predictions for temperate regions are the hardest to make due to the diversity of life history patterns, complexity of trophic relations, habitat variability and over-fishing (IPCC, 1997; Roessig et al., 2004).

2. Molecular mechanisms behind thermal tolerance and adaptation

2.1 Heat shock proteins

Throughout their lifetime, organisms are exposed to several stress factors. Environmental conditions tend to be stressful when they reach values outside the tolerance limits of the organism, causing a decrease in fitness. Fitness is dependent on the capacity of adaptation, which in turn can be related to the maintenance and integrity of the protein pool (Hofmann and Todgham, 2010) through heat shock response (HSR). Het shock response is characterized by the preferential synthesis of a group of proteins, the heat shock proteins (HSP's) (Ritossa, 1962), also called stress response proteins, that are molecular chaperones, helping proteins fold correctly during translation and facilitating their transport across membranes under non-stressful conditions (Frydman, 2001; Hartl and Hayer-Hartl, 2002). Under stressful conditions, molecular chaperones stabilize denaturing proteins and refold proteins that have already been denatured.

The molecular chaperone role of HSP's reflects the fact that protein conformation is a thermally sensitive weak-link in the macromolecular machinery of the cell that contributes to setting thermal tolerance limits (Somero, 2004). The HSR is thus an important biochemical indicator to assess levels of thermal stress and thermal tolerance limits.

This way, stressful conditions induce consequences at the cellular, physiological and individual levels. They can lead to great changes in the metabolic processes, disturbing vital functions and consequently survival, growth, reproduction, biological interactions and ultimately community and ecosystem's structure.

In general, stress response occurs at 3 levels:

Primary response – perception of an altered state and activation of the neuroendocrine/endocrine response, characterized by a rapid production of stress hormones (Iwama, 1999).

Secondary response – includes several physiological and biochemical adjustments and is regulated by stress hormones (adrenaline and cortisol) which activate metabolic pathways, which in turn lead to biochemical and hematological alterations (Barton and Iwama, 1991), changes in the hydromineral balance and cardiovascular, respiratory and immune functions (Barton, 2002). During stressful conditions, organisms mobilize their energy stores in order to provide energy for tissues, to deal with an increased need of energy.

Tertiary response – changes occurring at the organism and population levels, directly linked to the alterations that occurred due to the primary and secondary responses. If the organism cannot acclimate, adapt or maintain homeostasis, several changes may occur: at the behavioral level, resistance to disease, growth and reproduction capacity (Iwama, 1999; Barton, 2002). A severe or prolonged exposure to stress can eventually alter population demographics and dynamics. Impacts can be critical when it comes to larvae and juveniles because growth is of crucial importance to their fitness at these stages. If growth is fast, there are two advantages: there is a lower chance of being predated because a bigger size reduces the range of predators, and the first maturation will occur faster leading to a higher investment in reproduction (in iteroparous species). As such, if growth and reproduction become energetically compromised due to stressful conditions, it is reasonable to expect lower recruitment and production, altering the abundance and diversity of species in a community (Barton, 1997).

It is widely known that heat stress leads not only to ROS production and cellular damage in several components (mitochondria, Golgi complex, cytoskeleton, DNA and proteins – Dubois et al., 1991; Vidair et al., 1996; Snoeckx et al., 2001) but also to a slow-down or even shut-down of most original cellular functions (Csermely and Yahara, 2003). Chaperoning function of HSPs is a mechanism of defense in order to maintain cellular homeostasis. During stress, their production suggests that intrinsic mechanisms of defense have developed in tissues in order to recover or destroy damaged proteins.

Thus, heat shock proteins might be considered indirect biochemical indicators of the degree of damage and protein unfolding that is occurring in the cell (Hofmann, 2005). Studies concerning these proteins may give us clues about the temperature at which species become thermally stressed. Heat Shock Protein levels are related to past thermal history (Hofmann, 2005), thermal regime and its variability occurring in the habitat (Tomanek, 2010), which may partially explain species thermal limits and their resistance/vulnerability to increasing temperature.

2.2 Oxidative stress enzymes and cellular damage (Lipid peroxidation)

Oxygen buildup in Earth's atmosphere has an profound effect over organism´s physiology. Reactive oxygen species (ROS) production is prevalent in the world's oceans, as well as oxidative stress response in marine organisms exposed to a variety of environmental stressors such as thermal stress. The negative effects of ROS must also be balanced by their role in signal transduction, which facilitates processes such as apoptosis, autophagy and necrosis (Lesser, 2012).

Reactive oxygen species (ROS) are responsible for toxic effects of oxygen, and this is because in its ground-state oxygen is a distinctive element, as it is a biradical and has two unpaired electrons in its outer orbit (Asada and Takahashi, 1987; Cadenas, 1989; Fridovic, 1998; Halliwell and Gutteridge, 1999). The univalent reduction of molecular oxygen produces reactive intermediates such as O_2•-, H_2O_2, HO•, and finally water (Halliwell and Gutteridge, 1999).

The production of ROS beyond the capacity of an organism to quench these reactive species, can provoke lipid, protein and DNA damage (Halliwell and Gutteridge, 1999). The primary reason of antioxidant defense in biological systems is to quench O_2 at the site of production, and to quench or reduce the flux of other ROS such as $O_2{\bullet}^-$ and H_2O_2 to ultimately prevent the production of HO•, the most damaging of the ROS (Asada and Takahashi, 1987; Cadenas, 1989; Fridovich, 1998; Halliwell and Gutteridge, 1999).

Significant damage is caused by hydrogen peroxide (H_2O_2) since it's diffusion, within the cell, from its point of synthesis is less restrictive than other forms of ROS and it can enter into numerous other reactions. Exposure to H_2O_2 can damage directly many cellular constituents, such as DNA (Asada and Takahashi, 1987; Cadenas, 1989; Fridovich, 1998; Halliwell and Gutteridge, 1999), despite being also involved in cellular pathways such as programmed cell death or apoptosis (Halliwell and Gutteridge, 1999).

The reaction of ROS with lipids is considered one of the most prevalent cellular injury mechanism (Halliwell and Gutteridge, 1999). Lipid peroxidation involves three well-defined steps: initiation, propagation and termination (Yu, 1994; Halliwell and Gutteridge, 1999), ultimately forming ROO• (peroxyl radical). Degradation products of lipids peroxidation are aldehydes, such as malondialdehyde, and hydrocarbons, ethane and ethylene (Gutteridge and Halliwell, 1990; Freeman and Crapo, 1982). Lipid peroxidation in mitochondria is particularly cytotoxic, with multiple effects on enzyme activity and ATP production as well as on apoptosis initiation (Green and Reed, 1998).

There are several enzymatic antioxidants such as Superoxide dismutase (SOD), Catalase (CAT) and Glutathione S-Transferase (GST), SOD is the first line of defense, against oxidative stress. However, cells are not completely detoxified since it converts $O_2\bullet^-$ into H_2O_2 and oxygen. Because antioxidative enzymes are known to be intrinsically linked and dependent upon the activity of one another, Catalase removes H_2O_2, resulting from SOD activity, avoiding its accumulation in cells and tissues. As for Glutathione S-Transferase, it transforms xenobiotics into other conjugates (Lesser, 2006).

References

Addo-Bediako A, Chown SL, Gaston KJ (2000) Thermal tolerance, climatic variability and latitude. *Proc. R. Soc. Lond. B* 267: 739-745

Angilleta MJ, Oufiero CE, Leaché AD (2006) Direct and indirect effects of environmental temperature on the evolution of reproductive strategies: an information-theoretic approach. Am Nat 168(4): E123-E135.

Asada K, Takahashi M (1987) Production and scavenging of active oxygen in photosynthesis. In *Photoinhibition*, ed. DJ Kyle, CB Osmond, CJ Arntzen, pp. 228-87. Elsevier: Amsterdam.

Barton BA, Iwama GK (1991) Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann Rev Fish Dis 1: 3–26.

Barton BA (1997) Stress in finfish: Past, present, and future—a historical perspective. In Iwama GK, Pickering AD, Sumpter JP, Schreck CB (eds) Fish stress and health in aquaculture, pp.1-34. Cambridge University Press, Cambridge.

Barton BA (2002) Stress in Fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integ Comp Biol 42: 517–525.

Bennett W, Currier RJ, Beitinger TL (1997) Cold tolerance and potential overwinter of red-bellied piranha, Pygocentrus nattereri, in the United States. T Am Fish Soc 126(5): 841-849.

Brander, K.M.; Blom, G.; Borges, M.F.; Erzini, K.; Henderson, G.; MacKenzie, B.R.; Mendes, H.; Santos, A.M.P.; Toresen, P. (2003). Changes in fish distribution in the eastern North Atlantic: are we seeing a coherent response to changing temperature? ICES Mar. Sci. Symp. 219: 260-273

Cadenas E (1989) Biochemistry of oxygen toxicity. Annu. Rev. biochem. 58: 79-110.

Christian, K., Tracy, C.R., Porter, W.P., 1983. Seasonal shifts in body temperature and use of microhabitats by Galapagos land iguanas (*Conolophus pallidus*). Ecology 64, 463–468.

Csermely P, Yahara I (2003) Heat shock proteins. In: Keri G, Toth I (eds) Molecular pathomechanisms and new trends in drug research, Taylor & Francis Inc., London, pp 67-75.

Deutsch AC, Tewksbury JJ, Huey RB, Sheldon KS, Ghalambor CK, Haak D, Martin PR (2008) Impacts of climate warming on terrestrial ectotherms across latitude. PNAS 105: 6668-6672

Donelson JM, Munday PL, McCormick MI, Nilsson GE (2011) Acclimation to predicted ocean warming through developmental plasticity in a tropical reef fish. Global Change Biol 17: 1712–1719.

Dubois MF, Hovanessian AG, Bensaude O (1991) Heat shock-induced denaturation of proteins. J Biol Chem 266: 9707–9711.

Edwards M, Richardson AJ (2004) Impact of climate change on marine pelagic phenology and trophic mismatch. Nature 430: 881–884.

Farrell AP, Hinch SG, Cooke SJ, Patterson DA, Crossin GT, Lapointe M, Mathes MT (2008) Pacific Salmon in Hot Water: Applying Aerobic Scope Models and Biotelemetry to Predict the Success of Spawning Migrations. Physiological and Biochemical Zoology 81: 697-708

Fiedler GC (1998) Functional, simultaneous hermaphroditism in female-phase *Lysmata amboinensis* (Decapoda: Caridea: Hippolytidae). Pac Sci 52: 161-169.

Frederich M, Pörtner HO (2000). Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in the spider crab *Maja squinado*. Am J Physiol 279: R1531–R1538.

Freeman BA, Crapo JD (1982) Biology of disease, free radicals and tissues injury. Lab. Invest. 47: 412-26.

Fridovich I (1998) Oxygen toxicity: a radical explanation. J. exp. Boil. 201: 1203-9.

Frydman J (2001) Folding of newly translated proteins in vivo: the role of molecular chaperones. Annu. Rev. Biochem. 70, 603-649.

Ghalambor C, Huey RB, Martin PR, Tewksbury JJ, Wang G (2006) Are mountain passes higher in the tropics? Janzen's hypothesis revisited. Integrative and Comparative Biology 46: 5-17.

Grebmeier MJ, Overland EJ, Moore ES, Farley VE, Carmack CE, Cooper WL, Frey EK, Helle HJ, McLaughlin AF, McNutt LS (2006) A Major Ecosystem Shift in the Northern Bering Sea. Science 311: 1461-1463

Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309-12.

Gutteridge JMC, Halliwell B (1990) The measurement and the mechanism of lipid peroxidation in biological systems. Trends Biochem. Sci. 15: 129-35.

Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine. New York: Oxford Univ. Press. 936 pp.

Hartl FU, Hayer-Hartl M (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295, 1852-1858.

Helmuth B, Mieszkowska N, Moore P, Hawkins S (2006) Living on the edge of two changing worlds: forecasting the responses of rocky intertidal ecosystems to climate change. Annual Review of Ecology Evolution and Systematics 37: 373–404.

Hofmann GE, Todgham AE (2010) Living in the now: physiological mechanisms to tolerate a rapidly changing environment. Annu Rev Physiol 72: 22.1–22.19.

Hopkin RS, Qari S, Bowler K, Hyde D, Cuculescu M (2006) Seasonal thermal tolerance in marine Crustacea. Journal of Experimental Marine Biology and Ecology 331, 74–81.

Huey, R., 1991. Physiological consequences of habitat selection. American Naturalist 137, S91–S115.

Huey, R.B., Berrigan, D., 2001. Temperature, demography, and ectotherm fitness. American Naturalist 158, 204–210.

IPCC (1997) The Regional Impacts of Climate Change: An Assessment of Vulnerability. Cambridge University Press, UK.

IPCC (2001) Climate change 2001: impacts, adaptation and vulnerability. Contribution of Working Group II to the Third Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, USA.

Iwama GK, MM Vijayan, RB Forsyth, PA Ackenrian (1999) Heat shock proteins and physiological stress in fish. Am Zool 39: 901-909.

Kimball ME, Miller JM, Whitfield PE, Hare JA (2004) Thermal tolerance and potencial distribution of invasive lionfish (Pterois volitans/miles complex) on the east coast of the United States. Mar Ecol Prog Ser 283: 269-278.

Lesser MP (2006) Oxidative stress in marine environments: biochemistry and physiological ecology. Ann Rev Physiol 68: 253–278.

Levitus S, Antonov J, Boyer T (2005) Warming of the world ocean, 1955–2003. Geophys Res Lett 32.

Lutterschmidt WI, Hutchison VH (1997) The critical thermal maximum: history and critique. Can J Zool 75(10): 1561-1574.

MacKenzie BR, Schiedek D (2007) Daily ocean monitoring since the 1860 s shows record warming of northern European seas. Global Change Biol 13: 1335–1347.

Meehl GA, Stocker TF, Collins WD, others (2007) Global climate projections. In: Solomon S, Qin D, Manning M, eds. Climate Change 2007: The Physical Science Basis Cambridge: Cambridge University Press. pp 686–688.

Melzner, F; Mark, FC & Pörtner, H. (2007). Role of blood oxygen transport in thermal tolerance of the cuttlefish, *Sepia officinalis*. Integr Comp Biol 47(4): 645-655.

Mora C, Ospina F (2001) Thermal tolerance and potential impact of sea warming on reef fishes from Gorgona island (eastern Pacific Ocean). Mar Biol 139:765-769.

Morais S, Narciso L, Calado R, Nunes ML, Rosa R (2002) Lipid dynamics during the embryonic development of *Plesionika martia* (Decapoda; Pandalidae), *Palaemon serratus* and *Palaemon elegans* (Decapoda; Palaemonidae): relation to metabolic consumption. Mar Ecol Prog Ser 242: 195-204

Munday PL, Crawley N, Nilsson GE (2009) Interacting effects of elevated temperature and ocean acidification on the aerobic performance of coral reef fishes. Mar Ecol Prog Ser 388: 235–242.

Ospina-Álvarez N, Piferrer F (2008) Temperature-dependent sex determination in fish revisited: prevalence, a single sex-ratio response pattern, and possible effects of climate change. PLoS ONE 3(7): e2837.

Perry AL, Low PJ, Ellis JR, Reynolds JD (2005) Climate change and distribution shifts in marine fishes. Science 308: 1912–1915.

Porter, W.P., 1989. New animal models and experiments for calculating growth potentials at different elevations. Physiological Zoology 62, 286–313.

Pörtner HO, Mark FC, Bock C (2004) Oxygen limited thermal tolerance in fish? Answers obtained by nuclear magnetic resonance techniques. Resp. Physiol Neurobiol 141: 243–260

Pörtner HO, Knust R (2007) Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science 315: 95–97.

Pörtner HO, Bock C, Knust R, Lannig G, Lucassen M, Mark FC, Sartoris FJ (2008) Cod and climate in a latitudinal cline: physiological analyses of climate effects in marine fishes. Climate Research 37, 253–270.

Ritossa F (1962) A new puffing pattern induced by temperature shock and DNP in *Drosophila*. Experimentia 18-571.

Roessig JM, Woodley CM, Cech JJ, Hansen LJ (2004) Effects of global climate change on marine and estuarine fishes and fisheries. Reviews in Fish Biology and Fisheries 14, 251–275.

Rosa R, Seibel BA (2008) Synergistic effects of climate-related variables suggest future physiological impairment in a top oceanic predator. Proc Natl Acad Sci USA 105: 20776–20780.

Snoeckx LEH, Cornelussen RN, van Nieuwenhoven FA, Reneman RS, van der Vusse GJ (2001) Heat Shock Proteins and Cardiovascular Pathophysiology. Physiol Rev 81(4):1461-1485.

Sokolova IM, Sukhotin AA, Lannig G (2011) Stress effects on metabolism and energy budgets in mollusks. In: Abele D, Zenteno-Savı´n T, Vazquez-Medina J, eds. Oxidative stress in aquatic ecosystems. Chichester, UK: John Wiley & Sons. pp 261–280.

Somero GN (1969) Enzymic mechanisms of temperature compensation: immediate and evolutionary effects of temperature on enzymes of aquatic poikilotherms. American Naturalist 103, 517.

Somero GN (2004) Adaptation of enzymes to temperature: searching for basic ‘strategies’. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 139, 321-333.

Somero GN (2010) The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine ‘winners’ and ‘losers’. Journal of Experimental Biology 213, 912–920.

Stillman JH, Somero GN (2000) A comparative analysis of the upper thermal tolerance limits of eastern pacific porcelain crabs, genus *Petrolisthes*: influences of latitude, vertical zonation, acclimation, and phylogeny. Physiol Biochem Zool 73: 200–208.

Stillman JH (2002) Causes and consequences of thermal tolerance limits in rocky intertidal porcelain crabs, Genus *Petrolisthes*. Integrative and Comparative Biology 42, 790–796.

Tewksbury JJ, Huey RB, Deutsch CA (2008) Putting the heat on tropical animals. Science 320, 1296–1297.

Thomas CD, Cameron A, Green RE, Bakkenes M, Beaumont LJ, et al. (2004) Extinction risk from climate change. Nature 427: 145–148.

Tomanek L (2010) Variation in the heat shock response and its implication for predicting

Udekem d'Acoz C (1999) Inventaire et distribution des crustacés décapodes de l'Atlantique nord-oriental, de la Méditerranée et des eaux continentales adjacentes au nord de 25ºN. Patrimoines naturels (Mus Natl His Nat/ Serv Patrimoine Nat) 40: 1-383.

Vidair CA, Huang RN, Doxsey SJ (1996) Heat shock causes protein aggregation and reduces protein solubility at the centrosome and other cytoplasmic locations. Int J Hypertherm 12: 681–695.

Walsh SJ, Haney DC, Timmerman CM, Dorazio RM (1998) Physiological tolerances of juvenile robustred horse, *Moxostoma robustum*: conservation implications for animperiled species. Environ Biol Fishes 51: 429–444.

Witshire KH, Manly BF (2004) The warming trend at Helgoland Roads, North Sea: phytoplankton response. Helgol. Mar. Res. 58: 269-273.

CHAPTER 1

Heat tolerance limits and physiological responses of *Palaemon* shrimps to ocean warming

Abstract

The present work aimed to determine the upper thermal limits (LT_{50} and LT_{100}) of shrimps from the genus *Palaemon*, inhabiting intertidal (*Palaemon elegans*) and subtidal (*Palaemon serratus*) zones. Moreover, we investigate i) the expression of two molecular chaperones, heat shock cognate 70 (HSC70) and heat shock protein 70 (HSP70), ii) metabolic rates, iii) lipid peroxidation (indicator of cellular damage) and iv) activity of several antioxidant enzymes. It was found that intertidal species (*Palaemon elegans*) had higher thermal tolerance (LT_{50}) than subtidal congener, which was followed by higher HSC70/ HSP70 concentrations, as well as higher SOD, GST and CAT activities. Although there is some previous evidence that species inhabiting intertidal/supratidal zone may be at greater risk of global warming since they live closer to their upper thermal limit, the present findings suggest that they do not show yet such limited scope to tolerate further warming.

Key words: Thermal stress, *Palaemon serratus*, *Palaemon elegans*, shrimps, metabolism, respiratory enzymes, heat shock proteins, tidal zones

Introduction

During the past century, global temperatures have risen 0.6°C and are predicted to continue rising by 1.4-5.8°C over the next century (Houghton et al, 2001; Petes et al, 2007).

Temperature is one of the most important factors affecting organisms because it impacts the kinetic energy of molecules and biochemical reactions (Kennedy et al., 2002). Dynamic fluctuations of these abiotic variables can interfere and dominate life history, demographics and interspecies competition (Madeira et al, 2012), which could, to some extent, result in local if not total extinction of some species. In this manner, mobile species should be able to adjust their geographic distribution over time, while less mobile species may not (Kennedy et al, 2002).

Facing current concerns about climate change scenarios, the knowledge of thermal tolerance is the first step to understand how vulnerable species behave. However, not only there is a great diversity of responses but also global warming tends to vary regionally (Rivadeneira and Fernández, 2005; Madeira, 2012).

Additionally, when thermal stress is present, heat-shock proteins (HSPs) synthesis is initiated in order to promote refolding of denatured proteins and prevent further protein unfolding and aggregation (Dong et al., 2008; Morimoto and Santoro, 1998; Feder and Hofmann, 1999).

Another problem associated with ocean warming is oxidative stress. The production and accumulation of reactive oxygen species (ROS) beyond the organism capacity to quench them, can damage lipids, proteins and DNA (Lesser, 2006).

Under most physiological states, ROS production is closely matched by antioxidant responses (Rosa et al, 2012). Enzymatic antioxidants, such as superoxide dismutases, catalase and peroxidases, form an important part of the antioxidant response (Lesser, 2006).

The coastal margin is an environmentally dynamic habitat that experiences marked fluctuations in air and water temperature on a daily basis, as a consequence of tidal exchange (McElroy et al., 2012; Somero, 2010; Tomanek, 2010). The physiological tolerance of intertidal biota has long been focused by research (Somero, 2002; Pörtner et al., 2005).

The rocky intertidal zone is an ideal model system for examining the effects of environmental stress (Petes et al., 2007), once is one of the most physically harsh environments on earth. Within this habitat, environmental conditions range from fully aquatic to fully terrestrial over vertical distances of a few meters or less. Temperature and desiccation potential change seasonally and daily, depending on the tidal cycle and ambient weather conditions (Somero et al., 2008).

Intertidal organisms are routinely exposed to large variations in temperature and have evolved in terms of physiological tolerance to abiotic stresses, whose adaptations are important in determining the maximal vertical distributions of intertidal species (Stillman and Somero, 2000).

Studies of congener’s species allow to predict the effects of increasing temperatures, in order to establish which will be more vulnerable (Whiteley et al., 2011; Stillman, 2003; Stillman, 2010). As an example are shrimps from the genus *Palaemon*. While some species occur in the intertidal zone, in sea grass beds or in rocky tidal pools (up to a depth of 5 m), e.g. *Palaemon elegans* (Rathke, 1837), others inhabit subtidal zones, in rocky substrates with algae and zostera or in dark caves (up to a depth of 40 m), e.g. *Palaemon serratus* (Pennant, 1777) (Morais et al, 2002).

The aim of our study was to investigate heat tolerance limits (% survival, LT_{50}) and physiological responses (metabolic rates, heat shock proteins, lipid peroxidation and oxidative stress enzymes) of intertidal (*Palaemon elegans*) and subtidal (*Palaemon serratus*) caridean shrimps, in an ocean warming scenario.

Materials and methods

Specimen Collection and Maintenance

Specimens of *Palaemon elegans* and *Palaemon serratus* were collected in intertidal and subtidal zones at Cabo Raso (Cascais, Portugal) using dip nets and scuba diving, respectively. After collection, *Palaemon* spp. specimens where transferred to the aquaculture facilities in Laboratório Marítimo da Guia (Cascais, Portugal). Organisms were placed in two recirculating aquaculture systems (RAS) at 12°C (winter temperature of their habitat) and 20°C (summer temperature), for a time period of 30 days. During this time ammonia and nitrite levels were monitored daily and kept below detectable levels. Salinity and pH were kept at 34.0 ± 1.0 and 8.1 ± 0.1, respectively.

Thermal Tolerance Limits

The upper thermal tolerance limits of each species were determined according to Stillman and Somero (1996). One hundred and sixty shrimps, consisting in 80 individuals of each species and temperature, where placed into separated plastic containers, each containing 20 specimens comprising 3 replicates and a control for each species. Afterwards an acclimation temperature period was performed during 30–60 min. Thereafter, temperature was increased at a rate of 1°C/30 min. Every 30 min, aeration was provided to experimental beakers containing shrimps and temperature in each container checked (with thermocouple probes). Sensory antennules activity of each shrimp was visually monitored until they reached the end-point, in which no sensory antennules activity was noticed. The temperature at which 50% of the shrimps have died was calculated (LT_{50}), and used as the measure for the upper thermal tolerance limits. Additionally, the temperature at which 100% of the shrimps have died was used to determinate the maximal temperature these specimens can withstand and consequently survive.

Oxygen Consumption Rates and Thermal Sensitivity

Oxygen consumption measurements were determined according to Rosa et al. 2008, 2009; Pimentel et al, 2012, by individual shrimps incubation in sealed water-jacketed respirometry chambers (RC300 Respiration cell, Strathkelvin, North Lanarkshire, Scotland) containing filtered seawater (35 PSU), at the different incubation temperatures (winter temperature (12°C) and summer temperature (20°C)). Water volumes were adjusted in relation to animal mass (up to 250 mL) to avoid stress. Respiration chambers were placed in a temperature controlled water bath (Lauda, Lauda-Konigshofen, Germany). Oxygen concentrations were recorded with Clarke-type O2 electrodes connected to a multichannel oxygen interface (Strathkelvin 928, North Lanarkshire, Scotland), during 3 to 4 hours. Thermal sensitivity (Q_{10}) was determined using the standard equation:

$$Q10 = \left[\frac{R(T2)}{R(T1)}\right]^{\frac{10}{T2-T1}}$$

Where $R(T_2)$ and $R(T_1)$ represent the oxygen consumption rates at temperatures T_2 and T_1, respectively.

Preparation of tissues extracts

Approximately 100 mg of shrimp abdominal tissue was homogenized in 300 µl of homogenization buffer (Phosphate Buffer Saline solution, PBS, pH 7.3: 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4), using a glass Potter Elvejhem tissue grinder. Consequently, homogenates were centrifuged (20 min at 14 000 x g, 4°C) and enzyme activities and heat shock proteins were measured in the supernatant fraction.

All samples were analyzed for Glutathione S-Transferase, Catalase, Superoxide Dismutase, Lipid Peroxidation (MDA concentration) and HSP70/HSC70 levels.

Heat shock response (HSP70/HSC70)

HSP70/HSC70 content was assessed by Enzyme-Linked Immunosorbent Assay (ELISA), adapted from Njemini et al. (2005). Briefly, 10 µl of the homogenate's supernatant was diluted in 250 µl of PBS and 50 µl of the diluted sample was added to 96 well microplates (Nunc-Roskilde, Denmark) and allowed to incubate overnight at 4°C. Afterwards, microplates were washed three times in PBS 0.05% Tween-20 and 100 µl of blocking solution (1% BSA, Bovine Serum Albumin, Sigma-Aldrich, USA) was added to each well and left to incubate at room temperature for 2 hours. Following microplates washing (3x with 0.05% PBS-Tween-20), 50 µl of 5 µg mL^{-1} primary antibody (anti-HSP70/HSC70, Acris USA), detecting 72 and 73 kDa (molecular mass of inducible HSP70 and HSC70, respectively) proteins was added to each well and incubated overnight at 4°C. The non-linked antibody was removed from microplates by repeating the washing step (3X) which were then incubated for 2 hours at room temperature with 50 µl of 1 µg mL^{-1} of the secondary antibody, anti-mouse IgG, Fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA. After another wash, 100 µl of substrate (SIGMA FAST™ p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA) was added to each well and incubated 30 min at room temperature. Stop solution (3N NaOH) was then added (50 µl) to each well and then the absorbance read at 405 nm in a 96 well microplate reader (BIO-RAD, Benchmark, USA). Hsp70/Hsc70 sample quantifications were calculated by constructing a calibration curve obtained based on serial dilutions of purified HSP70 active protein (Acris, USA) to give a 0 to 2000 ng/mL range. The results were divided by the total amount of protein in the sample determined by the Bradford method (Bradford, 1976) and expressed as micrograms of total protein (ng hsp70/hsc70 mg^{-1} total protein).

Lipid peroxidation (determination of malondialdehyde, MDA)

Lipid peroxidation assay was adapted from the thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara, 1978) and it was determined by the quantification of a specific end-product of the oxidative degradation process of lipids (malondialdehyde (MDA)) in which thiobarbituric acid reacts with MDA yielding a fluorescent product that was detected spectrophotometrically (532 nm). Briefly, 5 µl of each sample were treated with 12.5 µl of dodecyl sulfate sodium (SDS 8.1%), 93.5 µl of trichloroacetic acid (20%, pH 3.5), and 93.5 µl of thiobarbituric acid (1%). Added to this mixture was 50.5 µl of Milli-Q grade ultrapure water and eppendorf's were insert in a vortex (30 s) and incubated in boiling water for 10 minutes. Then, they were placed on ice for 3 minutes to lower the temperature. Afterwards, 62.5 µL of Milli-Q water and 312.5 µL of n-butanol pyridine (15:1, v/v) were added and eppendorf's were placed in a vortex and centrifuged at 7000 rpm for 5 min. Duplicates of 150 µL of the supernatant of each reaction were inserted into a 96-well microplate and the absorbance read. To quantify lipid peroxides, an eight-point calibration curve (0-0.3 µM TBARS) was calculated using malondialdehyde bis (dimethylacetal) standards (Merck).

The results were divided by the total amount of protein in the sample calculated by the Bradford method (Bradford, 1976) and expressed as micrograms of total protein (nmol/mg total protein).

Enzymatic Analyses

1. Glutathione S-Transferase

Glutathione S-Transferase activity was determined spectrophotometrically (340 nm) in the supernatant, every 6 minutes, using a microplate reader (BIO-RAD, Benchmark, USA). Briefly, total GST activity was determined as described by Habig et al. (1974) by measuring the formation of the conjugate of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). Briefly, 180 µL of substrate solution (Dulbecco's Phosphate Buffered Saline with 200 mM L-glutathione reduced and 100 mM CDNB all from Sigma-Aldrich, Germany) were added to 20 µL of GST standard or sample into each well of a 96-well microplate. The total enzyme activity was determined at 340 nm by recording the absorbance at every minute for 6 minutes, using a microplate reader (BioRad Benchmark, USA). Equine liver GST (Sigma-Aldrich, Germany) was used as standard and positive control. The change in absorbance per minute (ΔA340) was estimated and the reaction rate at 340 nm was determined using CDNB extinction coefficient of 0.0096 µM-1cm-1. The results are expressed in relation to total protein concentration of the sample (nmol min^{-1} mg^{-1} total protein).

2. Determination of Catalase

A spectrophotometric method was applied, which consisted in measuring the absorbance at 240 nm (Aebi, 1983; Li and Schellhorn, 2007), in a time interval of 1 min 30 s, when the sample was added to hydrogen peroxide. The reaction is followed by a decrease in absorbance as the peroxide is transformed into oxygen and water. In order to perform this reaction 100 µl of each sample were added to 2900 µl of hydrogen peroxide (50 nmol/L potassium phosphate buffer (pH 7.0), 12.1 mmol/L H_2O_2) using quartz cuvettes. Peroxide consumption was monitored by reading the absorbance every 15 seconds. Standard catalase activity was measured using bovine catalase solution (Sigma-Aldric, Germany) of 1523.6 U/mL. Catalase activity was calculated using an absorption coefficient for H2O2 of 0.04 mmol^{-1} cm^{-1}, using the following equation:

$$\left(\frac{\Delta A240\ min^{-1}}{0.04}\right) \times \left(\frac{total\ volume}{sample\ volume}\right)$$

The results were divided by the total amount of protein in the sample calculated by the Bradford method (Bradford, 1976) and expressed as micrograms of total protein (nmol min^{-1} µg^{-1}).

3. Superoxide dismutase (SOD)

Superoxide Dismutase activity was determined spectrophotometrically (BIO-RAD, Banchmark, USA) in the supernatant at 550 nm. The adapted assay contained, at 25°C, 50 mM Potassium Phosphate Buffer (pH 7.8), 3 mM EDTA, 3 mM Xantine solution, 0.75 mM NBT (nitroblue tetrazolium), 100 mU XOD (Xanthine Oxidase Solution) and 1 U/µL SOD Enzyme solution all from Sigma-Aldrich (Germany). Superoxide Dismutase from bovine erythrocytes (Sigma-Aldrich, Germany) was used as standard and positive control. Enzymatic assay results are given in units of SOD activity per milligram of total protein in the sample (U mg^{-1} total protein), where one unit of SOD is defined as the amount of sample causing 50% inhibition of NBT reduction.

Statistical Analyses

A two-way analysis of variance (two-way ANOVA) was performed to test the effects of species and temperature on metabolism.

Three-way ANOVA’s were conducted in order to detect and analyze significant differences in HSP70/HSC70, GST, MDA, CAT and SOD contents between species, temperature and incubation time. Subsequently, post-hoc tests (Tukey HSD) were performed. All statistical analyses were performed for a significant level of 0.05, using Statistica software (version 10.0, StatSoft Inc., Tulsa, USA).

Results

Thermal tolerance limits

Thermal tolerance experiments showed that upper thermal tolerance limits were affected by acclimation temperature (Figure 1). Both LT_{50} and LT_{100} of *P. elegans* and *P. serratus* were higher after acclimation at 20°C than after acclimation at 12°C. LT_{50} and LT_{100} values for *P.elegans* were 34°C and 35°C at 12°C, and 37°C and 38°C at 20°C, respectively. While for *P. serratus* LT_{50} values were 33°C and 35°C, at 12°C and 20°C, respectively, and LT_{100} values were 34°C and 36°C, at 12°C and 20°C, respectively.

The acclimation temperature positively influenced both LT_{50} and LT_{100}, and such percent of increase were significantly higher in *P. elegans* than those observed for *P. serratus* (Figure 1).

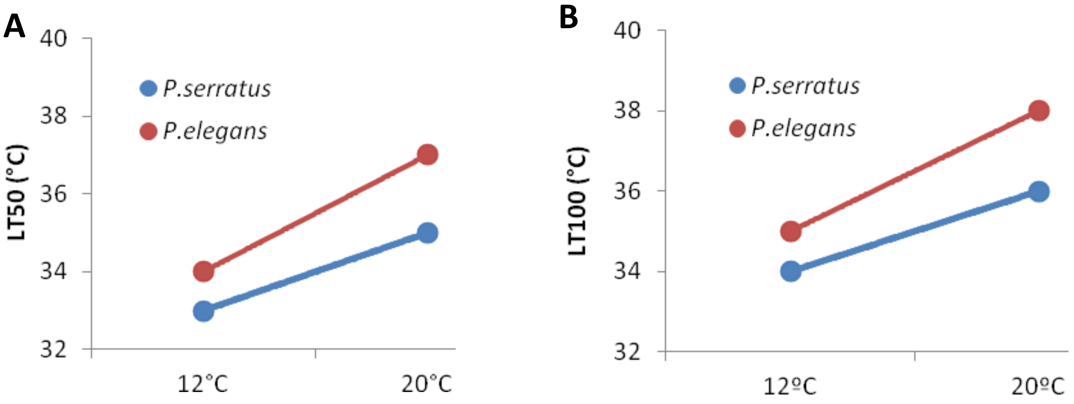

Figure 1. Thermal tolerance limits (A – LT50, °C; B – LT100, °C) of intertidal and subtidal shrimps, *P. elegans* and *P. serratus* respectively, at different temperature scenarios. Values represent means of triplicate experiments ± SD (=zero).

Metabolic Rates

Routine metabolic rates (RMR) were found to be significantly affected ($p < 0.05$) by temperature (Figure 2). *Palaemon elegans* displayed RMR values ranging from 9.0 µmol O_2/h/g at winter temperature (12°C) and 16.6 µmol O_2/h/g at summer temperature (20°C), while *Palaemon serratus* displayed lower RMR values than *P. elegans*, ranging from 8.6 µmol O_2/h/g at winter temperature (12°C) and 15.0 µmol O_2/h/g at summer temperature (20°C). There were no interspecific differences ($p > 0.05$). The same was observed for Q_{10} values, which were quite similar, ranging from around 2.14 for *P. elegans*, to 1.99, for *P. serratus*. It is worth noting that at normal operating temperatures, metabolic demand for oxygen increases with temperature with Q_{10} around 2-3.

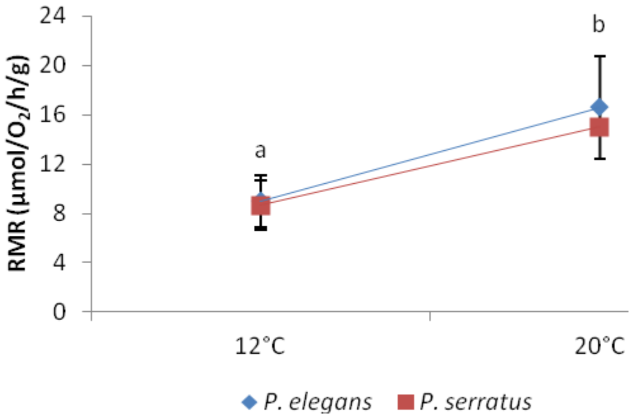

Figure 2. Routine metabolic rates (RMR, µmol/O_2/h/g) of *P. elegans* and *P. serratus* at the different temperature scenarios. Values are mean ± SD. Colored lines represent trendlines and different letters represent significant differences between temperatures. For more statistical details see Table 1.

Heat shock response and Lipid peroxidation

HSP were found to be significantly affected by temperature ($p < 0.05$) (Figure 3), being it’s expression higher at 20°C, for both studied species. Interestingly, compared to the winter condition, there was a significant augment in the HSP levels of *P. elegans* at the summer conditions, but stable during the three periods of incubation.

Regarding lipid peroxidation, i.e. MDA accumulation, there was a significant different between species ($p < 0.05$); MDA production was higher in *P. serratus*, mainly at 20°C (Figure 4).

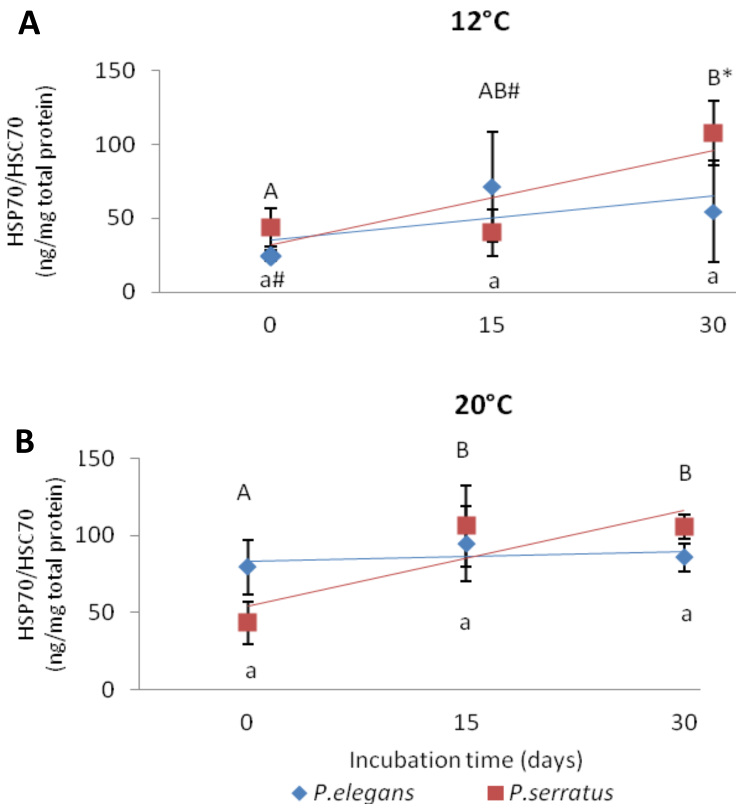

Figure 3. Impact of environmental warming on the HSP70/HSC70 concentrations (ng/mg total protein) of *P. elegans* and *P. serratus*. A) at 12°C; B) at 20°C. Values represent mean ± S.D. Colored lines represent trendlines and different letters (capital letters for *P. serratus* and lowercase letters for *P. elegans)*, asterisks and cardinals represent significant differences between incubation time, species and temperature scenarios. For more statistical details see Table 1.

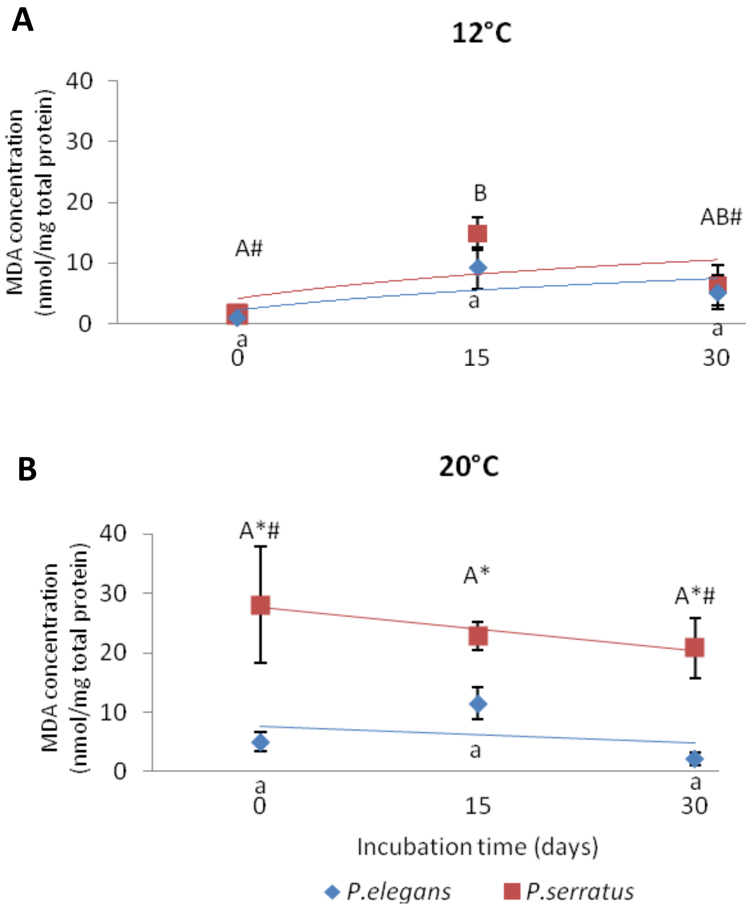

Figure 4. Impact of environmental warming on the MDA concentrations (nmol/mg total protein) of *P. elegans* and *P. serratus*. A) at 12°C; B) at 20°C. Values represent mean ± S.D. Colored lines represent trendlines and different letters (capital letters for *P. serratus* and lowercase letters for *P. elegans)*, asterisks and cardinals represent significant differences between incubation time, species and temperature scenarios. For more statistical details see Table 1.

Oxidative Stress tolerance

Glutathione S-Transferase (GST) activity varied significantly between species, being higher in *P. serratus*, mainly at 20°C (Figure 5).

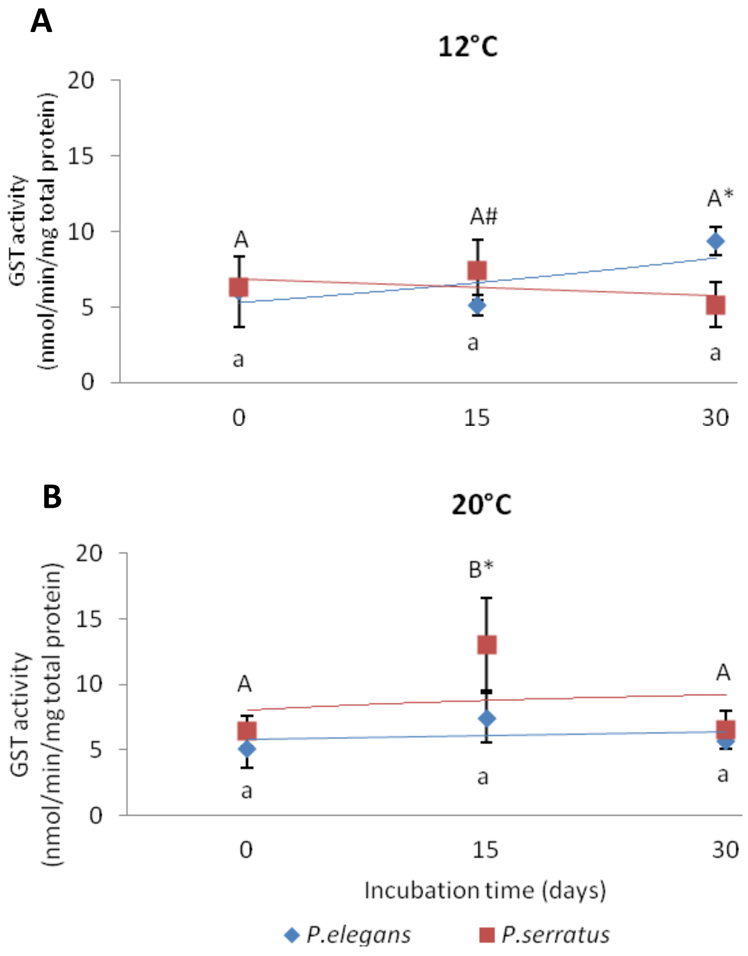

Figure 5. Impact of environmental warming on the GST concentrations (nmol/min/mg total protein) of *P. elegans* and *P. serratus*. A) at 12°C; B) at 20°C. Values represent mean ± S.D. Colored lines represent trendlines and different letters (capital letters for *P. serratus* and lowercase letters for *P. elegans)*, asterisks and cardinals represent significant differences between incubation time, species and temperature scenarios, respectively. For more statistical details see Table 1.

Regarding catalase, differences were found between the interaction species and temperatures ($p < 0.05$) for *P. serratus*, in which catalase activity was higher at 20°C (Figure 6), however there were no significant differences for *P.elegans*, across the thermal gradient.

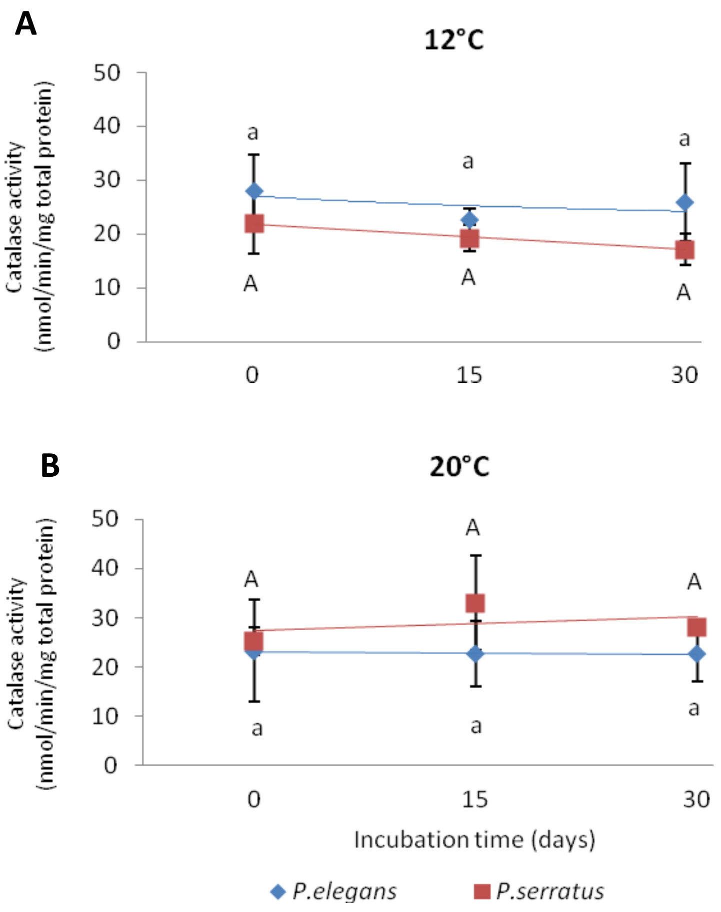

Figure 6. Impact of environmental warming on the Catalase concentrations (nmol/min/mg total protein) of *P. elegans* and *P. serratus*. A) at 12°C; B) at 20°C. Values represent mean ± S.D. Colored lines represent trendlines and different letters (capital letters for *P. serratus* and lowercase letters for *P. elegans)*, asterisks and cardinals represent significant differences between incubation time, species and temperature scenariosFor more statistical details see Table 1.

Regarding SOD activity, there were significant interspecific differences ($p < 0.05$); the production of this enzyme was greater for *P. elegans* than for *P. serratus*. However, for *P. elegans*, it's activity didn't change through the thermal gradient, while for *P. serratus* SOD activity was higher at 20°C (Figure 7).

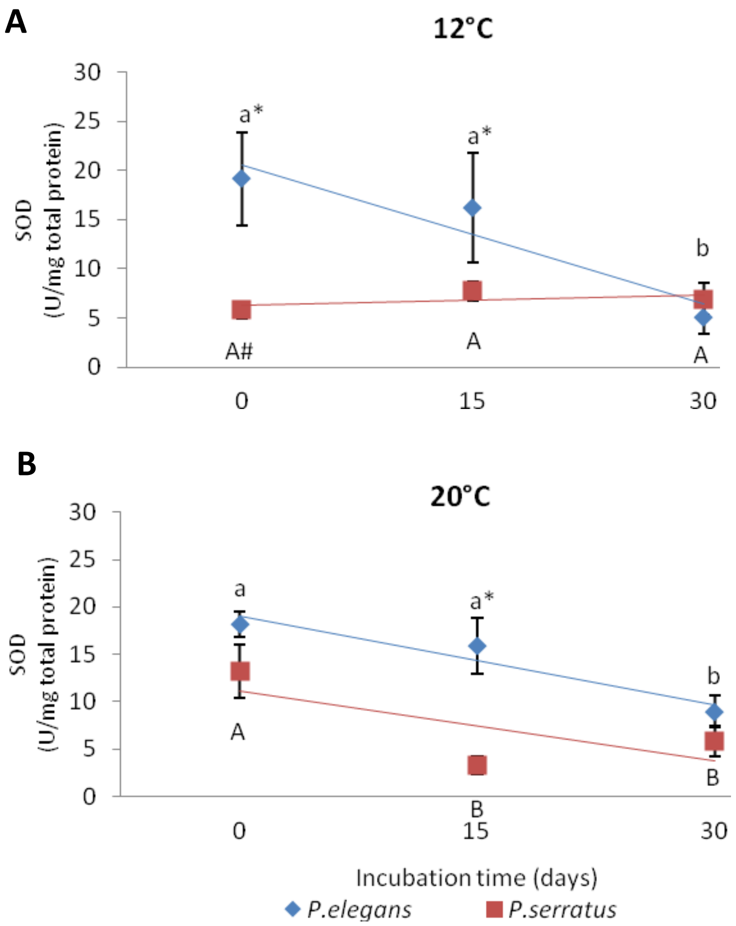

Figure 7. Impact of environmental warming on SOD concentrations (U/mg total protein)of *P. elegans* and *P. serratus*. A) at 12°C; B) at 20°C. Values represent mean ± S.D. Colored lines represent trendlines and different letters (capital letters for *P. serratus* and lowercase letters for *P. elegans)*, asterisks and cardinals represent significant differences between incubation time, species and temperature scenariosFor more statistical details see Table 1.

Discussion

Global warming may affect the distribution limits of intertidal species to a greater extent than for subtidal species (Stillman and Somero, 2000). Our results, as for other studies (e.g. Madeira et al., 2012;Danport and Davenport, 2005; Davenport and McAlister, 1996) follow the idea that organisms living in higher shore (intertidal zone) are more tolerant than those in the lower shore (subtidal zone) (Figure 1). Yet, the upper thermal tolerance limits of some intertidal species may be near current habitat maximum temperature. Here, the results showed that *Palaemon elegans* inhabit an extremely variable habitat in terms of temperature, salinity and dissolved oxygen ,will be more tolerant to global warming then their subtidal congener, *Palaemon serratus*. Subtidal species have a lower tolerance to temperature rising because they live in a relatively variable or more stable habitat, so they are exposed to lower temperatures, having physiological set-points lower in the temperature gradient (Davenport & Davenport, 2005; Davenport & McAlister, 1996). This indicates that species have adapted their upper thermal tolerance limits to coincide with microhabitat conditions.

Additionally and as expected, increased temperatures led to higher metabolic rates (Figure 2) (Pörtner and Knust, 2007) which were quite similar for the two species. As temperature risen similar Q_{10} values were obtained ranging from 2.14 for *P. elegans*, to 1.99 for *P. serratus.*

Our results, also shows that heat shock proteins were very sensitive to environmental stress (Figure 3), once both species expressed higher HSC70/HSP70 concentrations in summer temperature scenario (20°C). Heat shock proteins are a class of functionally related proteins involved in the folding and unfolding of other proteins and their expression increases when cells are exposed to elevated temperatures or other stress (Tomanek, 2010). Concomitantly, warming also lead to an increase of MDA concentrations, mainly for *P. serratus* (Figure 4), indicative of an enhancement of ROS action in organism’s lipids, called peroxidation, a process considered to be one of the most frequent cellular injury mechanisms (Lesser, 2006).

The antioxidative enzymes are known to be intrinsically linked and dependent upon the activity of one another, and therefore, one would expect to see correlative changes in their activity (Cooper et al., 2005). This effect was evident for *P. serratus* in summer warming scenario. In fact, increased MDA concentrations led to higher SOD activity (Figure 7) which indicates a superoxide production increase. At the same time, the increased SOD activity lead to higher GST and CAT activity (Figure 5 and 6, respectively), which catabolizes peroxide resulting from its activity.

In conclusion, ocean warming is expected to drive much more profound biological impacts on *Palaemon serratus*, once they have evolved in a relative stable and aseasonal environment when compared to *Palaemon elegans*.

References

Aebi HE (1983) Catalase. In: Bergmeyer HU, Bergmeyer J, Grassl JM (Eds) Methods of enzymatic analysis. VCH, Weinheim, pp. 273-286.

Cooper RU, Clough LM, Farwell MA, West TL (2002) Hypoxia-induced metabolic and antioxidant enzymatic activities in the estuarine fish *Leiostomus xanthurus*. J Exp Mar Biol Ecol 279: 1-20.

Davenport J, Davenport JL (2005) Effects of shore height, wave exposure and geographical distance on thermal niche width of intertidal fauna. Ecology Progress Series 292:41-50.

Davenport J, McAlister H (1996) Environmental conditions and physiological tolerances of intertidal fauna in relation to shore zonation at Husvik, South Georgia. Journal of the Marine Biological Association of the UK 76: 985-1002.

Feder ME, Hofman GE (1999) Heat-shock proteins, molecular chaperones and the stress response: evolutionary and ecological physiology. Ann Rev Physiol 61: 243-282.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases, the first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 249:7130–7139.

Kennedy VS, Twilley RR, Kleypas JA, Cowan JH, Hare SR (2002) Coastal and marine ecosystems & global climate change: Potential effects on U.S. resources. Pew Center on Global Climate Change.

Lesser MP (2006) Oxidative stress in marine environments: biochemistry and physiological ecology. Ann Rev Physiol 68: 253-278.

Li Y, Schellhorn HE (2007) Rapid kinetic microassay for catalase activity. J. Biomol. Technol. 18: 185-187.

Madeira D, Narciso L, Cabral HN, Vinagre C (2012) Thermal tolerance and potential impacts of climate change on coastal and estuarine organisms. Journal of Sea Research 70: 32-41.

Morimoto RI, Tissieres A, Georgopoulos C (1994) Progress and perspectives on the biology of heat shock proteins and molecular chaperones. In: Morimoto RI, Tissieres A, Georgopoulos C (eds) The Biology of Heat Shock Proteins and Molecular Chaperones, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Njeimini R, Demanet C, Mets T (2005) Comparison of two ELISAs for the determination of Hsp70 in serum. J Immunol Meth 306: 176-182.

Pörtner HO, Langenbuch M, Michaelidis B (2005) Synergistic effect of temperature extremes, hypoxia, and increases in CO_2 on marine animals: From Earth history to global change. J. Geophys. Res. 110.

Rivadeneira MM, Fernandéz M (2005) Shifts in southern endpoints of distribution in rocky intertidal species along the south-eastern Pacific coast. Journal of Biogeography 32: 203-209.

Somero GN (2002) Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. Integr. Comp. Biol. 42: 780-789.

Somero GN (2010) The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine “winners” and “losers”. Journal of Experimental Biology 213: 912-920.

Stillman JH (2002) Causes and consequences of thermal tolerance limits in rocky intertidal porcelain crabs, Genus *Petrolisthes*. Integrative and Comparative Biology 42: 790-796.

Stillman JH, Somero GN (1996) Adaptation to temperature stress and aerial exposure in congereric species of intertidal porcelain crabs (Genus *Petrolisthes*): correlation of physiology, biochemist and morphology with vertical distribution. The Journal of Experimental Biology 199: 1845-1855.

Stillman JH, Somero GN (2000) A comparative analysis of the upper thermal tolerance limits of eastern pacific porcelain crabs, genus *Petrolisthes*: influences of latitude, vertical zonation, acclimation, and phylogeny. Physiol Biochem Zool 73: 200–208.

Tomanek L (2010) Variation in the heat shock response and its implications for predicting the effect of global climate change on species’ biogeographic distribution ranges and metabolic costs. Journal of Experimental Biology 213: 971-979.

Uchiyama M, Mihara M (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem. 86: 271-278

Vinagre C, Madeira D, Narciso L. Cabral HN, Diniz M (2012) Effect of temperature on oxidative stress in fish: lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax*. Ecological Indicators 23: 274-279.

Whiteley NM, Rastrick SPS, Lunt DH, Rock J (2011) Latitudinal variations in the physiology of marine gammarid amphipods. Journal of Experimental Biology and Ecology 400: 70-77.

Attachments

Table 1 - Results of two-way ANOVA evaluating the effects of temperature and species *(P. elegans and P. serratus)* on routine metabolic rates (RMR), heat shock proteins (HSP) and antioxidant analyzes (GST, CAT, SOD).

	Df	MS	F	p
RMR				
Species (S)	1	5.702	0.8736	0.361117
Temperature (T)	1	292.091	44.9044	0,000002
S x T	1	2.250	0.3447	0,563711
Error	20	6.527		
HSP				
Species (S)	1	451.4	1.0045	0.322923
Temperature (T)	1	9935.6	22.1076	0.000037
S x T	1	706.0	1.5709	0.218154
Error	36	449.4		
LIPO				
Species (S)	1	1233.510	83.6305	0.000000
Temperature (T)	1	907.985	61.5603	0.000000
S x T	1	694.841	47.1094	0.000000
Error	36	14.750		
GST				
Species (S)	1	12.643	4.3809	0.043452
Temperature (T)	1	8.255	2.8603	0.099429
S x T	1	29.341	10.1670	0.002957
Error	36	2.886		
CAT				
Species (S)	1	0.23	0.0063	0.937155
Temperature (T)	1	137.58	3.8123	0.058688
S x T	1	424.24	11.7557	0.001535
Error	36	36.09		
SOD				
Species (S)	1	549.617	78.0490	0.000000
Temperature (T)	1	7.011	0.9956	0.325046
S x T	1	0.236	0,0336	0.855648
Error	36	7.042		

CHAPTER 2

Tropical and temperate (Lysmata) shrimps in a warming ocean: a physiological and biochemical comparison

Abstract

In the present study we investigate the physiological and biochemical responses of tropical (*Lysmata amboinensis*) and temperate (*Lysmata seticaudata*) shrimps to different warming scenarios (present-day temperatures, +3°C and +6°C warming scenarios), namely routine metabolic rates, thermal sensitivity (LT_{50}), heat shock protein expression (HSP70/HSC70), antioxidant enzyme activities (SOD, CAT, GST) and lipid peroxidation (namely MDA accumulation). Thermal sensitivity experiments showed that at +6°C scenario, the temperate shrimps showed higher thermal tolerance in comparison to tropical shrimps, with the latter also revealing metabolic suppression ($Q_{10} < 1.5$). The higher mass-specific metabolic rate of the temperate shrimp was accompanied by higher antioxidant enzyme activities and HSP response, with no increased cellular damage (lipid peroxidation). Thus, we provide evidence that the tropical cleaner shrimp *Lysmata amboinensis* has lower acclimation capacities and will be more vulnerable to global warming than the higher-latitude species, because the latter has evolved in a relatively unstable and seasonal environment.

Key words: shrimps, *Lysmata seticaudata*, *Lysmata amboinensis*, ocean warming, heat shock proteins, antioxidant enzymes, metabolism.

Introduction

Shrimps from the genus *Lysmata* are particularly peculiar organisms among other caridean shrimp genera due to their wide diversity of lifestyles (Calado and Dinis, 2007). While some species like the Monaco's shrimp *Lysmata seticaudata* (Risso, 1816), occur in dense aggregations and dwell freely both in rocky intertidal and/or subtidal habitats of temperate and subtropical zones, others such as *Lysmata amboinensis* (De Man, 1888) live as socially monogamous pairs (Fieldler, 1998), displaying associative cleaning services to tropical fishes. Studies of congeners allow predicting the effects of increasing temperatures in these species, in order to establish in which these impacts will be more severe (Whitely et al., 2011; Stillman, 2003; Somero, 2010).

Facing current concerns about future climate change scenarios, the knowledge of thermal tolerance is the first step to understand how vulnerable species are. However, not only there is a vast response diversity but also global warming tends to vary regionally (Rivadeneira and Fernández, 2005; Madeira et al., 2012).

Impacts of climate warming in the tropics, the cradle of biodiversity, are often predicted to be small in comparison to those in temperate regions (Parmesan, 2007; Root et al., 2003), because the rate of climate warming in the tropics is lower in comparison to higher latitudes (IPCC, 2007). However, predictions based upon the magnitude of climate change may be misleading (Tewksburry et al., 2008). Most literature has focused on tropical regions, possibly for the reasons that models that include organism´s physiology suggests that the impacts will be more severe in the tropics (Tewksburry et al., 2008), as well as predictions for temperate regions are the hardest to make due to the diversity of life history patterns, trophic relations complexity and habitat variability (IPCC, 1997; Madeira et al., 2012).

It is expected that impacts of climate warming ought to be greatest on thermal specialists (tropical ectotherms) which possess limited acclimation capacities (Hoegh-Guldberg et al., 2007), in comparison to higher-latitude species, once they have evolved in relatively constant, seasonal environments**.** In this manner, ecosystems that evolved in stable conditions for a long time, e.g. cold environments or tropical habitats, are especially at risk (Madeira et al., 2012).

The production and accumulation of reactive oxygen species (ROS), beyond the capacity of an organism to quench these reactive species, is considered to be an additional problem related to ocean warming, and it is called oxidative stress. Reactive oxygen species are responsible for the toxic effects of oxygen, because the univalent reduction of molecular oxygen produces reactive intermediates such as $O_2{\bullet}^-$, H_2O_2, HO•, and finally water (Lesser, 2006). They form as a natural by-product of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis (Cadenas, 1989). However, during times of environmental stress, e.g. heat exposure, ROS levels can increase dramatically (Cadenas, 1989; Vinagre at al., 2012). These free radicals can damage lipids, proteins, and DNA (Lesser, 2006).

Under most physiological states, ROS production is closely matched by antioxidant responses (Rosa et al., 2012), including superoxide dismutase (SOD), which converts O^{2-} into H_2O_2, catalase (CAT) which removes H_2O_2 avoiding its accumulation in cells and tissues, and glutathione-S-transferase (GST) that transforms xenobiotics into other conjugates (Lesser, 2006).

Additionally, thermal stress also induces heat shock protein (HSP) production (Hochachka and Somero, 2002; Tomanek, 2008; Tomanek, 2010; Rosa et al., 2012). HSP's provides a proteomic defense, which includes protein synthesis, in way to repair, refold, and eliminate damaged or denatured proteins (Tomanek, 2010; Rosa et al., 2012).

The aim of this study was to investigate heat tolerance limits (% survival, LT_{50} and LT_{100}) and physiological responses (metabolic rates, heat shock proteins expression, lipid peroxidation and activity of antioxidant enzymes) of temperate (*Lysmata seticaudata*) and tropical (*Lysmata amboinensis*) shrimp species in response to increasing ocean warming scenarios.

Materials and methods

Specimen Collection and Maintenance

Specimens of *Lysmata seticaudata* where collected in subtidal zones at Cabo Raso (Western coast of Portugal) using baited traps and scuba diving, while specimens of tropical cleaner shrimp *Lysmata amboinensis* were commercially acquired by TMC Iberia (Portugal) , from Sri Lanka.

After collection, organisms were immediately placed in recirculation aquaculture systems at the aquaculture facilities of Laboratório Marítimo da Guia (Cascais, Portugal). *Lysmata seticaudata* specimens were acclimated at three different temperatures, i.e. 18°C (present ocean temperature for this species), 21°C (+ 3°C warming scenario) and 24°C (+ 6°C warming scenario), while *Lysmata amboinensis* were stocked at 21°C (present ocean temperature for this species at the collection site), 24°C (+ 3°C warming scenario) and 27°C (+ 6°C warming scenario), for a time period of 30 days. Ammonia and nitrite levels were monitored on a daily basis and kept below detectable levels. Likewise salinity (34.0 ± 1.0) and pH (8.1 ± 0.1) were daily controlled.

Thermal Tolerance Limits

The upper thermal tolerance limits of each species were determined according to Stillman and Somero (1996). One hundred and sixty shrimps, comprising 80 of each species and for each temperature, where placed into separated plastic containers, each containing 20 specimens (n=3) and a control for each species. Afterwards, specimens were subject to the acclimation temperature for a period of 30–60 min. Subsequently, a temperature increase was performed at a rate of 1°C/30 min. Every 30 min, water bath aeration was performed as well as temperature in each container checked (with thermocouple probes). During thermal tolerance limits experiment, sensory antennules activity of each shrimp was visually checked until they reached the end-point, where no sensory antennules activity was noticeable. The temperatures at which 50% and 100% of the shrimps died were calculated, and used as the measurements for upper thermal tolerance limits, being referred from now onwards as LT_{50} and LT_{100} (maximal temperature this specimens can stand), respectively.

Oxygen Consumption Rates and Thermal Sensitivity

Oxygen consumption measurements were determined according to Rosa et al. 2008, 2009 and Pimentel et al, 2012, through incubation of individual shrimps in sealed water-jacketed respirometry chambers (RC300 Respiration cell, Strathkelvin, North Lanarkshire, Scotland) containing filtered seawater, at the different incubation temperatures for each species (present ocean temperature, + 3°C warming scenario and + 6°C warming scenario). Water volumes were adjusted in relation to animal mass (up to 250 mL), in order to avoid stress. Respiration chambers were placed in water baths (Lauda, Lauda-Konigshofen, Germany) to control temperature and oxygen concentrations were recorded with Clarke-type O_2 electrodes connected to a multichannel oxygen interface (Strathkelvin 929, North Lanarkshire, Scotland), during a 3 to 4 hours time period. Thermal sensitivity (Q_{10}) was determined using the standard equation:

$$Q10 = \left[\frac{R(T2)}{R(T1)}\right]^{\frac{10}{T2-T_1}}$$

where $R(T_2)$ and $R(T_1)$ represent the oxygen consumption rates at temperatures T_2 and T_1, respectively.

Preparation of tissue extracts

Approximately 60 mg of shrimp abdominal tissue was homogenized in 100 µl of Phosphate Buffer Saline solution (PBS, pH 7.3: 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4), using a glass Potter Elvejhem tissue grinder. Resulting homogenates were subsequently centrifuged (20 min at 14 000 x g at 4°C) and enzyme activity as well as heat shock proteins were quantified in the supernatant fraction.

All samples were assayed for Glutathione S-Transferase, Catalase, Superoxide Dismutase, Lipid Peroxidation (MDA content) and HSP70/HSC70 levels.

Heat shock response (HSP70/HSC70)

Heat Shock Protein 70 was quantified through Enzyme Linked Immunoabsorbent Assay (ELISA) (Njemini et al., 2005), using 96 well microplates (Nunc-Roskilde, Denmark). Briefly, 10 µl of the homogenate’s supernatant were diluted in 250 µl of PBS, and three replicates of the diluted sample (50 µl of each) were added to the microplate wells and allowed to incubate overnight, at 4°C. Afterwards, microplates were washed three tomes with PBS 0.05% Tween-20 and blocked by adding 100 µl of 1% BSA (Bovine Serum Albumin, Sigma-Aldrich, USA) to each well and left to incubate for 2 hours, at room temperature. Following microplates washing (3X with PBS 0.05% Tween-20), 50 µl of 5 µg mL^{-1} primary antibody (anti-HSP70/HSC70, Acris USA), detecting 72 and 73 kDa proteins corresponding to the molecular mass of inducible HSP70 and HSC70, was added to each well and then incubated overnight at 4°C. The non-linked antibody was removed through microplates washing (3X), which were incubated for 2 hours at room temperature, with 50 µl of 1 µg mL^{-1} of the secondary antibody (anti-mouse IgG, Fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA). After another wash, 100 µl of substrate (SIGMA FAST^{TM} p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA) was added to each well and incubated during 30 min at room temperature. Finally, 50 µl of stop solution (3N NaOH) was added to each well and the absorbance read at 405 nm in a 96 well microplate reader (BIO-RAD, Benchmark, USA). Hsp70/Hsc70 samples quantifications were calculated by constructing a calibration curve obtained based on serial dilutions of purified HSP70 active protein (Acris, USA), as to give a range from 0 to 2000 ng/mL. The results were then divided by the total protein quantify in the sample determined by the Bradford method (Bradford, 1976) and expressed as micrograms of total protein (ng hsp70/hsc70 mg^{-1} total protein).

Lipid peroxide assay (determination of malondialdehyde, MDA)

Lipid peroxide assay was adapted from the thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara, 1978) and it was determined by the quantification of a specific end-product of the lipid oxidative degradation process, the malondialdehyde (MDA) in which thiobarbituric acid reacts with MDA yielding a fluorescent product that was detected spectrophotometrically at 532 nm. Briefly, 5 µl of each sample were treated with 12.5 µl of dodecyl sulfate sodium (SDS 8.1%), 93.5 µl of trichloroacetic acid (20%, pH 3.5), and 93.5 µl of thiobarbituric acid (1%). To this mixture, 50.5 µl of Milli-Q grade ultrapure water were added and eppendorf's were placed in a vortex for 30 s and incubated in boiling water for 10 min. Thereafter, they were placed on ice for 3 min in order to lower temperature. Subsequently, 62.5 µL of Milli-Q water and 312.5 µL of n-butanol pyridine (15:1, v/v) were added and eppendorf's were placed in a vortex and centrifuged at 7000 rpm for 5 min. For each reaction, 150 µL duplicates of the supernatant were inserted into a 96-well microplates and the absorbance read. To quantify lipid peroxides, an eight-point calibration curve (0-0.3 µM TBARS) was calculated using malondialdehyde bis (dimethylacetal) (from Merck) standards.

The results were divided by the total amount of protein in the sample calculated by the Bradford method (Bradford, 1976) and expressed as micrograms of total protein (MDA nmol/mg total protein).

Enzymatic Analyses

1. Glutathione S-Transferase

The enzyme activity was determined spectrophotometrically in the supernatant at 340 nm, every 6 minutes using a microplate reader (BIO-RAD, Benchmark, USA). Briefly, total GST activity was determined as described by Habig et al. (1974) by measuring the formation of the conjugate of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). Briefly, 180 µL of substrate solution (Dulbecco's Phosphate Buffered Saline with 200 mM L-glutathione reduced and 100 mM CDNB all from Sigma-Aldrich, Germany) were added to 20 µL of GST standard or sample into each well of a 96-well microplate. The total enzyme activity was determined at 340 nm by recording the absorbance at every minute for 6 minutes, using a microplate reader (BioRad Benchmark, USA). Equine liver GST (Sigma-Aldrich, Germany) was used as standard and positive control. The change in absorbance per minute (ΔA340) was estimated and the reaction rate at 340 nm was determined using CDNB extinction coefficient of 0.0096 µM-1cm-1. The results are expressed in relation to total protein concentration of the sample(nmol min^{-1} mg^{-1} total protein).

2. Determination of Catalase

A spectrophotometric (BIO-RAD, Benchmark, USA) methodology was applied and consisted of measuring the absorbance at 240 nm (Aebi, 1983; Li and Schellhorn, 2007) in a time interval of 1 minute and 30 seconds.

When the sample is added to hydrogen peroxide the reaction can be followed by a decrease in absorbance as the peroxide is turned into oxygen and water. In order to perform this reaction 100 µl of each sample were added to 2900 µl of hydrogen peroxide (50 nmol/L potassium phosphate buffer (pH 7.0), 12.1 mmol/L H_2O_2) using quartz cuvettes. Peroxide consumption was monitored by absorbance reading, every 15 seconds. Standard catalase activity was measured using 1523.6 U/mL of bovine catalase solution (Sigma-Aldric, Germany). Catalase activity was calculated using an absorption coefficient for H_2O_2 of 0.04 $mmol^{-1}$ cm^{-1}, using the following equation:

$$\left(\frac{\Delta A240\ min^{-1}}{0.04}\right) \times \left(\frac{total\ volume}{sample\ volume}\right)$$

The results were divided by the total protein quantity in the sample (the total protein in samples was calculated using the Bradford method (Bradford, 1976) and expressed as micrograms of total protein) to obtain catalase activity in nmol min^{-1} μg^{-1}.

3. Superoxide dismutase (SOD) assay

SOD activity was determined spectrophotometrically in the supernatant at 25°C (BIO-RAD, Banchmark, USA) and 550 nm. The adapted assay contained, at 25°C, 50 mM of Potassium Phosphate Buffer (pH 7.8), 3 mM EDTA, 3 mM Xantine solution, 0.75 mM

NBT (nitroblue tetrazolium), 100 mU XOD (Xanthine Oxidase Solution) and 1 U/µL SOD Enzyme solution all from Sigma-Aldrich (Germany). Superoxide Dismutase from bovine erythrocytes (Sigma-Aldrich, Germany) was used as standard and positive control. The results of this enzymatic assay are given in units of SOD activity per milligram of total protein in the sample (U mg^{-1} total protein), where one unit of SOD is defined as the amount of sample causing 50% inhibition of NBT reduction.

Statistical Analyses

A two-way analysis of variance (two-way ANOVA) was performed to test the effects of species and temperature on metabolism.

Three-way ANOVA's were conducted to detect significant differences in HSP70/HSC70, GST, MDA, CAT and SOD contents between species, temperature and incubation time. Subsequently, post-hoc tests (Tukey HSD) were performed. All statistical analyses were performed for a significant level of 0.05, using Statistica software (version 10.0, StatSoft Inc., Tulsa, USA).

Results

Thermal tolerance limits

Both LT_{50} and LT_{100} were influenced by the acclimation temperature that specimens were exposed (Figure 1). When exposing to the +6°C scenario, the higher thermal tolerance was observed for the temperate shrimp (*Lysmata seticaudata*) (Figure 1).

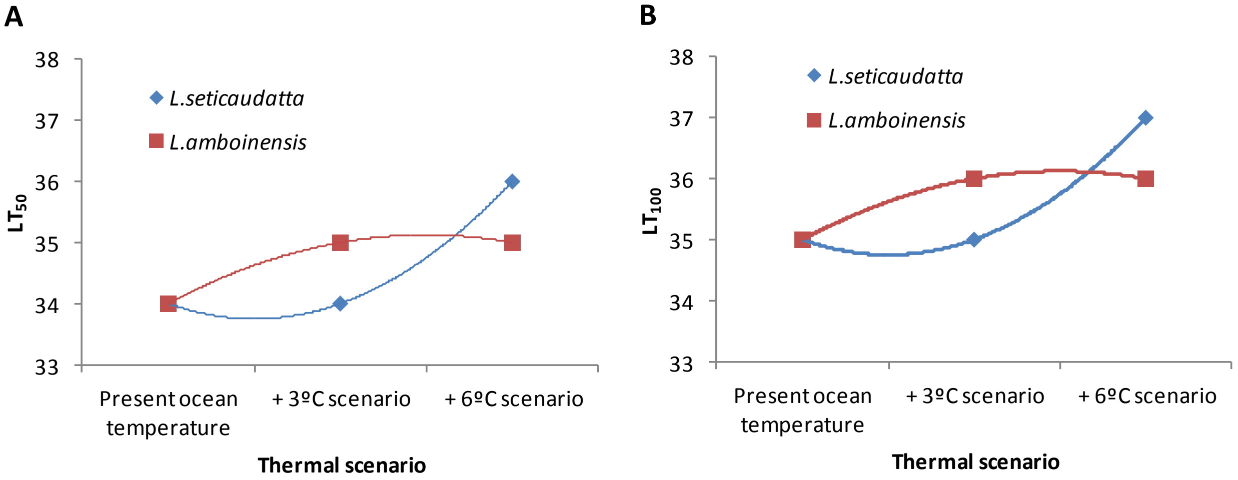

Figure 1. Thermal tolerance limits (A – LT_{50}, °C; B – LT_{100}, °C) of temperate (*L. seticaudata*) and tropical (*L. amboinensis*) shrimps, at different temperature scenarios. Values are means of triplicate experimens ± SD (=0). Colored lines represent trendlines.

Metabolic rates

Routine Metabolic Rates (RMR) was significantly affected by species and temperature ($p < 0.05$) (Figure 2). *Lysmata amboinensis* displayed RMR values ranging from 4.0 µmol O_2/h/g at present ocean temperature to 5.7 µmol O_2/h/g at + 3°C and 6.4 µmol O_2/h/g at + 6°C warming scenarios, while *Lysmata seticaudata* displayed higher RMR values than *Lysmata amboinensis*, ranging from 7.0 µmol O_2/h/g at present ocean temperature to 9.7 µmol O_2/h/g at + 3°C and 14.3 µmol O_2/h/g at + 6°C warming scenario. At the extreme thermal scenario (+ 6°C), *L. amboinensis* suppressed its metabolism, with a Q_{10} value of 1.5 (i.e. indicative of active metabolic suppression; Rosa and Seibel 2008, 2010, Rosa et al. 2012), while *L. seticaudata*, showed a Q_{10} value of 3.5 (Figure 3). At normal operating temperatures, metabolic demand for oxygen increases with temperature with Q_{10} around 2-3. Thus, *L. seticaudata*, increased its metabolic rates proportionally with temperature.

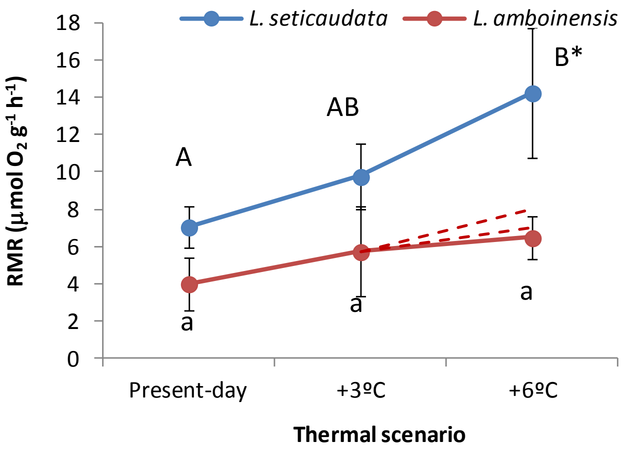

Figure 2. Routine metabolic rates (RMR, µmol O2 g-1 h-1) of temperate (Lysmata seticaudata) and tropical (*L. amboinensis*) shrimps at different temperature scenarios. Rates were standardized to 500mg shrimps assuming a scaling coefficient of -0.20. Values are mean ± SD. Different letters and asterisks represent significant differences between temperatures and species, respectively. For more statistical details see Table 1. Dashed lines represents the ''expected'' trend assuming a Q10 of 2 and 3.

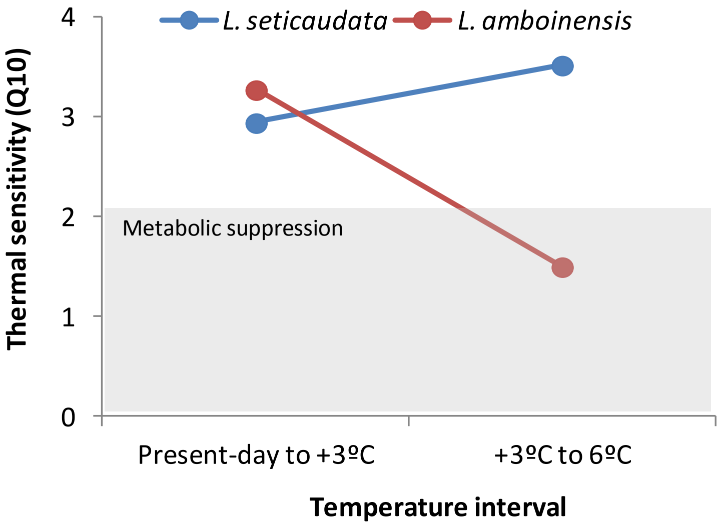

Figure 3. Thermal sensitivity (Q10) of temperate (*Lysmata seticaudata*) and tropical (*L. amboinensis*) shrimps at different temperature scenarios.

Heat shock proteins and lipid peroxidation

Concerning heat shock proteins (HSP) significant differences were found between species and thermal exposure scenarios ($p < 0.05$), *L. seticaudata* always showed higher HSP values in comparison to *L. amboinensis*, but in both species the values remained fairly stable at the different thermal scenarios and exposition time (Figure 4).

Considering MDA accumulation, there was no significant ($p > 0.05$) increased cellular damage, since the levels were fairly similar between the two species and thermal scenarios (Figure 5), with an exception in the beginning of the incubation of *L. amboensis* at the +3°C scenario.

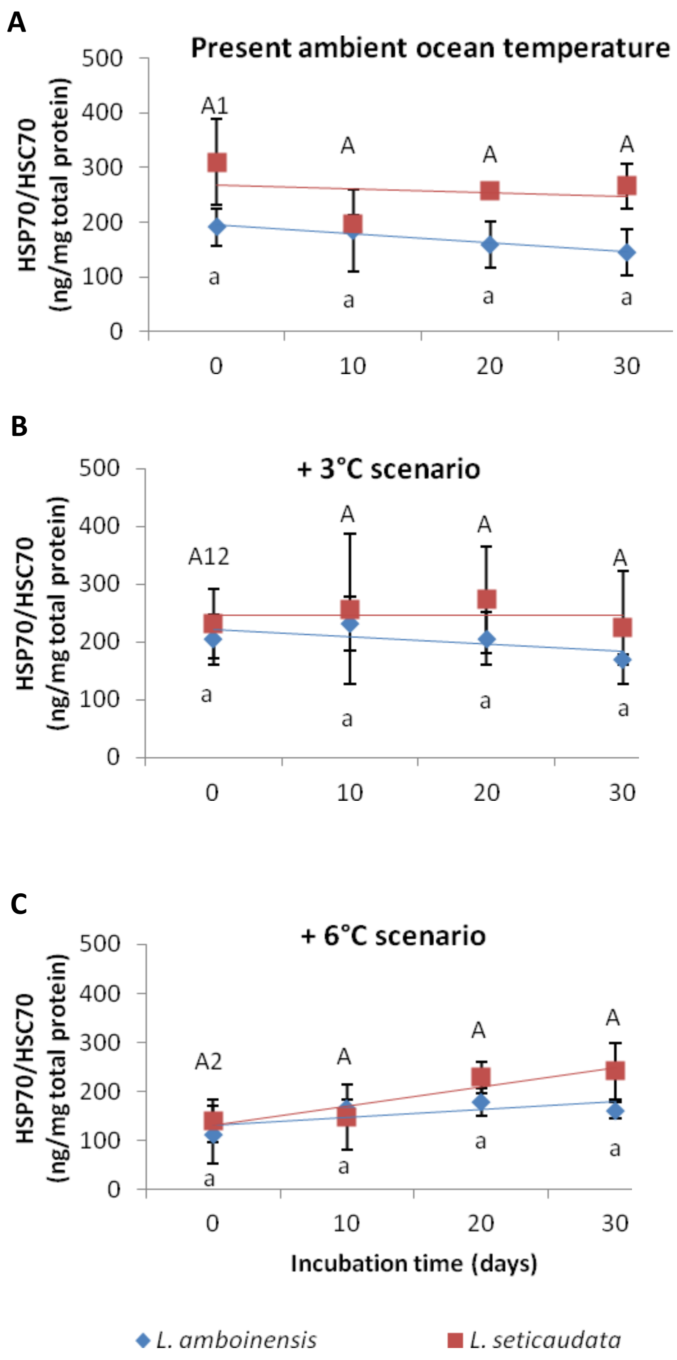

Figure 4. Impact of environmental warming on the HSP70/HSC70 concentrations (ng/mg total protein) of *L. amboinensis* and L. *seticaudata*. A) present ocean temperature, B) + 3°C warming scenario, C) + 6°C warming scenario. Values represent mean ± S.D. Colored lines represent trendlines and different letters (capital letters for *L. seticaudata* and lowercase letters for *L. amboinensis* and numbers represent significant differences between incubation time and temperature scenarios. For more statistical details see Table 1.

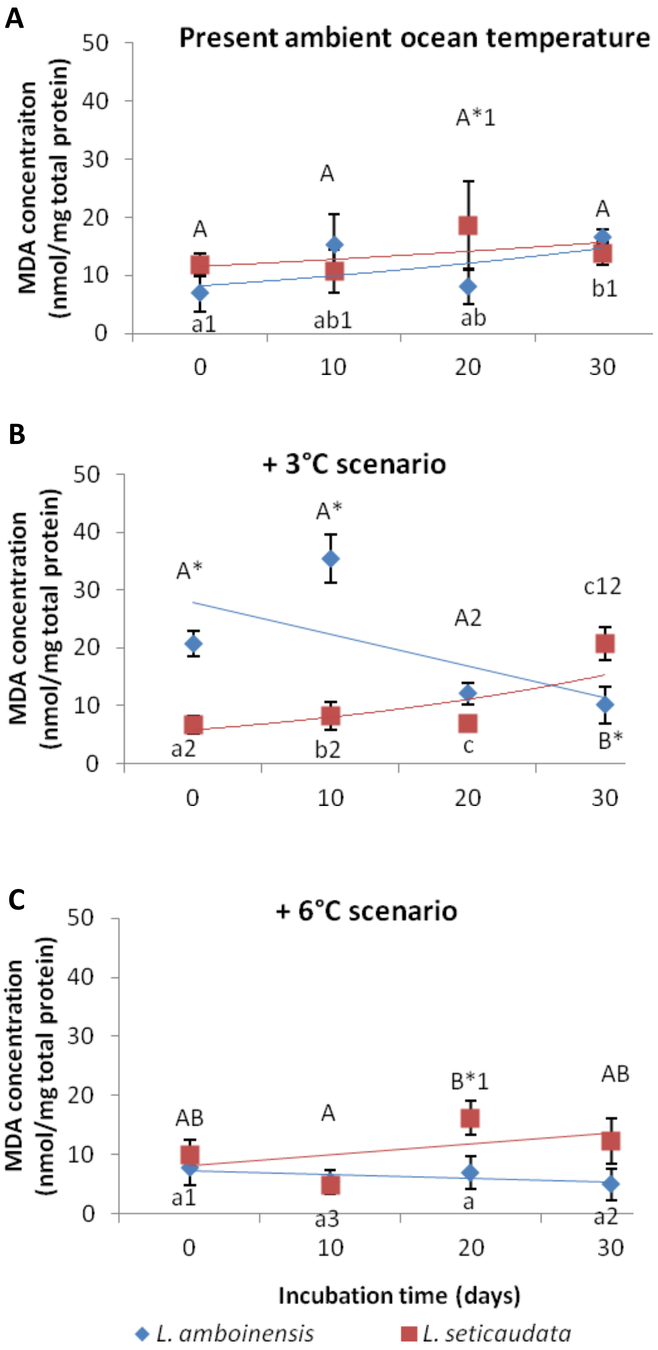

Figure 5. Impact of environmental warming on the MDA concentrations (nmol/mg total protein) of *L. amboinensis* and *L. seticaudata*. A) present ocean temperature, B) + 3°C warming scenario, C) + 6°C warming scenario. Values represent mean ± S.D. Colored lines represent trendlines and different letters (capital letters for *L. seticaudata* and lowercase letters for *L. amboinensis*, asterisks and numbers represent significant differences between incubation time, species and temperature scenarios. For more statistical details see Table 1.

Oxidative stress tolerance

Glutathione S-Transferase (GST) activity showed significant differences between species and temperature scenarios ($p < 0.05$). GST activity was always significantly higher for temperate shrimps (*L. seticaudata*). (Figure 6). Regarding catalase activity () there were also significant differences between species and experimental temperatures ($p < 0.05$). Interestingly, the same trend occurred, i.e., consistent higher activities in *L. seticaudata* (Figure 7).

Additionally, superoxide dismutase (SOD) activity also showed a significant difference between species and temperatures tested ($p < 0.05$). Although, the trends of variation are more complex that those observed in the previous enzymes, it is worth noting that SOD decreases significantly ($p < 0.05$) throughout incubation in the *L. amboensis* at all thermal scenarios.

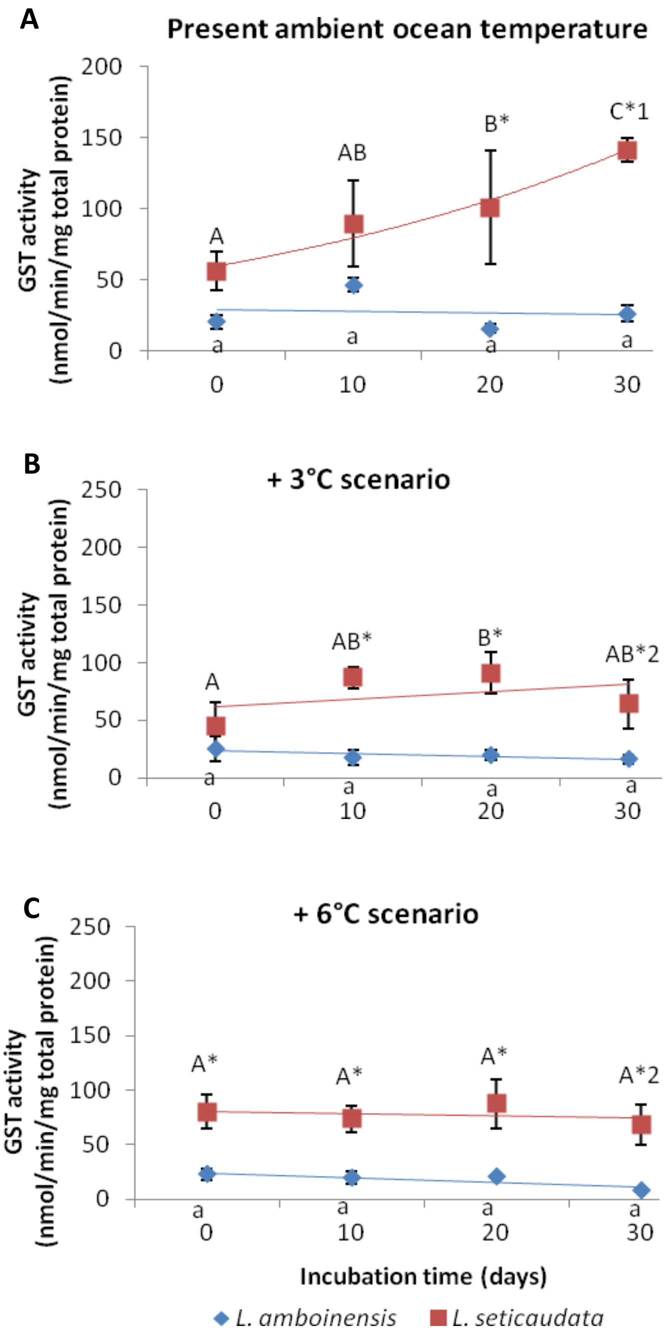

Figure 6. Impact of environmental warming on the GST concentrations (nmol/min/mg total protein) of *L. seticaudata* and *L. amboinensis*. A) present ocean temperature, B) +3°C warming scenario, C) + 6°C warming scenario. Values represent mean ± S.D. Colored lines represent trendlines and different letters (capital letters for *L. seticaudata* and lowercase letters for *L. amboinensis*, asterisks and numbers represent significant differences between incubation time, species and temperature scenarios. For more statistical details see Table 1.

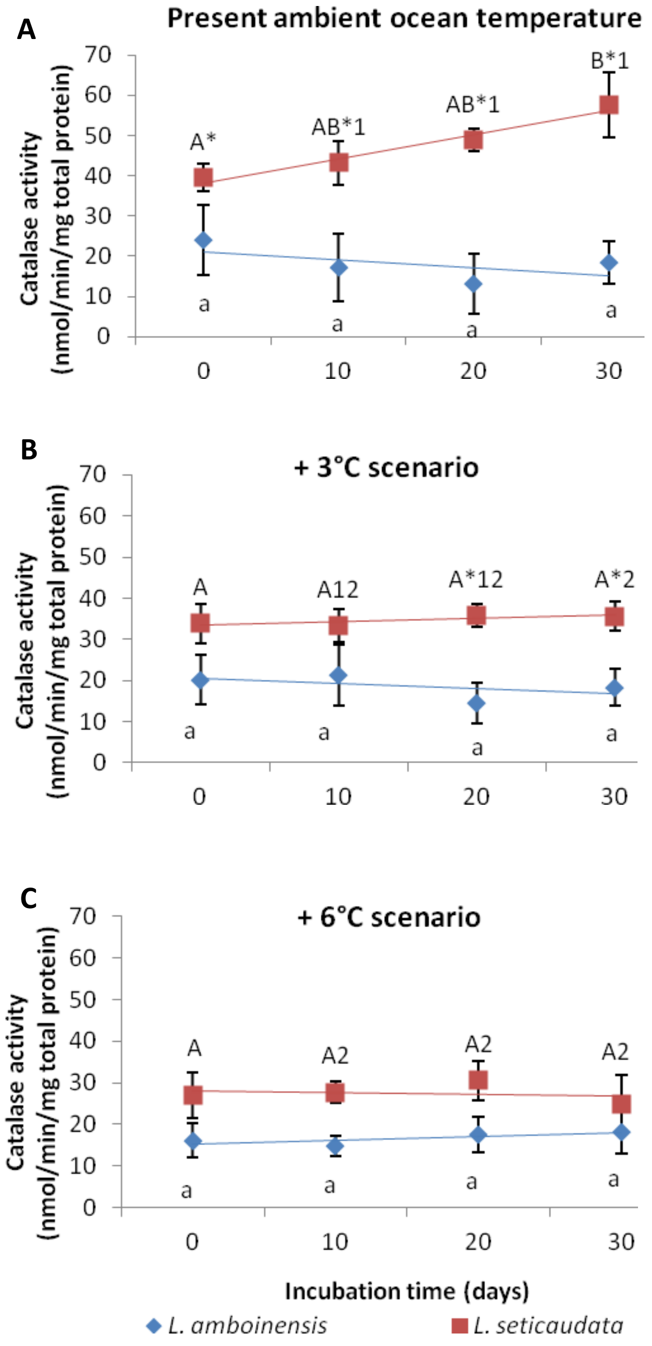

Figure 7. Impact of environmental warming on the Catalase concentrations (nmol/min/mg total protein) of *L. seticaudata* and *L. amboinensis*. A) present ocean temperature, B) + 3°C warming scenario, C) + 6°C warming scenario. Values represent mean ± S.D. Colored lines represent trendlines and different letters (capital letters for *L. seticaudata* and lowercase letters for *L. amboinensis*, asterisks and numbers represent significant differences between incubation time, species and temperature scenarios. For more statistical details see Table 1.

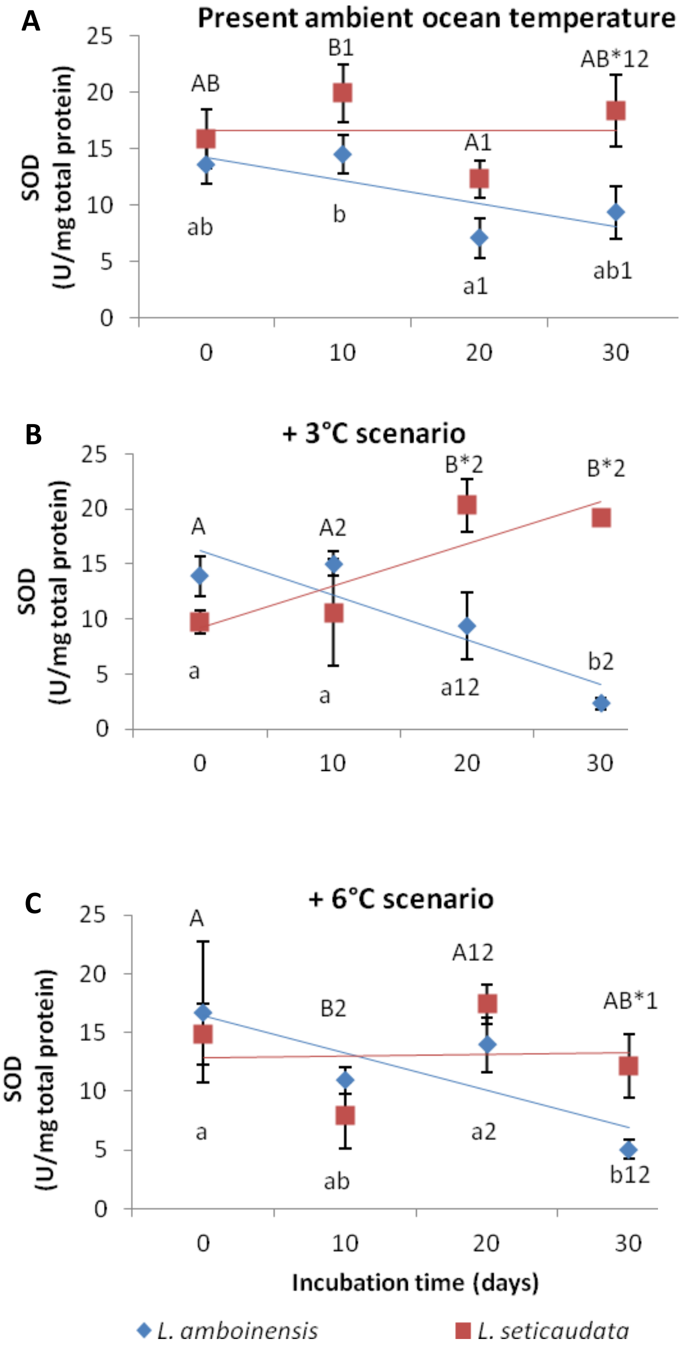

Figure 8. Impact of environmental warming on SOD concentrations (U/mg total protein) of *L. amboinensis* and *L. seticaudata*. A) present ocean temperature, B) + 3°C warming scenario, C) + 6°C warming scenario. Values represent mean ± S.D. Colored lines represent trendlines and different letters (capital letters for *L. seticaudata* and lowercase letters for *L. amboinensis*, asterisks and numbers represent significant differences between incubation time, species and temperature scenarios. For more statistical details see Table 1.

Discussion

It should be expectable that tropical organisms have a higher heat tolerance, since they are subject to far more warmer conditions throughout the year than temperate organisms. As also noticed in several studies (Pörtner and Farrell, 2003; Pörtner and Knust, 2007, Parmesan, 2007; Tewsksbury et al., 2008), we have observed that a shrimp species inhabiting the tropical zone will be more vulnerable to further increases in temperature than a temperate species. *Lysmata seticaudata* demonstrated a higher thermal tolerance limits than *L. amboinensis*, when exposed to a thermal setting of +6°C above their habitat temperature (Figure 1).

Tropical ectotherms are thermal specialists (Janzen, 1960), and have limited acclimation potential (Hoegh-Guldberg et al., 2007) in comparison to higher latitude species, as they inhabit in aseasonal environments (Tewksbury et al., 2008) and live close to their upper thermal limits (Jokiel and Coles, 1977; Sharp et al., 1997). Thereby, ecosystems that have evolved in stable conditions for a long time, e.g. cold environments and tropical habitats, are especially at risk.

Moreover, at the more extreme scenario (+ 6°C), the tropical shrimp suppressed it's metabolism ($Q_{10} < 1.5$) (see Figure 3). This depression means that, at such severe scenario, tropical shrimps do not prevent a performance decrement as a result of a consistently O_2 cell concentration falling to critical levels and CO_2 from rising. This trend seemed to reduce the animal capacity to perform aerobically (a drop in aerobic scope), with a progressive transition to an anaerobic mode of energy production (Rosa and Seibel, 2008, 2010, Rosa et al., 2012). Additionally, this decrease in aerobic scope was not caused by lower levels of environment avaible oxygen but through limited capacity of oxygen supply mechanisms, by ventilatory and circulatory systems, to meet animal's temperature-dependent oxygen demand (Pörtner & knust, 2007).

The higher mass-specific metabolic rate of the temperate shrimp was compensated by physiological mechanisms to minimize the negative effects of thermal stress over fitness. The HSP70/HSC70 concentrations, were quite similar for different thermal scenarios, with higher values obtained for *L. seticaudata (*Figure 4), although no increased cellular damage (Figure 5) (lipid peroxidation), a process considered to be one of the most frequent cellular injury mechanisms (Lesser, 2006), was observed.

This increased metabolic demands of *Lysmata seticaudata* lead to elevated ROS formation (Oliveira et al., 2005). In fact, the growing oxygen requirements for temperate shrimps led to an increase in SOD activity (Figure 8), due to an superoxide production increase, one of the most important ROS (Lesser, 2006).

Once the antioxidative response is linked and the activity of an enzyme is dependent upon the activity of one another (Cooper et al., 2002), this increase within SOD activity will lead to an increment of GST and CAT activity (Figures 6 and 7, respectively) as well, once they catabolize peroxide, which results of SOD action.

Our results suggest that heat shock proteins, SOD, CAT and GST production constituted an integrated stress response to ocean warming in temperate shrimps, but not in tropical ones.

We therefore conclude and provide evidence that tropical cleaner shrimp (*Lysmata amboinensis*) has lower acclimation capacities and will be more vulnerable to global warming than *Lysmata seticaudata*, partially due to the fact that the latter has evolved in a relatively unstable and seasonal environment.

References

Aebi HE (1983) Catalase. In: Bergmeyer HU, Bergmeyer J, Grassl JM (Eds) Methods of enzymatic analysis. VCH, Weinheim, pp. 273-286.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Cadenas E (1989) Biochemistry of oxygen toxicity. Annu. Rev. Biochem. 58: 79-110.

Calado R, Dinis MT (2007) Minimization of precocious sexual phase change during culture of juvenile ornamental shrimps *Lysmata seticaudata* (Decapoda: Hippolytidae). Aquaculture 269: 299-305.

Cooper RU, Clough LM, Farwell MA, West TL (2002) Hypoxia-induced metabolic and antioxidant enzymatic activities in the estuarine fish *Leiostomus xanthurus*. J Exp Mar Biol Ecol 279: 1-20.

Fiedler GC (1998) Functional, simultaneous hermaphroditism in female-phase *Lysmata amboinensis* (Decapoda: Caridea: Hippolytidae). Pac Sci 52: 161-169.

Gutowska MA, Melzner F (2009) Abiotic conditions in cephalopod (*Sepia officinalis*) eggs: embryonic development at low pH and high pCO_2. Mar. Biol. 156: 515-519.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases, the first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 249:7130–7139.

Hoegh-Guldberg O, Mumby PJ, Hooten AJ, Steneck RS, Greenfield P, Gomez E, Harvell CD, Sale PF, Edwards AJ, Caldeira K, Knowlton N, Eakin CM, Iglesias-Prieto R, Muthiga N, Bradbury RH, Dubi A, Hatziolos ME (2007) Coral reefs under rapid climate change and ocean acidification. Science 318: 1737-1742.

Hu MY, Sucre E, Charmantier-Daures M, Charmantier G, Lucassen M, et al. (2010) Localization of ion-regulatory epithelia in embryos and hatchlings of two cephalopods. Cell Tissue Res 339: 571-583.

IPCC (1997) The regional impacts of climate change: an assessment of vulnerability. Cambridge University Press. UK.

IPCC (2007) The physical science basis. Working group I contribution to the fourth assessment report of the IPCC. Cambridge Univ. Press. UK.

Jokiel PL, Coles SL (1977) Effects of temperature on the mortality and growth of Hawaiian reef corals. Marine Biology 4: 201-208.

Lesser MP (2006) Oxidative stress in marine environments: biochemistry and physiological ecology. Ann Rev Physiol 68: 253-278.

Li Y, Schellhorn HE (2007) Rapid kinetic microassay for catalase activity. J. Biomol. Technol. 18: 185-187.

Madeira D, Narciso L, Cabral HN, Vinagre C (2012) Thermal tolerance and potential impacts of climate change on coastal and estuarine organisms. Journal of Sea Research 70: 32-41.

Njeimini R, Demanet C, Mets T (2005) Comparison of two ELISAs for the determination of Hsp70 in serum. J Immunol Meth 306: 176-182.

Parmesan, C., 2007. Influences of species, latitudes and methodologies on estimates of phenological response to global warming. Global Change Biology 13: 1860–1872.

Pörtner HO, Farrell AP (2008) Physiology and climate change. Science 322: 690-691

Pörtner HO, Knust R (2007) Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science 315: 95-97.

Rivadeneira MM, Fernandéz M (2005) Shifts in southern endpoints of distribution in rocky intertidal species along the south-eastern Pacific coast. Journal of Biogeography 32: 203-209.

Root TL, Price JT, Hall RK, Schneider SH, Rosenzweig C, Pounds JA (2003) Fingerprints of global warming on wild animals and plants. *Nature* 421: 57–60

Rosa R, Pimentel MS, Boavida-Portugal J, Teixeira T, Trübenbach K, Diniz M (2012) Ocean warming enhances malformations, premature hatching, metabolic suppression and oxidative stress in the early life stages of a keystone squid. PLoS ONE 7(6): e38282.

Sharp VA, Brown BE, Miller D (1997) Heat shock protein (hsp 70) expression in the tropical reef coral *Goniopora djiboutiensis*. Journal of Thermal Biology 22: 11-20.

Somero GN (2010) The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine “winners” and “losers”. Journal of Experimental Biology 213: 912-920.

Stillman JH (2002) Causes and consequences of thermal tolerance limits in rocky intertidal porcelain crabs, Genus *Petrolisthes*. Integrative and Comparative Biology 42: 790-796.

Stillman JH, Somero GN (1996) Adaptation to temperature stress and aerial exposure in congereric species of intertidal porcelain crabs (Genus *Petrolisthes*):

correlation of physiology, biochemist and morphology with vertical distribution. The Journal of Experimental Biology 199: 1845-1855.

Tomanek L (2010) Variation in the heat shock response and its implication for predicting the effect of global climate change on species biogeographic distribution ranges and metabolic costs. Journal of Experimental Biology 213: 971-979.

Tweksbury JJ, Huey RB, Deutsch CA (2008) Putting the heat on tropical animals. Science 320: 1296-1297.

Uchiyama M, Mihara M (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem. 86: 271-278

Vinagre C, Madeira D, Narciso L. Cabral HN, Diniz M (2012) Effect of temperature on oxidative stress in fish: lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax*. Ecological Indicators 23: 274-279.

Whiteley NM, Rastrick SPS, Lunt DH, Rock J (2011) Latitudinal variations in the physiology of marine gammarid amphipods. Journal of Experimental Biology and Ecology 400: 70-77.

Attachments

Table 1 - Results of two-way ANOVA evaluating the effects of temperature and species *(L. amboinensis and L. seticaudata)* on routine metabolic rates (RMR), heat shock proteins (HSP) and antioxidant analyzes (GST, CAT, SOD).

	Df	MS	F	p
RMR				
Species (S)	1	200.043	243.1993	0.000038
Temperature (T)	2	58.737	24.5817	0.003207
S x T	2	15.542	7.2202	0.168305
Error	26	8.138		
HSP				
Species (S)	1	73917	22.594	0.000010
Temperature (T)	2	24665	7.539	0.001064
S x T	2	6366	1.946	0.150286
Error	72	3271		
LIPO				
Species (S)	1	14.72	1.470	0.229340
Temperature (T)	2	355.62	35.510	0.000000
S x T	2	414.73	41.413	0.000000
Error	72	10.01		
GST				
Species (S)	1	94270.1	346.7276	0.000000
Temperature (T)	2	3726.6	13.7066	0.000009
S x T	2	1229.8	4.5234	0.014109
Error	72	271.9		
CAT				
Species (S)	1	8378.52	280.549	0.000000
Temperature (T)	2	906.50	30.354	0.000000
S x T	2	712.20	23.848	0.000000
Error	72	29.86		
SOD				
Species (S)	1	359.83	55.519	0.000000
Temperature (T)	2	22.09	3.409	0.038573
S x T	2	36.83	5.682	0.005140
Error	71	6.48		

Final considerations

Crustaceans are excellent models to understand how environmental and endogenous factors shape complex life cycles. Factors such as ultraviolet light (UV), salinity, temperature, pollution and infections alter respiration and metabolism (Fanjul-Moles and Gonsebatt, 2012), as well as thermal sensitivity and ROS production.

Moreover, marine shrimps are ectotherms, which enables these organisms to be used as biomodels in order to study thermal stress response, once their body temperature follows ocean temperature, having a direct effect on their biochemistry, physiology and biogeographic distribution. Additionally, the study of thermal stress response, HSP70 and ROS production enables us to understand if species are resistant or vulnerable to temperature changes as well as to understand mechanisms they use to cope with those changes.

This study covered four shrimp species from different habitats in order to allow us a specific overview of stress response across a diverse set of ecological scenarios.

The purpose of the present work was to determined the thermal tolerance response of four species of shrimps, from different habitats, in order to compare the differences between two congener's from different tidal habitats (*Palaemon elegans* and *Palaemon serratus*, inhabiting intertidal and subtidal zones, respectively), and between two congener's who inhabit different geographic regions (*Lysmata amboinensis* and *Lysmata seticaudata*, which are found in tropical and temperate regions, respectively), to determine in which increasing temperatures will be more severe.

Our study showed that in tidal habitat, organisms living in intertidal zone are more tolerant than those in the lower shore (subtidal zone). In fact, *Palaemon elegans* showed higher tolerance values than *Palaemon serratus*, which means that although it inhabits an extremely variable habitat at several levels (such as temperature, salinity and dissolved oxygen) (Stillman, 2002; Tomanek, 2010), they are more tolerant to global warming then their subtidal congener, *Palaemon serratus*, since the latter live in a relatively variable or more stable habitat, so they are exposed to lower temperatures, having physiological set-points lower in the temperature gradient (Davenport & Davenport, 2005; Davenport & McAlister, 1996). .

With regard to differences between species of distinct geographic regions, it was found that, , species inhabiting the tropical zone seem to be more vulnerable to further increases in temperature than temperate shrimps, once *Lysmata seticaudata* demonstrated a higher thermal tolerance limit than *L. amboinensis*, when exposed to a thermal setting of + 6°C above their habitat temperature. This result may be explained, by the fact that tropical ectotherms are thermal specialists and have limited acclimation potential (Hoegh-Guldberg et al., 2007), in contrast to higher latitude species that inhabit aseasonal environments (Tewksbury et al., 2008) and do not live close to their upper thermal limits (Jokiel & Coles, 1977; Sharp et al., 1997).

Moreover, as expected, increased temperatures led to higher metabolic rates, however at extreme scenarios (+ 6°C), the tropical shrimp suppresses it's metabolism ($Q_{10} < 1.5$). This depression means that, at such severe scenario, tropical shrimps do not prevent a drop in aerobic scope, with a progressive transition to an anaerobic mode of energy production (Rosa and Seibel 2008, 2010, Rosa et al. 2012).

Heat shock proteins were a sensitive environmental stress marker. As expected, HSP production increased in way to protect cells against higher temperatures (Tomanek, 2010). Concomitantly, warming also lead to an augment of MDA concentrations, SOD, GST and CAT activity, mainly for *P. serratus*.

Regarding to latitudinal differences, the higher mass-specific metabolic rate of the temperate shrimp was compensated by physiological mechanisms to minimize the negative effects of thermal stress on fitness. This way, the HSP70 concentration and SOD, CAT and GST activities were higher *L. seticaudata*, however no increased cellular damage (lipid peroxidation), a process considering to be one of the most frequent cellular injury mechanisms (Lesser, 2006).

In conclusion, this work has shown that species have adapted their tolerance limits to coincide with (micro)habitat conditions, this way it was proved that ocean warming is expected to drive much more profound biological impacts on species that evolved in relatively stable and aseasonal environment (*P. serratus* and *L. amboinensis*) than in species inhabiting seasonal and unstable habitats (*P. elegans* and *L. seticaudata*).

References

Davenport J, Davenport JL (2005) Effects of shore height, wave exposure and geographical distance on thermal niche width of intertidal fauna. Ecology Progress Series 292:41-50.

Davenport J, McAlister H (1996) Environmental conditions and physiological tolerances of intertidal fauna in relation to shore zonation at Husvik, South Georgia. Journal of the Marine Biological Association of the UK 76: 985-1002.

Fanjul-Moles ML, Gonsebatt ME (2012) Oxidative stress and antioxidant systems in crustacean life cycles. In: Abele D, Vázquez-Medina JP, Zenteno-Savín T. oxidative stress in aquatic ecosystems. Blackwell Publishing Ltd. Pp 208-219.

Gutowska MA, Melzner F (2009) Abiotic conditions in cephalopod (*Sepia officinalis*) eggs: embryonic development at low pH and high pCO_2. Mar. Biol. 156: 515-519.

Hoegh-Guldberg O, Mumby PJ, Hooten AJ, Steneck RS, Greenfield P, Gomez E, Harvell CD, Sale PF, Edwards AJ, Caldeira K, Knowlton N, Eakin CM, Iglesias-Prieto R, Muthiga N, Bradbury RH, Dubi A, Hatziolos ME (2007) Coral reefs under rapid climate change and ocean acidification. Science 318: 1737-1742.

Hu MY, Sucre E, Charmantier-Daures M, Charmantier G, Lucassen M, et al. (2010) Localization of ion-regulatory epithelia in embryos and hatchlings of two cephalopods. Cell Tissue Res 339: 571-583.

Jokiel PL, Coles SL (1977) Effects of temperature on the mortality and growth of Hawaiian reef corals. Marine Biology 4: 201-208.

Lesser MP (2006) Oxidative stress in marine environments: biochemistry and physiological ecology. Ann Rev Physiol 68: 253-278.

Pörtner HO, Knust R (2007) Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science 315: 95-97.

Sharp VA, Brown BE, Miller D (1997) Heat shock protein (hsp 70) expression in the tropical reef coral *Goniopora djiboutiensis*. Journal of Thermal Biology 22: 11-20.

Stillman JH (2002) Causes and consequences of thermal tolerance limits in rocky intertidal porcelain crabs, Genus *Petrolisthes*. Integrative and Comparative Biology 42: 790-796.

Tomanek L (2010) Variation in the heat shock response and its implications for predicting the effect of global climate change on species' biogeographic distribution ranges and metabolic costs. Journal of Experimental Biology 213: 971-979.

Tweksbury JJ, Huey RB, Deutsch CA (2008) Putting the heat on tropical animals. Science 320: 1296-1297.