Universidade de Aveiro Departamento de Biologia
2018

Anthony Peter Moreira

Comparação da performance de espécies de ostra *Crassostrea* em Portugal e no Brasil no contexto de alterações climáticas

Comparative performance of *Crassostrea* species in Portugal and Brazil – Climate change implications

Universidade de Aveiro Departamento de Biologia
2018

Anthony Peter Moreira

Comparação da performance de espécies de ostra *Crassostrea* em Portugal e no Brasil no contexto de alterações climáticas

Comparative performance of *Crassostrea* species in Portugal and Brazil – Climate change implications

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Rosa Freitas (Investigadora Auxiliar do Departamento de Biologia da Universidade de Aveiro), da Doutora Etelvina Maria de Almeida Paula Figueira (Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro) e da Doutora Iracy Lea Pecora (Professora da Universidade Estadual Paulista "Júlio de Mesquita Filho")

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário através da atribuição da bolsa de doutoramento SFRH/BD/93107/2013.

o júri

Presidente/Chairman	Doutor António José Arsénia Nogueira Professor Catedrático da Universidade de Aveiro
Vogais/Other members	Doutor Francesco Regoli Professor Associado, Università Politecnica Delle Marche
	Doutor Roger John Henwood Herbert Equiparado a Professor Associado, Bournemouth University
	Doutor Amadeu Mortágua Velho da Maia Soares Professor Catedrático, Universidade de Aveiro
	Doutora Rosa de Fátima Lopes de Freitas Investigadora Auxiliar, Universidade de Aveiro

agradecimentos

Este trabalho não teria sido possível sem o apoio de inúmeras pessoas que me acompanharam durante um processo longo de aprendizagem que culmina no desenvolvimento desta tese de doutoramento.

Em primeiro lugar, gostaria de expressar um agradecimento especial às minhas orientadoras Rosa Freitas, Etelvina Figueira e Iracy Pecora, pelo apoio ao desenvolvimento deste trabalho a diversos níveis, desde o acompanhamento científico, técnico e pessoal que me prestaram, assim como uma paciência incansável e amizade demonstrada que não poderia deixar de referir.

Aos meus queridos colegas e amigos de laboratório (por ordem alfabética e portanto sem ordem de preferência): Adília, Ângela, Cátia, Carina, Francesca, José, Lucia, Luísa, Madalena, Paulo, Ricardo, Silvana, Simão e Stefania, entre outros que já partiram ou que estão a entrar, o mais sincero agradecimento também especial, pela mutua partilha de momentos de amizade, trabalho, dúvidas e de superação. Sem o companheirismo e amizade demonstrado por todos eles, toda esta experiêcia não teria sido igualmente gratificante.

À minha família que me apoiou e incentivou em todo este processo, e todos os outros amigos que foram importantes ao longo deste ciclo de estudos. O meu obrigado, e a promessa que terei várias oportunidades de lhes agradecer pessoalmente.

O mais especial agradecimento à Marta, minha companheira, amiga e motivo de orgulho, pelo apoio incondicional, carinho, paciência e compreensão fundamentais para o sucesso neste percurso.

Tenho a agradecer também a inúmeras pessoas que fui conhecendo ao longo destes anos pela partilha de conhecimentos técnicos, ciêntificos e de várias curiosidades essenciais ao desenvolvilento deste estudo:

Jaqueline Araújo, Francisco da Silva e Carlos Henriques do Laboratório de Moluscos Marinhos em Santa Catarina (Brasil), pelos conhecimentos em cultivo de microalgas e ciclo reprodutivo de ostra. Stefania Chiesa e Adília Pires pelos ensinamentos em análise filogenética. Professor Giovanni Libralato pelos conhecimentos em embriotoxicidade. Professora Maria João Bebianno pela introdução à proteómica. Aos senhores Pedro Pereira e Pedro Ferreira pela disponibilidade no fornecimento de exemplares de ostra na Ria de Aveiro e no Estuário do Sado, respectivamente. Ao Instituto para a Conservação da Natureza e das Florestas pelo apoio na recolha de exemplares de *C. angulata* na Reserva Natural do Estuário do Sado. Ao senhor Francisco Coutinho pela disponibilidade no fornecimento de exemplares de *C. brasiliana* no Estuário de Cananéia (Brasil). Ao Laboratório de Moluscos Marinhos pelo fornecimento de exemplares de *C. gigas* no Brasil.

Por fim gostaria de agradecer aos membros do júri, pelas sugestões e comentários construtivos, assim como pela revisão meticulosa do manuscrito aqui apresentado.

Palavras-chave

Ostra, alterações climáticas, *Crassostrea*, performance, estádios de vida, embriotoxicidade, ecotoxicologia, Arsénio, proteómica

Resumo

Ostras do Género *Crassostrea* são representadas por um grupo diverso de espécies com elevada importância ecológica e socioeconómica em ecossistemas costeiros e estuarinos da biosfera. Contudo, o aumento da frequência de fenómenos resultantes de alterações climáticas e de poluição no meio marinho (acidificação, alterações de salinidade e aumento da temperatura, contaminação por arsénio), propicia o aumento do nível de stresse ambiental que poderá colocar em perigo a diversidade de espécies de ostra à escala global. Deste modo, o estudo dos mecanismos de tolerância a diversos stressores e capacidade de resposta de diferentes espécies de ostra, bem como o conhecimento das características ecofisiológicas de cada espécie, poderão revelar-se importantes para a gestão destes importantes recursos biológicos marinhos.

Neste contexto, o objetivo desta tese visou comparar a performance de diferentes espécies de ostra quando expostas a diferentes cenários de alterações climáticas, no sentido de investigar se diferentes espécies apresentam diferentes capacidades de resposta a diferentes stressores, incluindo a presença de poluentes, e inferir no impacto que as alterações globais poderão vir a ter nessas espécies. Assim, um conjunto de ensaios laboratoriais foi efetuado para testar os efeitos da acidificação, alterações de salinidade e o aumento da temperatura, assim como o efeito combinado da exposição a arsénio, em diferentes espécies e estádios de vida de ostra (embriões, juvenis e adultos), através da análise da performance fisiológica, bioquímica e proteómica em resposta a estes stressores.

O estudo focou-se na análise comparativa de espécies de ostra atualmente presentes em ecossistemas de zonas temperadas, *C. angulata* e *C. gigas* (Portugal); e subtropicais, *C. brasiliana* e *C. gigas* (Brasil).

Através da análise comparativa das espécies *C. angulata* e *C. gigas* verificou-se que o desenvolvimento embrio-larvar da espécie *C. angulata* foi mais sensível a alterações de salinidade, temperatura e de arsénio (As) quando comparado com a espécie *C. gigas*. No estádio juvenil, foram também identificadas diferenças na performance de cada espécie aos efeitos da acidificação da água, tanto ao nível da resposta ao stresse oxidativo como ao nível de alterações no proteoma. Neste estádio de vida, os dados obtidos sugerem que a espécie *C. angulata* apresenta mecanismos que conferem maior plasticidade fenotípica em resposta a alterações do meio, que poderão conferir maior capacidade de resposta à acidificação do meio marinho. Contrariamente aos juvenis, os espécimes adultos de ambas as espécies apresentaram menor grau de resposta ao stresse oxidativo quando expostos aos mesmos stressores. Não obstante verificou-se que a capacidade de biomineralização foi potencialmente afetada nestes indivíduos quando expostos à acidificação. De um modo geral, os dados obtidos referentes aos juvenis e adultos sugerem que ambas as espécies apresentam capacidade para sobreviver à acidificação do meio aquático, embora através de mecanismos que acarretam custos energéticos assim como de capacidade de calcificação que poderão vir a ter impactos ao nível das populações. O estudo da resposta a alterações de salinidade revelou alterações relevantes ao nível metabólico, do stresse oxidativo e das reservas energéticas de espécimes adultos de *C. angulata* e *C. gigas*, sem diferenças a assinalar entre espécies. No compto geral, os dados obtidos para as espécies *C. angulata* e *C. gigas* sugerem que a fase de vida embrio-larval representa o estádio durante o qual as vantagens competitivas entre espécies são mais evidentes, sendo que durante este estádio de vida, populações da espécie *C. angulata* poderão vir a sofrer maiores impactos induzidos por alterações de salinidade, temperatura e contaminação por As, resultando na limitação do recrutamento de *C. angulata* a intervalos mais estreitos de condições abióticas.

Através da análise comparativa das espécies *C. brasiliana* e *C. gigas* no Brasil, verificou-se que cada stressor (acidificação e aumento de temperatura) induziu diferentes estratégias de resposta por parte de cada espécie. As diferenças observadas foram mais evidentes nos juvenis, para os quais a espécie *C. gigas* apresentou melhor condição fisiológica em resposta à acidificação, enquanto a espécie *C. brasiliana* demonstrou melhor performance bioquímica em resposta ao aumento de temperatura. Espécimes adultos de ambos os taxa apresentaram menor susceptibilidade aos stressores testados, apesar de demonstrarem estratégias de resposta semelhantes à dos juvenis. No compto geral, verificou-se que ambas as espécies possuem mecanismos de resposta aos stressores investigados, embora os dados obtidos sugerem que a duração e intensidade dos fenómenos extremos de stresse abiótico terão uma influência fulcral na performance das espécies estudadas.

Keywords

Oyster, climate change, *Crassostrea*, performance, life stage, embryotoxicity, ecotoxicology, Arsenic, proteomics

Abstract

Oysters are a diverse group of marine bivalves that present major ecological and socio-economic importance in coastal ecosystems worldwide. However, oyster species are becoming increasingly threatened by climate change related stressors (e.g. seawater acidification, salinity shifts and temperature rise) as well as anthropogenic pollution (e.g. arsenic). Therefore, it is important to increase the baseline knowledge on the stress response capacity of different oyster species towards environmental stress, in order to help predict and manage the fate of these important biological resources in the context of global change.

Hence, the aim of the present thesis was to investigate the comparative performance of different oyster species under several climate change related scenarios, and to project on the future of these species performance in light of global change. To achieve this goal, a series of laboratory based experiments were performed to simulate scenarios of seawater acidification, salinity shifts and temperature rise, as well as the combined exposure to arsenic (As), on different oyster species and life stages (embryo-larvae, juveniles and adults), followed by the assessment of embryo-larvae development, biochemical performance, and proteomic analysis.

Because different regions of the globe will face different climate change insult, this study focused on the comparison of the performance of oysters species from populations currently present in temperate (*C. angulata*, Portugal) and sub-tropical (*C. brasiliana*, Brazil) ecoregions, with that of a worldwide distributed species (*C. gigas*), to understand how climate driven stressors may define species competitive advantages in a changing environment.

Comparative performance of *C. angulata* and *C. gigas*, revealed that each stressor (seawater acidification, salinity and As exposures) induced similar stress response mechanisms in each species, although with differentiated capacities. Differences between species were more evident at the embryo-larvae stage, for which *C. angulata* was clearly more sensitive to changes in ambient salinity, temperature and As than *C. gigas.* At the juvenile life stage, differences in each species stress response signatures were revealed considering both oxidative stress response capacity and alterations at the proteome level. At this life stage, proteomic data suggested higher phenotypic plasticity of *C. angulata* in response to the investigated scenarios, thus likely attributing higher response capacity to seawater acidification in this species. In contrast to juveniles, adult *C. angulata* and *C. gigas* were less responsive to seawater acidification and As exposure, despite that data indicated that biomineralization capacity was impaired. As a corollary, data on juvenile and adults suggested that both *C. gigas* and *C. angulata* presented capacity to tolerate seawater acidification scenarios, despite that the stress response mechanisms involved present associated energetic and biomineralization costs that may have repercutions at the population levels. On the other hand, salinity showed to markedly influence oysters oxidative, metabolic and energetic status, with *C. angulata* likely presenting higher capacity to sustain osmotic stress. Hence, early development stages might represent the bottleneck that may define species competitive advantages towards one another.

Comparative performance of *C. brasiliana* and *C. gigas* (Brazil), revealed that each stressor (seawater acidification and temperature) induced different stress response strategies in each species. Differences between species were more evident at the juvenile stage, for which *C. gigas* presented higher capacity to sustain acidification than *C. brasiliana.* On the contrary, *C. brasiliana* presented better physiological fitness and oxidative status under thermal stress than *C. gigas.* Adult oysters were less susceptible to tested scenarios in both species, despite presenting overall similar response patterns than juveniles. The present data suggest that *C. brasiliana* is more tolerant to thermal stress than *C. gigas,* but in turn may be more sensitive to seawater acidification. Generally, both species presented stress response mechanisms that enabled for survival towards the tested stressors, however data obtained suggests that the time of duration and the intensity of climate change related phenomena such as seawater acidification and thermal stress, will have a crucial influence on both species performance.

Table of contents

Table of Figures

List of publications

Part of the results and figures presented in this thesis are published under the following references:

Moreira, A., Figueira, E., Soares, A.M.V.M., Freitas, R., 2016. The effects of arsenic and seawater acidification on antioxidant and biomineralization responses in two closely related *Crassostrea* species. Science of The Total Environment. 545–546, 569–581. doi.org/10.1016/j.scitotenv.2015.12.029

Moreira, A., Figueira, E., Soares, A.M.V.M., Freitas, R., 2016. Salinity influences the biochemical response of *Crassostrea angulata* to Arsenic. Environmental Pollution. 214, 756–766. doi.org/10.1016/j.envpol.2016.04.036

Moreira, A., Figueira, E., Pecora, I.L., Soares, A.M.V.M., Freitas, R., 2017. Biochemical alterations in native and exotic oyster species in Brazil in response to increasing temperature. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 191, 183–193. doi.org/10.1016/j.cbpc.2016.10.008

Moreira, A., Figueira, E., Pecora, I.L., Soares, A.M.V.M., Freitas, R., 2018. Native and exotic oysters in Brazil: Comparative tolerance to hypercapnia. Environmental Research. 161, 202–211. doi.org/10.1016/j.envres.2017.10.035

Moreira, A., Figueira, E., Libralato, G., Soares, A.M.V.M., Guida, M., Freitas, R., 2018. Comparative sensitivity of *Crassostrea angulata* and *Crassostrea gigas* embryo-larval development to As under varying salinity and temperature. Marine Environmental Research. 140, 135–144. doi.org/10.1016/j.marenvres.2018.06.003

Moreira, A., Figueira ,E., Mestre, N., Schrama, D., Soares, A.M.V.M, Freitas, R., Bebianno, M.J., 2018. Impacts of the combined exposure to Seawater Acidification and Arsenic on the proteome of *Crassostrea angulata* and *Crassostrea gigas*. Aquatic Toxicology. 203, 117–129. doi.org/10.1016/j.aquatox.2018.07.021

1 INTRODUCTION

1.1 Scope

For centuries, oyster reefs formed the dominant structural habitat in temperate estuarine systems of the world. Such biogenic structures are formed by one or few oyster species, that provide the structure for entire ecosystems (Beck et al., 2009). The ecosystem services that oyster reefs provide are broad, but the most important include habitat structure for commercially important fish species and water quality improvement (Coen et al., 2007; Grabowski et al., 2012), but also shoreline defence against coastal erosion (Ridge et al., 2017) and carbon dioxide source and storage (Fodrie et al., 2017). However, these ecosystems are globally threatened, and the majority classified as functionally extinct (Beck et al., 2011).

Factors that influence the loss of important oyster reefs around the globe include overexploitation and habitat degradation (Cranfield et al., 1999; Leniham & Peterson, 1998), pollution (Ruano, 1997), non-native species introduction (Miossec et al., 2009; Ruesink et al., 2005), and disease outbreaks (e.g. Comps et al., 1988; Virvilis & Angelidis, 2006). In addition, predictions for the upcoming decades regarding the threats of climate change related phenomena such as temperature rise, seawater acidification and salinity regime alterations will further challenge oyster species survival worldwide (Brander, 2007; Dekshenieks et al., 2000; Hoehg-Guldberg & Bruno, 2010; Knight, 2017; Levinton et al., 2011). Understanding the factors that will influence oyster species tolerance towards environmental change will increase the baseline knowledge that may enable to protect and manage this important biological resource.

As sessile organisms primarily inhabiting estuarine ecosystems, oysters are highly adapted to daily and seasonal fluctuations of abiotic parameters (e.g. temperature, salinity, CO_2, O_2, aerial exposure, contaminants) characteristic of these dynamic environments (reviewed by Zhang et al., 2016). However, different oyster species hold different ecophysiological traits that resulted from adaptive divergence to specific ecological niches (Ren et al., 2010). Thus, the environmental tolerance range of each species will define competitive advantages between species in a changing environment.

Cupped oysters of the genus *Crassostrea* include several species that together represent one of the most important shellfish cultured at the global level (FAO, 2015). The Portuguese oyster (*C. angulata*), the Pacific oyster (*C. gigas*) and the Mangrove oyster (*C. brasiliana* sin. *C. gasar*, Lazoski et al., 2011) are among the species that most contribute for global oyster aquaculture landings, exceeding 5.5 million tons per annum, approximately 35% of total mollusc aquaculture (Bayne, 2017).

1.2 Oyster species

1.2.1 *Crassostrea angulata* (Lamarck, 1819)

Crassostrea angulata, commonly known as the Portuguese oyster, was first thought to be native to the northeast Atlantic, before mitochondrial and nuclear DNA analysis concluded this species originated from Asia, and had been accidentally introduced in Portugal (Europe) via merchant ships during the 15th century (Foighil et al., 1998; Grade et al., 2016; Huvet, 2004). The expansion of the geographical distribution of the Portuguese oyster in Europe was strengthened during the late 19th century, when *C. angulata* was intentionally exported to France from productive oyster beds in the Tagus estuary (Portugal) to replace the native oyster (*Ostrea edulis*) (Héral, 1989). Few decades later *C. angulata* was the most productive species in Europe, when landings reached ca. 100 000 tons (Batista et al., 2015). However, massive disease epidemics, pushed the species European population to the break of extinction during the late 1960s, which coincided with the introduction of the Pacific oyster (*C. gigas*) to sustain growing demand (Comps et al., 1988).

At present, the geographical distribution of *C. angulata* in Europe is limited to the Iberian Peninsula (Buestel et al., 2009; Batista et al. 2015), where the most pristine population naturally occurs in the Sado estuary (Portugal). Fabioux et al. (2002) also reported the existence of *C. angulata* populations in Northern Africa. Most recently, Hsiao et al. (2016) showed that natural and cultuvated (marine farms) oyster populations in Taiwan and in the southern coast of China, previously thought to be *C. gigas*, were in fact *C. angulata*. These authors highlighted the need to protect this species genetic patrimony considering the eminent threat of environmental change, and suggested that *C. angulata* populations in this region should be most adapted to warmer waters than its closely related congener

C. angulata is considered to be a good model organism to study the effects of several stressors, a factor that has inclusively led researchers to sequence this species genome (Cross et al., 2014), to study the effects of climate change related stressors (Thiyagarajan & Ko, 2012), and of pollutants exposure (Macías-Mayorga et al., 2015; Zhang et al., 2013).

1.2.2 *Crassostrea gigas* (Thunberg, 1793)

The Pacific oyster *C. gigas* is native to coastal waters of Japan, but its current geographical distribution is widespread throughout the globe, mainly due to intentional introductions for aquaculture purposes (Padilla, 2010). The fast growth rate and ability to thrive under a wide range of environmental conditions have made this species preferential choice for worldwide cultivation (Guo, 2009). However, the same biological characteristics that confer this species with high aquaculture interest (high growth rates, adaptability, tolerance to physiological stress and high fertility), also enable for its establishment and posterior expansion into new environments, and consequent dominance over native species (Diederich et al., 2005; Mckindsey, 2007; Reise et al., 2017). The potential invasive nature of this species has therefore been topic of concern, by

either introducing pathogenic agents and consequent mortality events (Ruesink et al., 2005; McKindsey, 2007) or by outcompeting native oyster species (Krassoi et al., 2008).

High socio economic importance, ease of availability and other general characteristics common to bivalve molluscs (e.g. sessile, filter feeding), have led to the extensive study of the Pacific oyster, that include standard toxicity assessments, as well as the study of the combination of several abiotic factors in both early life stages and adults (e.g. Gagnaire et al., 2006; His et al., 1999; Zanette et al., 2011). Furthermore, *C. gigas* was the first mollusc species whose genome was entirely sequenced (Zhang et al., 2012), which in turn prompted the use of this species as model organism on the study of environmental resilience (e.g. Applebaum et al., 2014; Meng et al., 2013; Timmins-Schiffman et al., 2014; Zhang et al., 2015b, among others).

1.2.3 *Crassostrea brasiliana* (Lamarck, 1819)

The Mangrove oyster *C. brasiliana* (sin. *C. gasar*, Lazoski et al., 2011), is the most important native oyster species in Brazilian coastal waters, and is mainly extracted from the natural environment (Gomes et al., 2014; Lazoski et al., 2011; Neto et al., 2013). This species occurs from the northeast state of Pará to the southeast state of Santa Catarina, in rocky subtracts and attached to mangrove roots along the Brazilian coastline (Amaral & Simone, 2014). The most productive *C. brasiliana* natural banks are located in the southern coast of Brazil, in the Cananéia lagoon estuarine system (Galvão et al., 2013; Ristori et al., 2007), which is internationally recognized as one of the most productive ecosystems of the southern Atlantic, declared Natural Heritage Site for knowledge and conservation of human values in 1999, and included in the Atlantic Forest Biosphere by UNESCO in 2005. In this mangrove dominated ecosystem, *C. brasiliana* is one of the main fishery resources for the local communities, with high socio economic importance (Mendonça & Machado, 2010). The introduction of a non-native species (*C. gigas*) in the southern state of Santa Catarina (Brazil) (Melo et al., 2010) may threaten the native *C. brasiliana* given its potential to become invasive, as proven in several countries (e.g. Reise et al., 2017; Ruesink et al., 2005).

Several studies have considered the use of *C. brasiliana* as model organism to study environmental stress, including the assessment of biochemical markers in contaminated sites (Zanette et al., 2006), the impacts of xenobiotics on oysters transcriptome (Lüchmann et al., 2015), and the interactions of environmental salinity on oysters response to pollutants (Zhacci et al., 2017).

1.3 Environmental stressors affecting oysters performance

1.3.1 Climate change related factors

Climate change associated phenomena are considered major threats to coastal waters and low-lying areas of the world (e.g. estuaries, salt marshes, bays, mangroves), that are considered to be the most vulnerable marine ecosystems (Harley et al., 2006; Hoegh-Guldberg & Bruno, 2010). It is therefore expected that oyster species will become increasingly challenged under the eminence of climate change. The most important climate change related drivers that may affect oysters biological performance include seawater acidification, salinity alterations and temperature rise (Ko et al., 2014; Parker et al., 2010; Talmage & Gobler, 2011; Thiyagarajan & Ko, 2012).

Seawater acidification

The uptake of carbon dioxide (CO_2) by the world ocean surface waters is altering global seawater chemistry. Dissolution of CO_2 in seawater leads to a net increase of carbonic acid (H_2CO_3), hydrogen (H^+) and bicarbonate (HCO_3^-) ions concentrations, thus increasing seawater acidity (pH= -log [H^+]). These reactions are followed by a decrease of carbonate ion (CO_3^{2-}) concentration and lower saturation states of aragonite (ΩAr) and calcite (ΩCa). In short, this physico-chemical phenomenon is known as ocean acidification (OA) (Fabry et al., 2008). The International Panel on Climate Change (IPCC) forecasts pCO_2 levels in surface oceanic waters to reach between 490 and 1370 μatm (0.06 to 0.32 pH unit drop), depending on the atmospheric emission scenario (IPCC, 2014), while other authors refer that pH in the ocean will decrease up to 0.5 units by year 2100 (Caldeira & Wickett, 2003; Raven et al., 2005). The impacts of OA are expected to be amplified in low lying marine ecosystems (estuaries, bays, mangroves), where daily and seasonal pH and pCO_2 fluctuations are common features (Miller et al., 2009; Tomanek et al., 2011), where higher amplitude of pH fluctuations (Ringwood & Keppler, 2002) and potentially higher CO_2 levels (e.g. ca. 4000 μatm pCO_2) (Melzner et al., 2013) are expected to be exacerbated by OA.

Because OA may affect the capacity of calcifying organisms to produce and maintain shell or skeleton integrity (Feely et al., 2009), extensive research on the impacts of OA on marine bivalves have been published. Alterations in calcification, shell and skeleton dissolution rates, metabolic shifts and larvae survival are among the most prevailing effects observed in bivalves exposed to OA (reviewed by Gazeau et al., 2013; Parker et al., 2013). Concerning oysters, so far studies showed a great variability on the effects of OA to different species and life stages, namely considering the calcification properties and shell deposition mechanisms, that also depend on ambient pCO_2 (Amaral et al., 2012; Beniash et al., 2010; Dickinson et al., 2012; Ries et al., 2009; Talmage & Gobler, 2011). Adding to effects on calcification dynamics, other studies evidenced alterations in oxidative stress status and basal metabolic costs in different oyster species, namely in *Crassostrea virginica* (Beniash et al., 2010; Tomanek et al., 2011), and *C. gigas* (Wang et al., 2016).

Salinity shifts

Global warming is causing alterations in global atmospheric circulation and precipitation patterns (IPCC, 2014), as well as increased freshwater input into the marine environment by melting ice sheets, glaciers and ice caps (Philippart et al., 2011; Raper & Braithwaite, 2006). Such alterations in planetary hydrological cycle inevitably affects salinity regimes at global and regional scales, with particular impacts to coastal ecosystems (Antonov, 2002; Boyer et al., 2005; IPCC, 2014). The global pattern of salinity shifts indicates that at tropical and sub-tropical latitudes, surface seawater is getting saltier, whereas at higher latitudes seawater is becoming fresher (reviewed by Aretxabaleta et al., 2017).

Salinity is the major environmental factor limiting species survival, richness, biomass and distribution within estuarine systems (Gosling, 2008; Telesh & Khlebovich, 2010). It is likely that climate change induced changes in salinity patterns may affect oyster species performance, including the interactive effects with other stressors (e.g. Schiedek et al., 2007; Zanette et al., 2011). Oysters are osmoconformers, with no ability to osmoregulate the extracellular fluid. Hence, to maintain isosmotic balance, oysters regulate cell volume by accumulating or releasing organic osmolytes (e.g. taurine, betaine) in response to changing salinity. Salinity fluctuations may therefore result in energetically costly processes to maintain isosmotic balance (Eierman & Hare, 2014), thus altering the delicate balance between metabolic performance, oxidative stress and energetic fitness that define organisms acclimation capacity (reviewed by Rivera-Ingraham & Lignot, 2017). Accordingly, recent studies have shown the influence of salinity on oyster species metabolic, oxidative stress and energetic fitness (Fuhrmann et al., 2018; Sokolov & Sokolova, 2018).

Temperature rise

Rising anthropogenic greenhouse gas (e.g. CO_2) emissions have proven to lead to unprecedented oscillations in temperature regimes in several terrestrial and marine ecosystems (Greco et al., 2011; IPCC, 2014). Projections for temperature rise by the end of the 21st century of between 2 and 4 ºC (Hansen et al. 2013; IPCC, 2014) and 2.4 to 6.4 ºC (Smith et al. 2009), are of particular concern regarding the impacts on aquatic ecosystems biodiversity and functions (Brierley & Kingsford, 2009; Doney et al., 2012). Temperature is considered to be the key factor determining zoogeographical patterns, with intertidal ecosystems showing to be particularly susceptible to pronounced and rapid changes (Somero, 2012). Additionally, organisms inhabiting tropical or sub-tropical ecoregions are expected to be more susceptible to temperature rise, mainly because they live closer to their upper-thermal tolerance limit (Tewksbury et al., 2008).

Studies have pointed out several biological consequences of global/regional temperature rise in marine invertebrate species, such as changes in reproduction timing and success (Byrne et al., 2012 and references therein), growth (Talmage et al., 2011), mortality (Garrabou et al., 2009) and shifts in species geographical distribution (Cheung et al., 2009; Somero, 2012). Generally, temperature rise causes a decrement in organisms biological performance, that is tightly linked to aerobic scope (the increase in oxygen consumption rate from resting to maximal)

(Pörtner & Farrell, 2008). In ectothermic metazoans such as oysters, any substantial deviation from the optimum environmental temperature induces the disappearance of aerobic scope, and the onset of partial anaerobiosis, as the organisms performance decays due to a limited capacity for oxygen supply. Shifts in metabolic strategies have implications in energetic fitness and defence mechanisms modulation, that have been integrated in what is called the oxygen- and capacity-limited thermal tolerance concept (Sokolova et al., 2012, and references therein). Importantly, differences in oyster species performance in response to thermal stress have been demonstrated, in what regards to metabolic modulation capacity, energetic trade-offs and oxidative stress response (Li et al., 2017), with likely implications at the population levels under the eminent threat of temperature rise.

1.3.2 Pollutants

In addition to physico-chemical stressors associated to estuaries (e.g. salinity, temperature and pH), these ecosystems are also characterized by anthropogenic pollution, that include inorganic contaminants such as metals and metalloids (Riba et al., 2004; Schropp et al., 1990). Although most research on the impacts of environmental changes has focused on the study of single stressors, such as the effects of salinity, or acidification (e.g. Hamer et al., 2008; Parker et al., 2012), the need to study the interactive effects between natural stressors and contaminants toxicity has been highlighted (Noyes et al., 2009).

Climate change associated phenomena may alter pollutants geochemical cycles, through changes in atmospheric circulation and precipitation patterns (Bromirski et al., 2003; Pisias et al., 2001), that can increase pollutant input (Harley et al., 2006; Robins et al., 2016), thus increasing their bioavailability. Changes in seawater chemistry (e.g. pH) can alter contaminants' chemical speciation, thus potentially altering their toxicity (Sharma & Sohn, 2009). Moreover, alterations in organisms physiological status from changes in seawater physico-chemical characteristics, can also affect oysters stress response capacity to pollutant exposure (Zacchi et al., 2017; Zanette et al., 2011).

Examples of recent studies on the effects of multiple stressors include the assessment of the effects of pharmaceutical drugs at different seawater acidification levels (Freitas et al., 2016a), combined effects of metal/metalloid pollution and ocean acidification (Ivanina & Sokolova, 2013), and interactive effects of metals and temperature (Sokolova & Lannig, 2008), interactive effects of seawater acidification, temperature and metals (Nardi et al., 2017), changes in seawater salinity and its combined effects with contaminants on aquatic organisms performance (Freitas et al. 2016b; Wu et al., 2013).

Arsenic (As), is one of the most widely distributed pollutants, and is highly mobile in nature (Mandal & Suzuki, 2002). Anthropogenic activities (e.g. mining, agricultural pesticides, coal burning) have been increasing environmental As concentrations worldwide (Leermakers et al., 2006), raising public concern due to its high toxicity and carcinogenic properties (Aposhian et al., 2004). In the marine environment, in both seawater and sediment (Fattorini et al., 2006; Neff,

1997), As occurs mainly in its inorganic and more toxic forms (arsenite and arsenate) (Fattorini & Regoli, 2004). Arsenic induced toxicity can be generally attributed to alterations in cellular homeostatic imbalance between prooxidant and antioxidant status, leading to oxidative stress (Samuel et al., 2005). Although oysters are capable of biotransforming inorganic arsenicals into less toxic organic forms (Zhang et al., 2015a) the effects induced by this metalloid can be affected by environmental seawater characteristics. Such has been shown in *Ruditapes philippinarum* clams under different salinities (Velez et al., 2016) and *Mytilus galloprovincialis* mussels (Coppola et al., 2018).

1.4 Oyster response to environmental stress

1.4.1 Embryo-larvae development

Several groups of marine invertebrates such as oysters, present early free-living benthic (at a very early stage after fertilisation) and planktonic life stages (Pechenik, 1999). During the early life stages, larvae sensitivity to environmental stressors is generally higher than juveniles and adults (Beiras and His, 1994; His et al., 1999; Przeslawski et al., 2008). Impacts on these stages can impair the recruitment of adult populations, and thus endanger the populations survival (Byrne et al., 2012).

Under the eminent threat of global climate change, the ability of marine species early life stages to cope with a multitude of environmental stressors will condition species survival and ecosystem functioning (Byrne et al., 2011). Changes in abiotic factors such as salinity and temperature can directly influence the development of marine invertebrates early life stages (Carbalheira et al., 2011; Verween et al., 2007), and can also affect the sensitivity of these stages to pollutants (Coglianese, 1982; His et al. 1999; His et al., 1989).

In oysters species of the *Crassostrea* genus, early free-living life stages include gametes, embryos and larvae (Kasyanov et al., 1998). The early development of *C. gigas* embryos into straight hinged larvae (D-shape) is a process of intense cellular activity, during which any impairment within a series of biochemical and physiological mechanisms can result in malformed larvae (Leverett & Thain, 2013). Due to the cost effectiveness of *C. gigas in vitro* fertilization, ecological relevance and high sensitivity of embryos to common contaminants (Beiras & His, 1994), several embryotoxicity protocols have been developed: Thain (1991), USEPA (1996), His et al. (1997), ASTM (2004), and ISO (2015). Further applications include sediment quality assessment (Geffard et al., 2001; Ghirardini et al., 2005; Libralato et al., 2008), wastewater toxicity (Libralato et al., 2010; Mamindy-Pajany et al., 2010), emergent pollutant screening (Fabbri et al., 2014) and climate change stressors (Gamain et al., 2016).

1.4.2 Biochemical response

The use of enzymatic and non-enzymatic biomarkers as indicators of organisms response to environmental stress have been extensively used, namely through the assessment of oxidative stress status of organisms exposed to pollutants (Monserrat et al., 2007, Regoli & Giuliana, 2014), and most recently to climate change related factors (Matozzo et al., 2013; Velez et al., 2016) including in oysters (Matoo et al., 2013). Oxidative stress is an inevitable consequence of aerobic life, because reactive oxygen species (ROS) are constantly generated as byproducts of aerobic metabolism (Davies, 2000). The most important ROS generated during cellular metabolism are singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$) (Regoli & Giuliana, 2014), which can readily interact with macromolecules (lipids, protein and DNA) and thus impair critical cellular processes (Livingstone, 2003). Organisms have developed adequate antioxidant mechanisms to protect cells from oxidative injury. The onset of oxidative stress is generally described as an imbalance between ROS production rates and the antioxidant defense system (reviewed in Metcalfe & Alonso-Alvarez, 2010).

The antioxidant system comprises a complex network of several antioxidant scavengers that enable cells for aerobic respiration, avoiding the toxic effects of ROS. These include enzymatic and non-enzymatic scavengers. Antioxidant enzymes have been widely used as biomarkers of environmental stress in bivalve species from a wide range of stressors including the exposure to xenobiotics (e.g. Freitas et al., 2014; Regoli et al., 1997), climate change related stressors (e.g. Matoo et al., 2013; Matozzo et al., 2013), as well as to the combined effects of both xenobiotics and climate change parameters (e.g. Moreira et al., 2016; Verlecar et al., 2007). Because organisms hold the capacity to modulate the activity of antioxidant enzymes in response to stress related enhanced ROS formation to prevent oxidative damage, the measurement of antioxidant enzymes (e.g. superoxide dismutase and catalase) activity give indications on the oxidative challenge organisms are subjected under a given scenario (Valavanidis et al., 2006).

Among non-enzymatic antioxidants, glutathione is the most abundant ROS scavenger that plays a central role in cellular redox status maintenance and oxidative stress repair (Rahman, 2007). Reduced glutathione (GSH) serves as electron donor in numerous reductive processes, either directly reducing oxidized molecules, or indirectly as cofactor of several antioxidant/biotransformation enzymes (e.g. Glutathione *S*-transferases, Glutathione peroxidase). The ratio of reduced (GSH) to oxidized (GSSG) glutathione gives a measure of the oxidative stress status of a given organism.

The onset of oxidative damage occurs when the antioxidant system is insufficient or impaired, leading to the reaction of ROS with membrane lipids. This is one of the most important mechanisms of cellular injury, that involves a cascade of oxidation reactions generally termed lipid peroxidation (Halliwell & Gutterige, 1999). Aldehydes such as malonaldehyde (MDA) are among the most important degradation products of the lipid peroxidation chain reaction, and therefore MDA concentration has been used as a measure of cellular membrane peroxidation

resulting from oxidative stress in aquatic organisms including oysters (Amiard-Triquet et al., 2016; Géret et al., 2002; Zanette et al., 2011).

Recent studies have also highlighted the importance of metabolic modulation and energetic fitness in marine invertebrates stress response (Rivera-Ingraham & Lignot, 2017; Sokolova, 2013; Tomanek, 2015). For instance, alterations in mitochondria respiration capacity and electron transport flow through the electron transport chain to modulate ROS production (Harms et al., 2014; Gibbin et al., 2017), and such alterations may be induced as a defence mechanism to prevent ROS mediated damage (Abele et al., 2007; Tomanek, 2015). In line with these notions, metabolic status and energetic fitness have been proposed as important biomarkers to assess environmental stress (Sokolova et al., 2012). Accordingly, biomarkers related to metabolism, such as the electron transport system (ETS) activity, has successfully been used as a measure of metabolic potential in different organisms (Berridge et al., 2005), including the assessment of the effects of environmental factors such as anoxia, temperature, salinity and pollutants in oyster species (García-Esquivel et al., 2001; Le Moullac et al., 2007; Samain & McCombie, 2008). Because the energetic fitness dictates the available energy for antioxidant defence and repair systems (Sokolova et al., 2012), the study of organisms energetic status has gained importance in the field of ecotoxicology and climate change (Rivera-Ingraham & Lignot, 2017). In bivalves, glycogen (GLY) is the most important carbohydrate stored in tissues (Beukema, 1997), and can be utilised as an energy source to sustain several physiological processes (Deslous-Paoli & Héral, 1988). Several studies have assessed GLY content as a measure of bivalves physiological response to environmental stressors such as salinity shifts, pollutant contamination and high *p*CO2 (Carregosa et al., 2014; Velez et al., 2016).

Apart from oxidative stress, metabolic and energy related responses, the biomineralization capacity is an important feature affecting oyster species, especially considering OA (Gazeau et al., 2013). Carbonic anhydrase (CA), an ubiquitous metalloenzyme that plays key roles in a variety of physiological mechanisms involving CO_2 and HCO_3^-, such as osmoregulation, gas exchange, nitrogen metabolism and biomineralization (Lionetto et al., 2000, 2006; Monserrat et al., 2007), is sensitive to pollutant exposure and has been proposed as biomarker for environmental monitoring (Lionetto et al., 2012). This enzyme is especially important in bivalve molluscs, because it participates in calcification and biomineralization processes (Gazeau et al., 2013; Le Roy et al., 2014), and has been identified as one of the most important shell formation enzymes in *C. gigas* (Zhang et al., 2012). In line with these features, CA has been measured as a biomarker for biomineralization in oyster species exposed to environmental stress such as OA and hypoxia (e.g. Beniash et al., 2010; David et al., 2005; Dickinson et al., 2012).

1.4.3 Proteomics

The term “Proteomics” can be generally defined as the analysis of the protein complement of a cell, tissue type or organism, under a specific condition (Yu et al., 2010), including the characterization of protein function, as well as the interactions of these proteins with other molecules (Bradshaw & Burlingame, 2005). The term proteomics can also be applied in more specialized contexts, such as “cell map proteomics” (characterizes all proteins within particular organelles to gain insight in cellular architecture and protein function), and “functional proteomics” (characterizes proteins in a cell, tissue or organisms that undergo changes in response to a specific biological condition) (Smith, 2009). The recent development of Proteomics as a central field of biological research has been largely based on advances in high throughput technologies, namely two-dimensional gel electrophoresis (2DE), and mass spectrometry (e.g. matrix-assisted laser desorption ionization mass, MALDI) (Bradshaw & Burlingame, 2005). Two-dimensional gel electrophoresis (2DE) separates proteins from complex samples in two dimensions: molecular charge and molecular size. Each protein is therefore resolved at a unique isoelectric point/molecular size coordinate, enabling to visualise and measure differential expression of each protein with specialized software. Proteins of interest can then be excised from the gel and identified by mass spectrometry sequence analysis and database comparison (Smith, 2009). A large number of proteins can therefore be analysed simultaneously, providing complex protein expression signatures, together with quantitative information on the response of individual proteins and the underlying biological functions associated to those proteins (Thompson et al., 2012).

Because proteins constitute the organisms biochemical machinery, and thus represent the functional processes towards environmental stress, proteomic approaches have been recently applied to study the modes of action (MoA) involved in stress adaptation of marine bivalves in response to external stimuli (reviewed in Tomanek 2014, 2015). In fact, an extensive body of research on oyster species proteomic response to climate change associated stressors, as well as to pollutants, have been published (e.g. Harney et al., 2016; Muralidharan et al., 2012; Timmins-Schiffman et al., 2014; Tomanek et al., 2011; Zhang et al., 2015b; among others). Additionally, due to the high throughput of such approaches, proteomics allows comparisons between closely related congeners and discriminate if different environmental stressors induce different stress response signatures (Tomanek, 2015). For instance, proteomics has been applied to assess closely related *Mytilus* congeners (Tomanek et al., 2012), and different breeding lines of *Saccostrea glomerata* oysters (Stapp et al., 2017; Thompson et al., 2016) in response to different environmental stressors (e.g. thermal, osmotic and hypercapnic stress).

1.5 Aims of the present thesis

In context with the topics outlined in the previous sections, the present thesis aimed to answer the following questions: i) How do different oyster species respond to climate change related phenomena? ii) How does the combination of climate change and pollutant exposure influence oyster species performance? iii) Do different life stages present different susceptibilities to these stressors?

To answer these questions, a series of laboratory based experiments were conducted on different oyster species and life stages, to simulate scenarios of seawater acidification, salinity shifts, temperature rise, and the combined effects of a model pollutant. The following endpoints were selected to infer on species comparative performance: embryo-larval development; biomarkers of oxidative stress, biomineralization capacity, metabolic potential and energetic fitness; and 2DE-Proteomics.

2 MATERIAL & METHODS

2.1 Study organisms

Crassostrea angulata specimens were collected in the Sado estuary – Portugal. Progenitors for embryo-larvae experiments, and juveniles (2.6-3.8 cm shell height, 1.9-2.3 cm shell length) for exposures were provided by an aquaculture facility operating in the Sado estuary, Portugal. Adult specimens were collected in natural intertidal banks, where oyster agregations grow on top of soft sediment (6.9-7.6 cm shell height, 4.4-5.3 cm shell length).

Crassostrea gigas progenitors for embryo-larvae experiments were obtained from Guernsey Sea Farms (United Kingdom). Juveniles (2.6-3.8 cm shell height, 1.9-2.3 cm shell length) were obtained from an aquaculture facility operating in the Sado estuary – Portugal. Adult oysters were provided by an aquaculture facility operating in the Ria de Aveiro estuary – Portugal (7.2-8.1 cm shell height, 4.5-4.9 cm shell length).

Crassostrea brasiliana specimens were collected from submerged oyster racks in the Cananéia estuary (25°00′29.50″S 48°01′29.35″W) in the Extractive Reserve of the Mandira – Brazil. Average shell height of *C. brasiliana* juveniles was 4.0 ± 0.8 cm, and 7.2 ± 0.4 cm in adults.

Crassostrea gigas specimens studied in Brazil were provided by the Laboratory of Marine Molluscs of the University of Santa Catarina – Brazil. Average shell height of *C. gigas* juveniles was 4.2 ± 0.2 cm, and 7.8 ± 0.3 cm in adults.

Phylogenetic lineage confirmation

Due to morphological resemblance between oyster species, the phylogenetic lineage of each species batch studied (subsamples, n=10) was confirmed, based on mitochondrial cytochrome oxidase subunit 1 (COI) gene sequencing and analysis (excluding *C. gigas* progenitors from Guernsey Sea Farms). Genomic DNA extractions were made with NZY Tissue gDNA Isolation Kit (NZYTech) using oysters adductor muscle.

C. angulata and *C. gigas* COI was amplified with primers designed by Folmer et al. (1994) and amplification cycles described in Reece et al. (2008). *C. brasiliana* COI was amplified with primers and amplification cycles described in Melo et al. (2010). Nucleotide sequencing of each purified Polymerase Chain Reaction (PCR) product was commercially performed by STABvida (Portugal). Blast and multiple alignments of sense and antisense sequences were conducted with MEGA v6, using default alignment settings of the CLUSTALW algorithm (Tamura et al., 2013). Phylogenetic relationships were calculated by Maximum likelihood (ML) reconstruction method (Jukes-Cantor model), with MEGA v6 software. Node support was assessed by 1000 bootstrap value replicates.

Analysed COI sequences were deposited in GenBank, under assession numbers KT932101 to KT932019 (*C. angulata*); KT932093 to KT932100 (*C. gigas* – Portugal); KX436134 to KX4361421 (*C. brasiliana*); KX436124 to KX436133 (*C. gigas* – Brazil)

2.2 Experimental assays

2.2.1 *Crassostrea angulata* and *Crassostrea gigas* (Portugal)

2.2.1.1 Embryo-larvae development

The sensitivity of *C. angulata* and *C. gigas* embryo-larval development was assessed considering various exposure scenarios including changes in arsenic (As) concentration, salinity and temperature levels. Embryotoxicity tests on *C. angulata* and *C. gigas* were carried out considering a range of As concentrations (30, 60, 120, 240, 480, 960 and 1920 µg L^{-1}), different salinity (20, 26 and 33) and temperature (20, 24 and 28 ºC) levels, as well as different time of exposure (24 and 48 h) to investigate: i) the effect of varying salinity and temperature on embryo-larval development; ii) As embryotoxicity; and iii) the effects of varying salinity and temperature on embryos sensitivity to As.

Experimental setup

Experiments were conducted using previously cleaned and sterilised glassware for the entire experimental setup. Analytical grade artificial seawater (Tropic Marine Sea Salt) from the same batch was used for exposure media preparation and spawning, prepared according to the manufacturer's instructions using reverse osmosis (RO) water, 3 days before the experiments took place too achieve a salinity of 33 (i.e. reference salinity) (Leverett & Thain, 2013). After complete salt dissolution and equilibration (24 h) seawater was filtered (0.2 µm) through cellulose acetate filters (Millipore) using a vacuum filtration unit. Seawater salinity was adjusted to obtain 3 separate batches at three different salinity levels (20, 26 and 33 ± 1) for exposure media preparation using RO water as dilution media (measured pH for all batches of seawater used ranged from 8.16 – 8.29 (Hanna-Instruments)). Salinity levels were in accordance to Moreira et al. (2018), that showed As toxicity to *C. gigas* embryos could restrict embryo-larval development within 20-33 of salinity.

A stock solution of sodium arsenate (Na_3AsO_4) (CAS no. 10048-95-0, Sigma-Aldrich) was prepared in ultra-pure water and spiked in separate volumetric vessels to achieve nominal As concentrations of 30, 60, 120, 240, 480, 960 and 1920 ® L^{-1}, for each target salinity. Exposure concentrations were chosen based on previous studies on *C. gigas* embryonic development and As exposure (Mamindy-Pajany et al., 2013; Martin et al., 1981). Exposure solutions were distributed in 24-well sterile capped polystyrene microplates (VWR), giving one microplate per salinity level, 3 wells (3 mL each) per exposure condition (As concentration) including negative controls. Each microplate corresponding to salinity and As conditions, were 3 fold replicated for incubations at different temperatures (20, 24 and 28 ºC) to test ± 4 °C from control temperature (24 ºC), within projected global surface temperature rise by the end of the 21st century relative to years 1986–2005 (RCP8.5) (IPCC, 2014), and within a temperature range that would allow to infer on embryo development under As exposure based on previous data (Moreira et al., 2018).

All the microplates were further replicated twice, to test all conditions after two different timeframes of embryonic development, to allow for the assessment of As induced delayed development to oyster embryos (Moreira et al., 2018), while testing valid exposure time criteria of 24 h (His et al., 1999) and 48 h (Knezovich et al., 1981) for oyster species (*C. gigas*). Each microplate was incubated at the desired temperature overnight in separate climatic chambers (±1 ºC) before spawning induction took place to stabilize testing media at the target temperature. Copper ($Cu(NO_3)_2$) was used as reference toxicant (positive control) (Libralato et at., 2009) at 3, 6, 12, 18 and, 30 µg L^{-1} of Cu, for which incubations took place at standard salinity 33 and temperature 24 ºC.

Each stock of seawater used for the embryotoxicity assays (at every combination of salinity and As concentration) were analysed by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) to determine effective As concentrations for each condition. Multi-Element Standard IV - 71A (Inorganic Ventures) was used as standard for As quantification, and calibration curve verified with standard reference material (NIST SRM 1643f).

Spawning and fertilization

Spawning was induced by thermal stimulation, by consecutively changing oysters from seawater baths set at 18 and 28 ºC in 30 min consecutive intervals (Libralato et al., 2007). Gamete emission and quality (oocyte shape and sperm motility) were visually inspected under a microscope. Six male and five female *C. angulata* were selected for fertilization and spawning, while four male and three female *C. gigas* were selected.

Selected females were left to spawn in separate beakers, oocyte suspensions were filtered through a 100 µm nylon mesh and mixed in a final 500 mL suspension (salinity 33, 24 ºC). Male gametes were collected in separate, filtered through a 45 µm nylon mesh into a mixed suspension, and left to activate for 20 min. Oocyte suspensions were fertilized by adding approximately 1 to 10^6 oocyte-to-sperm ratio, and fertilization success verified by microscopy.

Zygotes were immediately transferred to microplates containing the exposure media to reach approximately 200 embryos per well. Microplates with the exposure media (0, 30, 60, 120, 240, 480, 960 and 1920 µg L^{-1} of As) at different salinities (20, 26, 33) and fertilized oocytes were left to incubate in the dark, at different temperatures (20, 24 and 28 ±1 ºC) for 24 and 48 h post fertilization.

After incubation, embryo-larval development was stopped by adding buffered formalin (4%). Analysis followed visual inspection of embryo-larval development under an inverted microscope and camera (Leica: DMIL-1; MC170 HD) by counting 100 embryos per well, and characterizing the relative frequencies of different types of development (D-shape, pre-D, protruded mantle, kidney shape, indented shell and dead larvae) according to His et al. (1997) (Figure 1).

The validity of the embryotoxicity experiments was inferred by results obtained in the negative and positive controls. In the negative control (salinity 33, 24 ºC, 24 h) frequencies of well-developed D-shape larvae (>70%), were within the accepted values described in standard

embryotoxicity protocols that consider acceptable frequencies of normal developed larvae (D-shape) should be ≥ 70% (ASTM, 2004; His et al., 1999), after 24 h incubation at standard salinity (33) and temperature (24 ºC). Results obtained in positive controls (Cu) (11.40 (9.59-13.54) µg L^{-1} of Cu) were within the toxicity thresholds previously reported for Cu in oyster *C. gigas* (9.47 µg $L^{-1} \leq$ EC50 ≤ 21.72 µg L^{-1}) (Libralato et al., 2009).

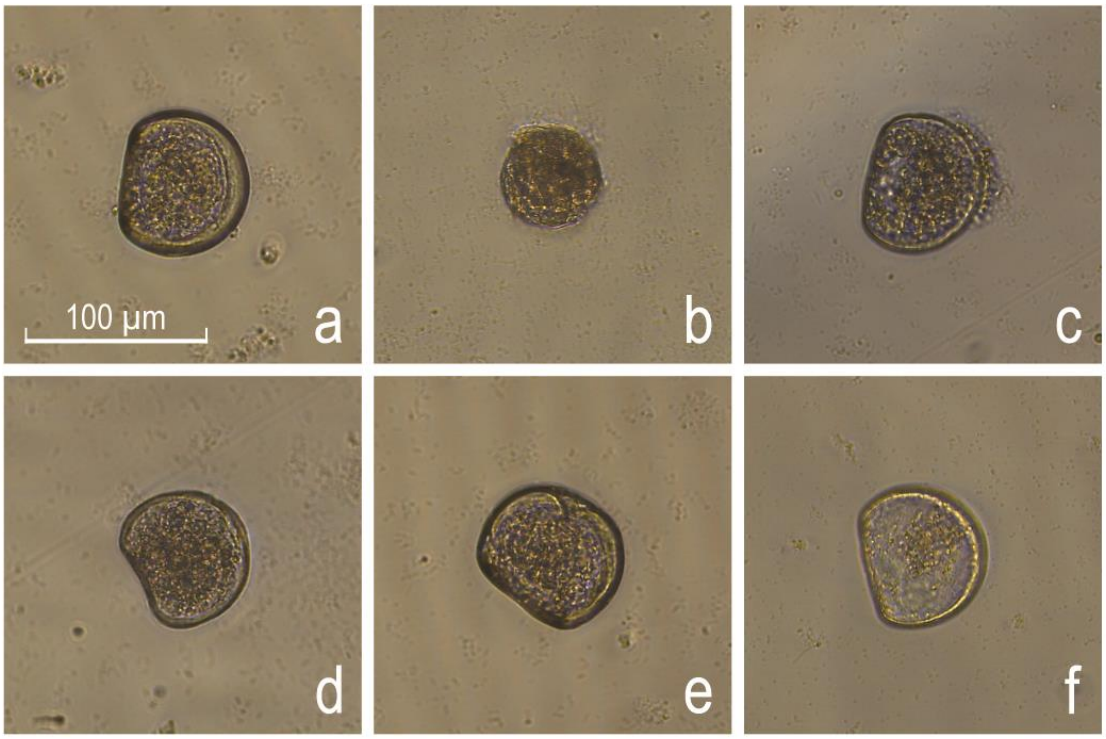

Figure 1 - Embryo-larvae types observed in *Crassostrea angulata* and *C. gigas*.

Photographic record of embryo-larvae types observed in *C. angulata* and *C. gigas* after embryotoxicity exposures: a) D-shape; b) pre-D; c) protruded mantle; d) kidney shape; e) indented shell; and f) dead larvae after His et al. (1997).

2.2.1.2 Chronic assays with juveniles and adults

The performance of *C. angulata* and *C. gigas* (juveniles and adults) was assessed considering several exposure scenarios that included simulation of seawater acidification, salinity shifts, and the combinations of the former stressors with As exposure.

Chronic assays (28 days) were conducted on juvenile and adult specimens considering simulated seawater acidification (-0.4 or -0.5 pH units towards control pH); different salinity (10, 20, 30, 40), in the presence or absence of As (0.5 or 4 mg L^{-1} in juveniles and adults, respectively).

Endpoints assessed after laboratory exposures included: determination of total As accumulation in oyster tissues, biochemical parameter assessment and 2-DE proteomic analysis, to investigate: i) the effects of seawater acidification on oyster species performance (in the presence or absence of As); ii) the effects of salinity on oyster species performance (in the presence or absence of As).

Seawater acidification and Arsenic exposure

Experimental setup - juveniles

Juvenile *C. angulata* and *C. gigas* were acclimated to laboratory conditions for 6 weeks in 400 L tanks with recirculated artificial seawater (Tropic Marine Sea Salt) set at 30 ± 1 salinity, 17 ºC (Hailea), UV filtration (TMC Vecton^2), protein skimming (Deltec), and a total recirculation rate of 3000 L h^{-1} (Eheim). During the acclimation period seawater was partially renewed every 2 days (30%) and completely renewed every week. After the first week of acclimation, oysters were fed live microalgae (*Isochrysis galbana* and *Chaetoceros calcitrans*) 5 days per week, at a daily ration of ca. 10^9 cells L^{-1} day^{-1} (T-*Iso* equivalents cells).

After acclimation, oysters were transferred to 20 L aquaria (10 oysters per aquarium) in a triplicate design. Aquaria were filled with fresh seawater and were used to simulate 4 separate conditions: control (CTL), As exposure (As), seawater acidification (Low pH) and the combination of seawater acidification and As exposure (Low pH+As). Oysters that were subjected to seawater acidification (Low pH and Low pH+As) were previously acclimated to acidification by -0.2 pH units per day until targeted pH value was achieved.

For As exposure, a stock solution of sodium arsenate (Na_3AO_4) (CAS no. 10048-95-0) was directly spiked into aquaria to achieve a final concentration of 500 µg As L^{-1}. Arsenic exposure concentration was chosen considering: i) previous studies that showed sublethal effects of As to adult oysters (Moreira et al., 2016; Zhang et al., 2015a); median effect concentrations to oyster embryo-larval development (Mamindy-Pajany et al., 2013; Martin et al., 1986; Moreira et al., 2018); iii) data on resuspended As concentrations in the water column (Ereira et al., 2015).

Seawater acidification was achieved by pH manipulation using a pH stat system (Aquamedic) targeting a 0.4 pH unit decrease from CTL conditions (pH = 8.0) to pH 7.6. The chosen pH was based on: i) the average pH in the Sado Estuary sampling area (Amaral e Costa, 1999); ii) present global oceanic seawater pH and predicted pH levels by year 2100 (Caldeira and Whickett, 2003; Raven et al., 2005); and pCO_2 levels (ca. 600 and 1650 µatm) within those

predicted for estuarine systems under present and future acidification conditions (Melzner et al., 2013; Tomanek et al., 2011). To achieve this, independent pH probes were used to constantly monitor each aquaria pH in all acidified conditions. Probes were linked to a central computer (Aquamedic) that enabled to automatically switch on or off a dedicated solenoid valve for each aquarium, enabling for CO_2 gas to flow through glass diffusers and maintain targeted pH levels. Each pH electrode measurement was crosschecked with that of an independent probe (Hanna Instruments) at least every two days, and the pH stat system electrodes recalibrated if necessary.

Experimental exposures followed 28 days. Maintenance procedures included daily faecal debris removal (ca. 5% water change), feeding (*I. galbana* and *C. calcitrans* at 10^9 T-*Iso* equivalent cells L^{-1}) at least 5 days per week, As concentration replenishment, pH monitoring and total weekly water renewals. Prior to complete water renewals pH, salinity and temperature were measured (Hanna Instruments), and seawater samples collected from each aquarium to determine total alkalinity (TA). TA was determined by potentiometric titration (Gran, 1952) for each aquarium every week, and data obtained were plotted together with pH, temperature and salinity measurements corresponding to the time of each sample collection in CO2SYS Calc software (Robbins, 2010) to further characterize seawater carbonate system parameters using K1 and K2 CO_2 dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and $KHSO_4$ from Dickson (1990) (Table I). After 28 days exposure period, oysters were immediately frozen in liquid nitrogen, and stored at -80 ºC for further analysis.

Table I - Carbonate system parameters of the chronic assay on juvenile *Crassostrea angulata* and *C. gigas* exposed to seawater acidification and As. pH, total alkalinity (TA), partial CO_2 pressure (pCO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ion concentrations, calcite (ΩCal) and aragonite (ΩAg) saturated states (mean ± standard deviation, n=4).

C. angulata							
	pH	TA ($\mu mol\ kg^{-1}$)	pCO_2 (μatm)	HCO_3^- ($\mu mol\ kg^{-1}$)	CO_3^- ($\mu mol\ kg^{-1}$)	ΩAr	ΩCa
CTL	8.01±0.03	2046±54	576±34	1776±49	110.2±5.2	2.7±0.1	1.8±0.09
As	7.99±0.02	2103±41	599±28	1830±37	110.2±4.9	2.7±0.1	1.7±0.08
Low pH	7.59±0.03	2087±61	1633±110	1970±56	46.4±3.6	1.1±0.08	0.7±0.05
Low pH+As	7.59±0.02	2129±44	1734±99	2015±42	46.4±2.2	1.1±0.05	0.7±0.03
C. gigas							
	pH	TA ($\mu mol\ kg^{-1}$)	pCO_2 (μatm)	HCO_3^- ($\mu mol\ kg^{-1}$)	CO_3^- ($\mu mol\ kg^{-1}$)	ΩAr	ΩCa
CTL	7.99±0.02	2001±58	586±21	1743±49	102.8±4.7	2.5±0.1	1.6±0.07
As	7.98±0.04	1996±59	577±13	1739±46	102.8±5.9	2.5±0.1	1.6±0.09
Low pH	7.57±0.01	2040±64	1732±84	1933±63	42.7±1.7	1.1±0.04	0.7±0.03
Low pH+As	7.58±0.02	2027±62	1654±62	1920±55	43.8±3.2	1.1±0.08	0.7±0.05

Experimental setup – adults

Acclimation to laboratory conditions followed one month prior to the beginning of exposures. During this period, species were maintained in separate tanks, 110 L tanks (0.4 ind L^{-1}), with constant filtration and water circulation at 1200 L h^{-1} in artificial seawater (Tropic Marin) salinity 29, 20±1 ºC, fed 5 days per week *I. galbana* and *C. calcitrans* ca. 10^9 cells L^{-1} (T-iso equivalent cells).

After the acclimation period, oysters were transferred to 20 L aquaria (3 oysters per aquarium) in a triplicate design. Aquaria were filled with fresh seawater and were used to simulate 4 separate conditions: control (CTL), As exposure (As), seawater acidification (Low pH) and the combination of seawater acidification and As exposure (Low pH+As). Oysters that were subjected to seawater acidification (Low pH and Low pH+As) were previously acclimated to acidification by -0.2 pH units per day until targeted pH value was achieved

For As exposure, a stock solution of sodium arsenate (Na_3AO_4) (CAS no. 10048-95-0) was directly spiked into the aquaria to achieve a final concentration of 4 mg L^{-1}, a sublethal As concentration to another oyster species (Zhang et al. 2015).

Seawater acidification was achieved by pH manipulation using a pH stat system (Aquamedic) targeting a 0.5 pH unit decrease from CTL conditions (pH = 7.8) to pH 7.3 (3100 µatm pCO_2). The chosen pH was based on: i) the average pH in the Sado Estuary and Ria de Aveiro of the sampling area (Amaral & Costa, 1999; Coelho et al., 2014); ii) predicted pH levels by year 2100 (Caldeira and Whickett, 2003; Raven et al., 2005); and pCO_2 levels (ca. 780 and 3100 µatm pCO_2) within those predicted for estuarine systems under present and future acidification conditions (Melzner et al., 2013; Tomanek et al., 2011). To achieve this, independent pH probes were used to constantly monitor each aquaria pH in all acidified conditions. Probes were linked to a central computer (Aquamedic) that enabled to automatically switch on or off a dedicated solenoid valve for each aquarium, enabling for CO_2 gas to flow through glass diffusers and maintain targeted pH levels. Each pH electrode measurements were crosschecked with an independent probe (Hanna Instruments) at least every two days, and the pH stat system electrodes recalibrated if necessary.

Experimental exposures followed 28 days. Maintenance procedures included daily faecal debris removal (ca. 5% water change), feeding (*I. galbana* and *C. calcitrans* at 10^9 T-*Iso* equivalent cells L^{-1}) at least 5 days per week, As concentration replenishment, pH monitoring and total weekly water renewals. Prior to complete water renewals pH, salinity and temperature were measured (Hanna Instruments), and seawater samples collected from each aquarium to determine total alkalinity (TA). TA was determined by potentiometric titration (Gran, 1952) for each aquarium every week and data obtained were plotted together with pH, temperature and salinity measurements corresponding to the time of each sample collection in CO2SYS Calc software (Robbins, 2010) to further characterize seawater carbonate system parameters using K1 and K2 CO_2 dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero

(1987) and $KHSO_4$ from Dickson (1990) (Table II). After 28 days exposure period, oysters were immediately frozen in liquid nitrogen, and stored at -80 ºC for further analysis.

Table II - Carbonate system parameters of the chronic assay on adult *Crassostrea angulata* and *C. gigas* exposed to seawater acidification and As. pH, total alkalinity (TA), partial CO_2 pressure (pCO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ion concentrations, calcite (ΩCal) and aragonite (ΩAg) saturated states (mean ± standard deviation, n=4).

C. angulata							
	pH	TA ($\mu mol\ kg^{-1}$)	pCO_2 (μatm)	HCO_3^- ($\mu mol\ kg^{-1}$)	CO_3^- ($\mu mol\ kg^{-1}$)	ΩAr	ΩCa
CTL	7.79±0.04	2180±122	769±102	1946±120	96.9±9.6	1.5±0.1	2.4±0.2
As	7.82±0.03	2274±181	728±47	2008±150	108.5±15.7	1.7±0.2	2.7±0.4
Low pH	7.27±0.03	2574±228	3213±390	2488±223	36.1±3.4	0.6±0.05	0.9±0.08
Low pH+As	7.28±0.03	2524±178	3100±393	2438±176	35.8±1.5	0.6±0.02	0.9±0.09
C. gigas							
	pH	TA ($\mu mol\ kg^{-1}$)	pCO_2 (μatm)	HCO_3^- ($\mu mol\ kg^{-1}$)	CO_3^- ($\mu mol\ kg^{-1}$)	ΩAr	ΩCa
CTL	7.76±0.04	2133±130	797±124	1911±133	89.4±5.9	1.4±0.09	2.2±0.2
As	7.82±0.03	2270±119	728±65	2010±100	106.4±13.1	1.7±0.2	2.6±0.3
Low pH	7.27±0.04	2533±237	3178±446	2446±231	36.1±3.9	0.6±0.06	0.9±0.1
Low pH+As	7.27±0.03	2550±247	3180±393	2464±241	36.1±3.6	0.6±0.06	0.9±0.04

Salinity and Arsenic exposure

Experimental setup - adults

Laboratory exposures were performed with *Crassostrea angulata* and *C*. *gigas* to infer on species stress response to different salinity levels in the presence or absence of As.

Acclimation to laboratory conditions followed 6 weeks prior to the beginning of exposures. During this period, species were maintained in separate tanks, 110 L tanks (0.4 ind L^{-1}), with constant filtration and water circulation at 1200 L h^{-1} in artificial seawater (Tropic Marin) salinity 30, 20±1 ºC, and were daily fed (*I. galbana* and *C. calcitrans* (10^9 cells L^{-1} initial cell density).

After the acclimation period, oysters were transferred to 20 L aquaria (3 oysters per aquarium) in a triplicate design. Aquaria were filled with fresh seawater (salinity 30) and were used to simulate 4 salinity levels (10, 20, 30 and 40), in both the presence or absence of As. Acclimation to salinity levels was previously conducted by increasing, or lowering salinity at a rate of 2 units every 2 days to achieve testing salinities 10, 20 and 40, in the respective testing groups after 6 weeks acclimation.

For As exposure, a stock solution of sodium arsenate (Na_3AO_4) (CAS no. 10048-95-0) was directly spiked into the aquaria to achieve a final concentration of 4 mg L^{-1}, a sublethal As concentration to another oyster species (Zhang et al., 2015a).

Oysters were exposed to each condition for a period of 28 days. Daily procedures included fecal debris removal during 10% partial water changes, proportional contamination replenishment for As exposure aquaria, and feeding. Live microalgae were provided 5 days a week, as described above. At the end of the experiment, each oyster was opened, tissue was immediately frozen using liquid nitrogen, and samples stored at -80 ºC. Samples were manually homogenized in liquid nitrogen, using a mortar and pestle. Each specimens homogenate was further separated in 0.5 g aliquots for biochemical analyses.

2.2.2 *Crassostrea brasiliana* and *Crassostrea gigas* (Brazil)

2.2.2.1 Chronic assays with juveniles and adults

The performance of *C. brasiliana* and *C. gigas* (juveniles and adults) was assessed considering several exposure scenarios that included simulation of seawater acidification, and thermal stress.

Chronic assays (28 days) were conducted on juvenile and adult specimens considering different acidification levels (pH 7.8, 7.4 and 7.0) and temperature (24, 28 and 32 ºC). Endpoints assessed after laboratory exposures included the determination several biochemical parameters to investigate: i) the effects of acidification on oyster species performance; ii) the effects of thermal stress on oyster species performance.

Acclimation to laboratory conditions followed one week prior to the beginning of exposures. During this period, juvenile and adult specimens were maintained in separate tanks, in recirculated artificial seawater (Ocean Fish – Prodac) (pH 7.8; temperature 24 ºC, salinity 25) and daily fed with AlgaMac Protein Plus (10^9 cells L^{-1} initial cell density).

Seawater acidification

Experimental setup – juveniles and adults

After acclimation, oysters were randomly distributed in 50 L aquaria with individual filters and circulation pumps (500 L^{-1} total water flow). Each condition was replicated in three separate aquaria, stocked with 4 adults and 8 juveniles each (n=12 adults and n=24 juvenile). Oysters that were exposed to intermediate (pH 7.4) and to high acidification (pH 7.0) were progressively acclimated to acifified seawater by lowering 0.2 pH units per day until targeted pH values were achieved. Three different pH levels were tested, that corresponded to pCO_2 of 1000 µatm (pH 7.8), 4000 µatm (pH 7.4) and 10 000 µatm (pH 7.0). Acidification levels were selected based on maximum pH recorded during summer (pH 7.8) in submerged oyster beds in the Cananéia estuary (i.e.: pH 7.85, Miraldo and Valenti, unpublished data); an intermediate acidification level pH 7.4 (4000 pCO_2) (Melzner et al., 2013); and high acidification pH 7.0 (10 000 µatm pCO_2) based on reported *p*CO2 in estuarine systems worldwide (Cai, 2011).

To achieve targeted pH levels, food grade CO_2 was diffused into each aquarium (conditions pH 7.4 and pH 7.0) through bubble-counter CO_2 diffusers, at gas releasing rates that were pre-established for each condition, and regulated through six-needle valves (ISTA Products) allowing for constant and stable gas flow (Duarte et al., 2014). During the entire experimental procedures, pH of each tank was measured and checked three times per day (Hanna Instruments).

After pH equilibration in testing aquaria exposures carried on for 28 days. During this period water parameters (temperature, dissolved oxygen, salinity) were daily monitored (YSI Pro plus). Faecal debris were removed prior to feeding (AlgaMac Protein Plus) 5 days a week, giving partial water renewals of 5%. Oysters were checked for mortality on a daily basis. Water samples were collected every week, prior to total water renewals to determine total alkalinity (TA) for each

aquarium by potentiometric titration (Gran, 1952) with an automatic titrator (Mettler Toledo). Determined TA for each aquarium was plotted against pH, temperature and salinity average values measured during each week on CO2SYS Calc software, to determine carbonate system variables (Robbins et al., 2010), using dissociation constants K1 and K2 from Mehrbach et al. (1973) refit by Dickson and Millero et al. (1987) and KSO_4 from Dickinson (1990) (Table III). At the end of the experiment (28 days), oysters were frozen at -80 ºC until further analysis.

Table III - Carbonate system parameters of the chronic assay on juvenile and adult *Crassostrea brasiliana* and *C. gigas* exposed to hypercapnia. pH, total alkalinity (TA), partial CO_2 pressure (pCO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ion concentrations, calcite (ΩCal) and aragonite (ΩAg) saturated states (mean ± std, n=4).

C. brasiliana							
Condition	pH	TA ($\mu mol.Kg^{-1}$)	pCO_2 (µatm)	HCO_3^- ($\mu mol.kg^{-1}$)	CO_3^{2-} ($\mu mol.kg^{-1}$)	ΩCal	ΩAra
pH 7.8	7.78±0.02	1920±106	1068±65	1764±99	63.6±4.8	1.7±0.1	1.1±0.09
pH 7.4	7.38±0.03	2508±184	3751±282	2428±176	34.4±4.2	0.9±0.1	0.6±0.07
pH 7.0	7.00±0.03	2789±96	9992±1023	2751±97	16.7±1.4	0.4±0.04	0.3±0.02
C. gigas							
Condition	pH	TA ($\mu mol.Kg^{-1}$)	pCO_2 (µatm)	HCO_3^- ($\mu mol.kg^{-1}$)	CO_3^{2-} ($\mu mol.kg^{-1}$)	ΩCal	ΩAra
pH 7.8	7.78±0.02	2087±91	1182±80	1937±99	69.0±5.6	1.8±0.2	1.1±0.1
pH 7.4	7.38±0.02	2679±83	3927±246	2591±81	37.5±2.1	1.0±0.1	0.6±0.04
pH 7.0	7.01±0.04	2881±103	10101±934	2840±101	17.7±1.8	0.5±0.05	0.3±0.03

Thermal stress

Experimental setup – juveniles and adults

Oysters were acclimated to laboratory conditions upon arrival, during one week prior to the beginning of exposures. During this period, juvenile and adult specimens were maintained in separate tanks, in aerated artificial seawater (Ocean Fish – Prodac) (Temperature 24 ºC, Salinity 29) and daily fed with AlgaMac Protein Plus (initial cell density of 10^9 cells per L^{-1}). After one-week acclimation, oysters were randomly distributed into testing chambers, that consisted of 10 L tanks with individual air flow biological filters. Juveniles and adults were maintained in separate (4 juveniles and 2 adults per chamber). Three different temperature levels were tested (24, 28 and 32 ºC) in a triplicate design. Acclimation to increased temperature levels (28 and 32 ºC), was achieved by increasing 1 ºC per day until the chosen testing values were reached in all testing groups. Temperature levels were chosen based on present (24 ºC), and maximum (28 ºC) temperature at the Cananéia estuary (Brazil); and a future scenario (32 ºC) considering forecasted surface temperature rise of 4 ºC by the end of the 21st century (Solomon, 2007). Experimental temperature levels were controlled using thermostats in water baths surrounding each group of chambers.

Exposures to different temperatures were conducted during 28 days. Water parameters (temperature, dissolved oxygen and salinity), were daily monitored (YSI Pro plus). Chambers were daily checked for mortality. Faecal debris were removed prior to feeding (AlgaMac Protein Plus) 5 days a week, giving partial water renewals of approximately 10%. Total water renewals were performed weekly. At the end of the experiment (28 days) oysters were frozen at -80 ºC until further analysis.

2.3 Biochemical analysis

A suit of biochemical biomarkers were assessed in oyster samples of juvenile and adult specimens after laboratory exposures. These included the determination of antioxidant enzymes activity: superoxide dismutase (SOD) and catalase (CAT); biotransformation capacity: glutathione *S*-transferases (GSTs) activity; redox status: ratio between reduced (GSH) and oxidized (GSSG) glutathione; membrane damage: lipid peroxidation (LPO) levels; metabolic potential: electron transport system (ETS) activity; energetic fitness: glycogen (GLY) content; biomineralization capacity: carbonic anhydrase (CA) activity; and protein (PROT) content.

Tissue samples for biochemical markers determination were extracted in specific buffers in a 2:1 ratio (buffer volume : sample weight), and the mixtures sonicated for 15 seconds (55 watts cm^{-2}, on ice), and posteriorly centrifuged at 3000 *g* (for the ETS activity assay) or at 10 000 *g* (for the remaining biomarkers) for 15 min at 4 ºC.

For SOD, CAT, GSTs activities measurement, as well as GLY and PROT content determination, supernatants were extracted in phosphate buffer (50 mM sodium dihydrogen phosphate monohydrate; 50 mM disodium hydrogen phosphate dehydrate; 1 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (v/v) (PVP); 1 mM dithiothreitol (DTT) (pH 7.0). For the electron transport system (ETS) activity assay, supernatants were extracted in 0.1 M Tris–HCl buffer (15% (w/v) polyvinylpyrrolidone (PVP); 153 mM magnesium sulfate ($MgSO_4$); 0.2% (v/v) Triton X-100) (pH 8.5). For GSH and GSSG quantification, extraction buffer consisted of 0.6% sulfosalicylic acid in potassium phosphate buffer (0.1 M dipotassium phosphate, 0.1 M potassium dihydrogen phosphate, 5 mM EDTA, 0.1% Triton X-100, pH 7.5). For LPO determinations, supernatants were extracted in 20% (v/v) trichloroacetic acid (TCA). For CA assay, extractions were made in 100 mM Tris-HCL buffer (pH 8.3): 0.1 mM EDTA 1% (w/v) 0.5% PVP (v/v) 2% Triton X-100 (v/v).

2.3.1 Antioxidant and biotransformation enzymes

Superoxide dismutase (SOD)

Determination of SOD activity was performed following Beauchamp and Fridovich (1971) using SOD standard 0.25–60 U mL^{-1}(superoxide dismuthase from bovine liver, Sigma-Aldrich). Reaction mixture consisted of phosphate buffer 50 mM (pH 8.0), 68.4 µM NBT (nitroblue tetrazolium chloride), 0.1 mM DTPA (diethylenetriamineppent-acetic acid), 0.1 mM hypoxanthine. Enzyme activity was determined at 560 nm in a microplate reader after adding xanthine oxidase (5 mU), diluted in phosphate buffer 50 mM (pH 8.0). Absorbance was measured after 20 min incubation at 22 ºC, and the rate of NBT reduction determined. SOD activity was expressed in U g^{-1} fresh weight (fw) (U = µmol min^{-1}).

Catalase (CAT)

Measurement of CAT activity was performed according to Johansson and Borg (1988), using formaldehyde as standard (0–150 µM). Reaction was made in phosphate buffer (pH 7.0), 5.6 M methanol, and the presence of 35.28 mM H_2O_2. Reaction was stopped by adding 10 M KOH and 34.2 mM purpald. Absorbance was measured at 540 nm in a microplate reader. CAT activity was expressed in U g^{-1} fw (U = nmol min^{-1}).

Glutathione *S*-transferases (GSTs)

Determination of GSTs activity was performed spectrophotometrically at room temperature following Habig et al. (1974) with modifications described in Carregosa et al. (2014). Absorbance was measured at 340 nm (ε = 9.6 mM^{-1} cm^{-1}) in a microplate reader, in intervals of 10 s during 5 min. The GSTs activity was expressed in U per g fw (U = µmol min^{-1}).

2.3.2 Redox status

Reduced (GSH) and oxidized glutathione (GSSG)

Quantification of GSH and GSSG concentrations was performed spectrophotometrically at 412 nm following Rahman (2007), using analytical grade (GSH and GSSG, Sigma-Aldrich) standards (0-60 µmol L^{-1}). GSH and GSSG concentrations (nmol g^{-1} fw) were further expressed as a ratio (GSH/GSSG) considering the number of thiol equivalents (GSH/GSSG = [GSH] / 2 x [GSSG]) (Rahman, 2007).

2.3.3 Membrane damage

Lipid peroxidation (LPO)

Quantification of LPO levels followed an adaptation of the thiobarbituric acid (TBA) assay from Buege and Aust (1978). Reaction mixture consisted of TBA at 5% (v/v) in TCA at 20% (v/v). Samples were incubated at 96 ºC for 30 min and then cooled on ice. Absorbance was measured at 535 nm, and MDA concentrations determined based on ε = 156 mM^{-1} cm^{-1}. LPO was expressed in nmol MDA g^{-1} fw.

2.3.4 Metabolic and energy related biomarkers

Electron transport system (ETS)

Measurement of the ETS activity was performed according to King and Packard (1975) and modifications introduced by Coen and Janssen (1997). Reaction mixture consisted of 0.13 M Tris-HCL buffer (pH 8.5, 0.3% (v/v) Triton X-100), 0.25 mM NADH, 36.5 µM NADPH, and 2.3 mM INT (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium Chloride). Formazan production rate was determined spectrophotometrically at 490 nm during 10 min (25 s intervals), and determined based on ε = 15,900 M^{-1} cm^{-1}. Results were expressed in nmol min^{-1} mg^{-1} fw.

Glycogen content (GLY)

Quantification of GLY concentrations in samples was performed following Yoshikawa (1959), using glucose (Sigma-Aldrich) as standard (0 to 5 mg/mL). Samples were incubated at room temperature for 30 min after reacting with phenol (5%) and sulphuric acid (98%). Absorbance was measured at 492 nm, and GLY content expressed in mg g^{-1} fw.

2.3.5 Biomineralization capacity

Carbonic anhydrase (CA)

Carbonic anhydrase (CA) activity was measured spectrophotometrically, following a microplate adaptation of the titrimetric method described by Warrier et al. (2014) as follows: 20 µL extracted supernatant in TRIS HCL buffer (pH 8.3) samples were placed in microplate wells, with 80 µL TRIS buffer (0.1 M) with 20 ppm Bromothymol Blue. 200 µL of CO_2 saturated dH_2O (Weis, 1991) was added to each sample. Bromothymol Blue conversion rate to yellow was immediately measured at 436 nm on a microplate reader during 1 min, and $\Delta ABS \cdot min^{-1}$ determined in triplicate for each sample. Non-enzymatic reaction rate was also determined in triplicate, following the same procedure, after denaturing samples at 100 °C for 5 min. All samples and reagents were kept and mixed at 4 °C. Mean nonenzymatic reaction rate from each sample was subtracted to each sample mean. Results were expressed in $\Delta pH\ min^{-1}\ g^{-1}$ fw. Conversion of $\Delta ABS\ min^{-1}$ to $\Delta pH\ min^{-1}$ was determined by running a set of samples through a conventional pH assay (Weis and Reynolds, 1999), and calculating the linear regression between $\Delta ABS\ min^{-1}$ and $\Delta pH\ min^{-1}$ ($\Delta \mathrm{pH} = \frac{\Delta ABS + 0.0125}{0{,}1944}$, r^2=0.9993).

2.3.6 Protein content

Quantification of PROT concentration in each sample was performed following the Biuret method (Robinson and Hogden, 1940), using bovine serum albumin (BSA) as standard.

Results on enzymatic activities (ETS, SOD, CAT) and glutathione (GSH and GSSG) content from exposure assays on *C. brasiliana* and *C. gigas* (Brazil), were standardized by PROT concentration of each sample.

2.4 Proteomic analysis

2.4.1 Protein extract preparation for proteomic analysis

Oysters whole soft tissue were used for protein extractions. For each species, the soft tissue of two oysters per condition replicate were pooled together, and 3 individual samples (n=3) per experimental condition were analysed. Samples were weighted and homogenised (1:3 w/v) in 10 mM HEPES buffer, 250 mM sucrose, 1 mM DTT, 1 mM Na_2EDTA, 1 mM PMSF, and protease inhibitor (Sigma-Aldrich Tablets) with an Ultra-Turrax homogenizer on ice (4 ºC). Homogenates were centrifuged at 15 000 g (4 ºC) for 2 h, and the cytosolic fraction (supernatant) collected, separated in aliquots and stored (-80 ºC) or immediately used for protein concentration determination following the Bradford method using BSA as standard (Bradford, 1976).

Posteriorly, supernatant volume corresponding to 150 μg protein from each sample was precipitated in 10% TCA in acetone solution at 1:9 (v/v) for 2 h at -20 ºC and centrifuged at 10 000 *g* for 30 min (4 ºC). After this procedure the pellet containing precipitated protein was washed with ice-cold acetone. Washing procedure was repeated three times, and the pellet left to dry for 3-5 min. The pellet containing dried protein extracts of each sample were reconstituted in 300 μL lyse buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.8% (w/v) pharmalyte, 65 Mm DTT, protease inhibitor (Sigma-Aldrich Tablets), and traces of bromophenol blue) for 30 min, centrifuged at 14 000 *g* for 10 min. Samples were immediately used or stored at -80 ºC for further analysis.

2.4.2 Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed twice for each sample, in order to obtain 6 gel images representative of each condition. For the first-dimension protein separation by isoelectric focusing (IEF), the sample (150 μg protein in 300 μL lyse buffer) was loaded into 24 cm ceramic immobilized pH gradient (IPG) holders (GE Healthcare). IPG strips (GE Healthcare Immobiline DryStrip pH 4-7, 18 cm) were carefully introduced over each sample. The IPG ceramic holders were sealed with dedicated mineral oil (GE Healthcare DryStrip Cover Fluid) and lids, holding each sample and the IPG strip inside, were placed. IEF was performed on an Ettan IPGphor 3 System (GE Healthcare) set to develop the following programme: 6:30 h at 0 V (rehydration); 6:30 h at 50 V (fast); 1 h at 1000 V (linear); 1 h at 4000 V (fast); 1 h at 8000 V (linear); 1 h 8000 V (fast). After IEF, IPG strips containing separated proteins by their isoelectric point, were removed from the ceramic holders and stored at -80 ºC.

Before the second-dimension protein separation, IPG strips were equilibrated by consecutive emersion in two SDS equilibration buffers (6 M urea, 75 mM Tris-HCl pH 8.8; 29.3% glycerol; 4% SDS) for 15 min each: the first SDS equilibration buffer contained 1% (w/v) DTT, 0.1 mM EDTA, and a trace amount of bromophenol blue; the second SDS equilibration buffer contained 4% (w/v) iodoacetamide, 0.1 mM EDTA, and a trace amount of bromophenol blue. After equilibration, IPG strips were carefully placed on 10% (v/v) polyacrylamide gels (made from 40% Acrylamide/Bis Solution 37.5:1, Biorad) containing 0.1% SDS (w/v), sealed using 0.5% agarose,

and run under denaturing conditions in an Ettan Dalt 6 (GE Healthcare) refrigerated at 20 ºC following a two-step program: 15 mA and 5 w per gel (120V) for 30 min; plus 60 mA and 17 W per gel (500 V) for ca. 5 h (until the front die reached the end of the gel). After second dimension separation, gels were fixed overnight in 40% (v/v) ethanol and 10% (v/v) acetic acid. Gels were silver stained following Blum et al. (1987). After staining, gels were scanned with a densitometer (Bio-Rad, GS-800). A total of 6 gel images were obtained representative of each condition for each species (3 experimental replicates times 2 duplicates).

2.4.3 Protein identification by mass spectrometry

In-gel protein digestion was performed as outlined by Shevchenko et al. (2006). Before MS analysis, samples were resuspended in 10 µL of formic acid 0.3% (v/v) and 0.5 µL of sample was hand-spotted onto a MALDI target plate (384-spot ground steel plate). Posteriorly, 1 µL of a 7 mg/mL solution of α-cyano-4-hydroxycinnamic acid matrix in 50% (vol/vol) acetonitrile in aqueous trifluoroacetic acid 0.1% (vol/vol) was added and allowed to dry. Mass spectra were acquired on an Ultraflex II MALDI TOF mass spectrometer (Bruker) operated in positive ion mode using a reflectron (m/z range of 600–3500). A total of 500 spectra were acquired for each sample at a laser frequency of 50 Hz. External calibration was performed with $[M + H]^+$ monoisotopic peaks of bradykinin 1–7 (m/z 757.3992), angiotensin II (m/z 1046.5418), angiotensin I (m/z 1296.6848) substance P (m/z 1758.9326), ACTH clip 1–17 (m/z 2093.0862), ACTH18–39 (m/z 2465.1983) and somatostatin 28 (m/z 3147.4710). Peptide mass fingerprints (PMF) were searched via MASCOT search engine set for the following parameters: (i) NCBInr *Crassostrea* (138572 sequences; 81064928 residues); (ii) molecular weight of protein: all; (iii) two missed cleavage; (iv) fixed modifications: carbamidomethylation (C); (v) variable modifications: oxidation of methionine and (vi) peptide tolerance up to 100 ppm. The significance threshold was set to a minimum of 95% ($p \leq 0.05$). Protein matching was considered successful when protein identification score was located out of the random region (Mascot score ≥ 64).

2.5 Elements quantification

2.5.1 Trace element quantification in embryos

The zygote suspension that remained unused from embryotoxicity assays were analysed for trace elements (As, Cd, Cr, Cu, Hg, Ni, Pb, Sn and Zn), following an adaptation of Weng & Wang (2017) protocol as follows. The zygotes suspensions were centrifuged at 3000 *g* for 20 min (4 ºC) followed by pellet collection. The pellets were freeze dried, and posteriorly digested overnight with analytical grade nitric acid and hydrogen peroxide (30% w/w), alongside with TORT-3 (Lobster Hepatopancreas Reference Material for Trace Metals, NRC Canada) for analytical control by elements recovery percentages calculation. The digested samples were analysed for As, Cd, Cr, Cu, Hg, Ni, Sn and Zn by ICP-MS, with IV - 71A (Inorganic Ventures) as standard, and calibration curves verified with standard reference material (NIST SRM 1643f),

calculated measure of trueness over 90%. Recovery percentages based on TORT-3 reference values varied between 93.7 and 111.6%. Results were expressed in μg g^{-1} dry weight (dw).

2.5.2 Arsenic quantification in juveniles

Total As concentration in oysters was quantified in whole soft tissues homogenates of 3 oysters from each experimental condition replicate, including in control following the protocol described in Freitas et al. (2012). Briefly, each specimens soft tissue was homogenised using a mortar and pestle under liquid nitrogen. Known weight aliquots (ca. 0.3 g) from each oyster were digested overnight in Teflon bombs with analytical grade HNO_3 and H_2O_2 (30% w/w). The digested samples were analysed by ICP-MS and total As determined based on IV-71A standard (Inorganic Ventures). Calibration curve was verified with standard reference material (NIST SRM 1643) (calculated measure of trueness over 90%). The recovery percentage of the digestion procedure was verified by parallel digestion of reference standard material TORT-3 (Lobster Hepatopancreas, NRC Canada) and recovery percentages determined for As (103.7%). Results were expressed in μg g^{-1} fw.

2.5.3 Arsenic quantification in adults

Total As concentration in adult oysters exposed to As at different pH levels, and different salinities, were quantified in whole soft tissues homogenates in one individual from each experimental replica (n=3), following the protocol described in Freitas et al. (2012). Each specimens soft tissue was homogenised using a mortar and pestle under liquid nitrogen. Known weight aliquots (ca. 0.5 g) from each oyster were digested overnight in Teflon bombs with analytical grade HNO_3 and H_2O_2 (30% w/w). The digested samples were analysed by ICP-MS and total As determined based on IV-71A standard (Inorganic Ventures). Calibration curve was verified with standard reference material (NIST SRM 1643) (calculated measure of trueness over 90%). The recovery percentage of the digestion procedure was verified by parallel digestion of reference standard material TORT-2 (Lobster Hepatopancreas, NRC Canada). Results were expressed in μg g^{-1} fw.

2.6 Data analysis

2.6.1 Embryo-larval development

Results obtained were analysed as percentages of abnormal larvae (including pre-D and other malformations) according to His et al. (1999), and the validity of the experiments verified by results from negative and positive controls, considering the acceptability ranges proposed by ASTM (2004) for negative controls, and Libralato et al. (2009) for positive controls.

To represent embryo-larval development under different salinity and temperature levels, frequencies of malformed larvae observed at different salinities and temperature (negative

controls), after 24 and 48 h development were represented in Contour plots using SigmaPlot version 11.0.

To assess As embryotoxicity, data on abnormal development observed in As exposures were corrected for the effects observed in the respective negative control (salinity and temperature) using the Abbott's formula (ASTM, 2004). Data were submitted to non-linear regression analysis using GraphPad Prism version 6.01. Effective As concentrations retrieved from ICP-MS for each condition were used for toxicity calculations, after lognormal transformation. The best fit dose-response curve values allowed to estimate the median effect concentrations (EC50s) and respective 95% confidence intervals for conditions for which larvae development was successful. To test the effects of salinity and temperature on embryos sensitivities to As, the following null hypotheses were tested: i) H0': no differences existed among EC50s at different salinity levels considering fixed temperature; ii) H0'': no differences existed among EC50s at different temperature levels considering fixed salinity; iii) H0''': no differences existed between EC50s at 24 and 48 h for each combination of salinity and temperature. To test for differences among EC50 values between conditions, analysis of variance was performed using one-way ANOVA, followed by Tukey's multiple comparisons tests, based on values of Log(EC50) and the associated standard error returned by the software for each estimated curve (GraphPad Prism version 6.01). EC50 values are provided for each species in separate graphs showing the relative 95% confidence intervals, and significant differences ($p \leq 0.05$) were represented with different letters: lower-case letters for comparisons of EC50s at different temperature and different salinities after 24 h exposure; upper-case letters for comparisons of EC50s at different temperature levels and different salinities, after 48 h exposure; asterisks (*) when statistical differences between 24 h and 48 h exposures at each fixed combination of salinity and temperature were observed.

2.6.2 Biochemical markers

Data on biochemical parameters were submitted to hypothesis testing using permutational analysis of variance, employing the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008). Parameters were analyzed following a one-way hierarchical design, with each tested condition as the main fixed factor. Concerning each descriptor, the null hypothesis tested was: H_0') in each species and life stage (juvenile or adult) no significant differences existed among tested conditions. Data for each biomarker are presented as mean ± standard error. Significant differences ($p \leq 0.05$) among groups representing each condition were identified in figures with different symbols.

2.6.3 Proteomic data analysis

Gel images (2-DE maps) analysis and statistics were performed using PDQuest software version 8.0.1 (Bio-Rad). Master gels representative of each condition were constructed based on 6 gels (3 experimental replicates times 2 duplicates). Protein spot intensity of each 2-DE map was normalized by total density of each gel image. Each protein spot assignment and matching between gels were verified using the software matching and group consensus tools. Reproducibility of the 2-DE process was verified and accepted for mean coefficients of variation among gels representative of the same condition over 70%. Analysis followed pairwise comparisons between all conditions by overlaying master gels 2-DE maps. The analysis was performed in separate for each species, to test the following null hypothesis: H_0': no significant differences existed between each protein spot quantity between conditions.

Protein expression was considered differentially altered between conditions whenever the intersection of quantitative (2-fold or higher) and statistically different ($p \leq 0.05$, Student's t-Test) spot intensity changes were observed. Differentially expressed protein spots were ranked by highest fold change in each condition, and the most important proteins (top 10 up or downregulated proteins) for each condition were selected for excision and mass spectrometry analysis.

2.6.4 Arsenic quantification

Statistical analysis was performed to verify the existence of significant differences concerning accumulated As concentrations among conditions using permutational analysis of variance (PERMANOVA) with PRIMER v6 software (Anderson et al., 2008). Briefly, hypotheses testing were performed on Euclidean distances similarity matrix constructed based on As concentration data of each sample. "experimental condition" and "species" were defined as the fixed factors. One-way hierarchical designs considering 9999 permutations were followed to test the null hypothesis: H_0' for each species no significant differences existed in total accumulated As among conditions. Significant differences ($p \leq 0.05$) among conditions were represented with different letters.

3 RESULTS & DISCUSSION

3.1 *Crassostrea angulata* and *Crassostrea gigas* (Portugal)

3.1.1 Embryo-larvae

3.1.1.1 *Crassostrea angulata*

Influence of salinity and temperature

Results on *C. angulata* embryo-larvae development under different combinations of salinity and temperature are depicted in Figure 2 A and B (24 and 48 h post fertilization respectively). Contour plots show that *C. angulata* presented low frequencies of malformed larvae (<20%) at intermediate and high salinity levels (26 and 32) and temperatures of 24 and 28 ºC, after 24 h development (Figure 2A). On the other hand, *C. angulata* presented high frequencies of malformed larvae at every salinity level combined to low temperature (20 ºC) (100% identified as Pre-D) after 24 h development (Figure 2A). At higher temperatures and low salinity (20), results showed higher percentages of malformed larvae of 90% (28 ºC) and 75% (24 ºC). After 48 h of embryo-larval development, *C. angulata* showed lower frequencies of malformed larvae at the intermediate salinity (26) at all temperature levels (Figure 2B), while relatively higher malformation percentages were observed at the highest salinity (33), at both 24 and 28 ºC after 48 h development, compared to results obtained at 24 h (Figure 2A). At the lowest salinity (20), low frequencies of embryo-larval development were observed at the lowest (20 ºC) and highest (28 ºC) temperature after 48 h of exposure (Figure 2B).

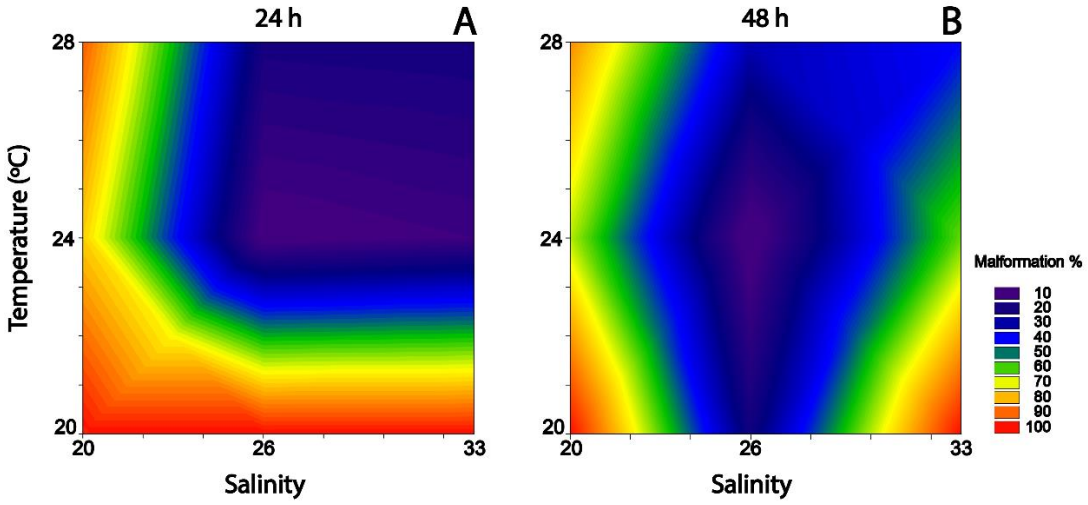

Figure 2 - *Crassostrea angulata* embryo-larvae development under varying salinity and temperature.

Contour plots representing the observed percentage of malformations at different combinations of salinity and temperature, for *C. angulata* after 24 h (A) and 48 h (B) development. Each colour represents a different category of percent frequency of malformation.

Studies concerning *C. angulata* teratogenesis date back to the 19th century, when the first trials on artificial fecundation of *C. angulata* were described for full salinity and 22 ºC (Bouchon-Brandely, 1882), however scarce information is available on the effects of varying salinity and temperature on this species embryonic development in laboratory conditions. Even so, early studies by Amemiya (1926) found that the lowest limit of salinity for *C. angulata* embryonic development was 21, and optimum salinity to be between 28 and 35 (studies performed at 20-23.5 ºC, 24 h). Similarly, our data showed that low salinity (20) induced poor embryonic development (75-100% malformations) at all tested temperatures, and malformation frequency decreased with the increase of salinity.

The relative increase of the range of temperature and salinity at which embryos successfully developed into D-shape larvae observed between 24 and 48 h, reflected differences in developing rates that depend on the surrounding media temperature and salinity, because increasing the time of exposure allowed for embryo development completion, even at suboptimal temperature and salinity conditions. Both these environmental factors affect oyster embryo development rate. For instance, studies on *Pinctata imbricata* showed that suboptimal temperature and salinity delayed embryonic development (O'Connor & Lawler, 2004). This delayed effect is also observed in later stages, namely D-shape larvae of *C. angulata* (Thiyagarajan & Ko, 2012), *C. gigas* (His et al., 1989) and *P. imbricata* (Dove & O'Connor et al. (2007). Moreover, data obtained in the present study show that intermediate salinity (26) is likely closest to the optimum salinity for this species under laboratory conditions, the salinity at which the widest thermal tolerance range was observed (Le Dantec, 1868) (most noticed after 48 h development). This is in accordance with studies by Thiyagarajan & Ko (2012) that described optimum salinity for *C. angulata* D-shape larvae development to be between 24 and 27. Also in line with environmental studies showing higher survival rates of *C. angulata* larvae at temperatures over 22 ºC and salinities between 28 and 32 (Le Dantec, 1868).

Influence of salinity and temperature on oysters sensitivity to Arsenic

The effects of salinity and temperature on embryos sensitivity to As, measured as the median effective concentration (EC50) at every combination of salinity and temperature tested are depicted in Table IV and Figure 3.

As toxicity measured in terms of EC50 considering standard toxicity assay conditions with oyster embryos (24 ºC, 33 salinity, 24 h) for *C. angulata* was 39.2 µg L^{-1} As (18.7 µg L^{-1} As at 48 h) (Table IV), and is likely the first report on the effects of contaminants to *C. angulata* embryonic development. The toxicity of As (EC50) measured in the present study for *C. angulata* was in the lower range of previously reported toxicity threshold values for related oyster species such as *Crassostrea virginica* (with LC50 of 7.2 mg L^{-1}) (Calabrese et al., 1973) and *C. gigas* (EC50 ranging from ca. 1 to 920 µg L^{-1} of As) (Moreira et al., 2018 and Mamindy-Pajany et al., 2013).

Table IV - Embryotoxicity of As to *C. angulata* at different combinations of salinity, temperature and time of exposure.

Summary table of median effect concentrations (EC50s in µg L^{-1}) of As and relative 95% confidence intervals at different combinations of salinity and temperature, after 24 and 48 h exposures. n.c – EC50 value not calculated due to low or zero percentage of developed larvae (D-shape).n.d – 95% confidence interval not determined due to few points for curve fit parameter estimation.

T (ºC)	Salinity	EC50 (µg As L^{-1})	
		24 h	48 h
20	20	n.c	n.c
	26	n.c	31.0 (n.d)
	33	n.c	n.c
24	20	n.c	n.c
	26	29.8 (29.8-29.8)	41.8 (38.7-45.0)
	33	39.2 (38.8-39.6)	18.7 (17.1-20.5)
28	20	n.c	nc
	26	39.6 (39.5-39.7)	50.5 (44.0-58.1)
	33	36.2 (28.5-46.1)	15.9 (14.6-17.3)

To assess the effects of salinity on embryos sensitivity to As, pairwise comparisons among EC50 values obtained at each salinity were performed considering fixed temperature. Pairwise comparisons showed that at 24 h, EC50 values between salinities were significantly different only at 24 ºC, for which EC50 was higher at salinity 33 than at salinity 26. On the other hand, results for *C. angulata* embryos exposed for 48 h, showed significantly higher EC50 values at salinity 26 at both compared temperature levels (24 and 28 ºC) (Figure 3). These findings illustrate that different salinities as well as time of exposure altered the sensitivity of embryos to As.

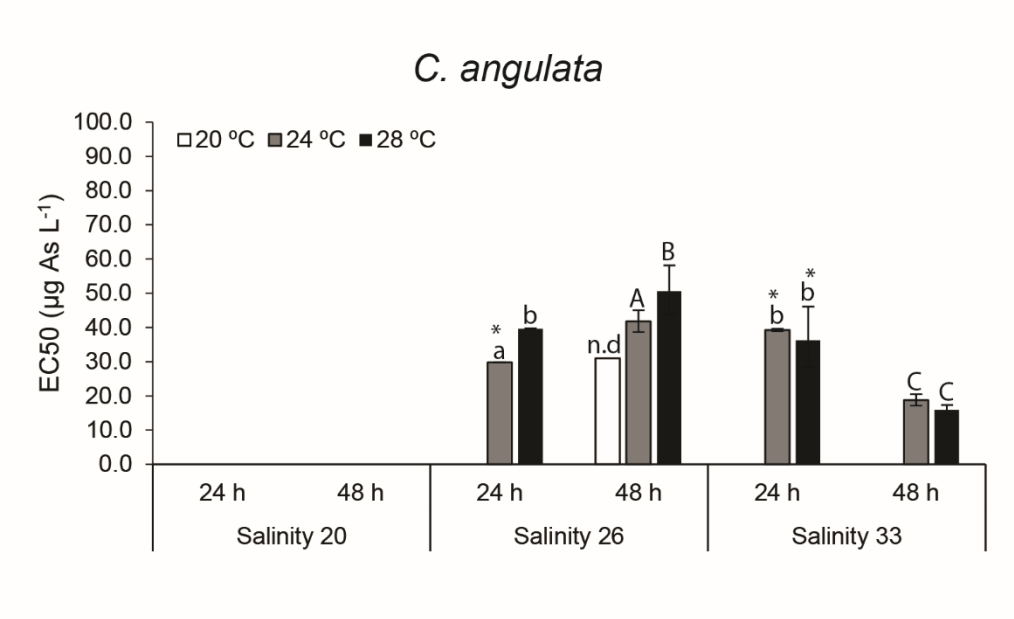

Figure 3 – Arsenic embryotoxicity under different combinations of salinity and temperature to *C. angulata*.

Median effect concentrations (EC50s) of As and relative 95% confidence intervals at different salinity and temperature conditions, after 24 and 48 h exposure. Different letters represent statistical differences between tested groups ($p \leq 0.05$): lower-case letters for comparisons between combinations of salinity and temperature conditions after 24 h; upper-case letters for comparisons between combinations of salinity and temperature conditions after 48 h; * for statistical differences between 24 and 48 h at each combination of salinity and temperature. Values are EC50 ± 95% confidence interval (n.d. – undetermined 95% confidence interval due to few points for curve fit parameter estimation).

Salinity can influence oyster embryo development rate, by increasing the rate with the increase of salinity (O'Connor and Lawler, 2004), and therefore the retardation effect of As previously described (Moreira et al., 2018) at intermediate salinity (26) could be more noticeable than at higher salinity (33), as observed after (24 h). However, these differences were counteracted after 48 h exposure, for which significantly higher EC50 values were observed at salinity 26 comparing to salinity 33, for both comparable temperature levels (24 and 28 ºC). These results, showed in one hand that extending the exposure period from 24 to 48 h had a beneficial effect at salinity 26 (significant increase of EC50 at 24 ºC), corroborating the above stated hypothesis that developing rates influenced final As toxicity. On the other hand, sensitivity to As (lower EC50) increased significantly at higher salinity (33) comparing 24 and 48 h exposures, likely resulting from poor physiological status at higher salinities (Thiyagarajan and Ko, 2012), also supported by results obtained in negative controls at the same conditions for which we observed relatively high frequencies of dead larvae (data not shown).

To evaluate the effect of temperature on embryos sensitivity to As, pairwise comparisons between EC50 values obtained at different temperatures, within each salinity level were performed. Results showed significant increases of EC50s with the increase of temperature (24 and 28 ºC) at salinity 26 for both 24 and 48 h exposures. At salinity 33 no significant differences were observed between temperature levels, at either 24 and 48 h exposures (Figure 3).

Studies on the effects of temperature on the toxicity of pollutants to bivalve embryos, have reported contrasting results. For instance, increased toxicity with the increase of temperature has been shown for Cu and Ag (Boukadida et al. (2016), and for Pb (Hrs-Brencko, 1977) in *Mytillus galloprovincialis*. However, studies on *C. virginica* showed that Cu was more toxic at lower temperatures, presumably because closer to suboptimal temperature conditions would induce higher toxicity (MacInnes and Calabrese, 1979). Assuming that higher temperature increases the rate of chemical reactions in the media, the uptake of contaminants through biological membranes can be promoted at higher temperatures (Hazel, 1997), hence toxicity could be expected to increase at higher temperatures.

In the present study results obtained for *C. angulata* at optimum salinity (26) and both exposure periods (24 and 48 h) showed the opposite trend, for which the effect of increasing temperature resulted in lower As toxicity. These results were likely related to a counteractive effect of increased rates of embryo development at higher temperatures, with the retarding effect of As (Moreira et al., 2018), thus resulting in higher EC50s at higher temperatures.

To assess the effects of salinity on embryo sensitivity to As, pairwise comparisons among EC50 values obtained at each salinity were performed considering fixed temperature. Pairwise comparisons showed that at 24 h, EC50 values between salinities were significantly different only at 24 ºC, for which EC50 was higher at salinity 33 than at salinity 26. On the other hand, results for *C. angulata* embryos exposed for 48 h, showed significantly higher EC50 values at salinity 26 at both compared temperature levels (24 and 28 ºC) (Figure 3).

3.1.1.2 *Crassostrea gigas*

Influence of salinity and temperature

Crassostrea gigas embryo-larvae development under different salinity and temperature combinations is depicted in Figure 4 A and B (24 and 48 h respectively). Contour plots show that *C. gigas* presented low frequencies of malformed larvae (<30%) at all combinations of salinity and temperature after 24 h development, except for embryos exposed to low salinity (20) and low temperature (20 ºC), where high percentages of malformed larvae were observed (100% identified as pre-D, supplementary table S2) (Figure 4A).

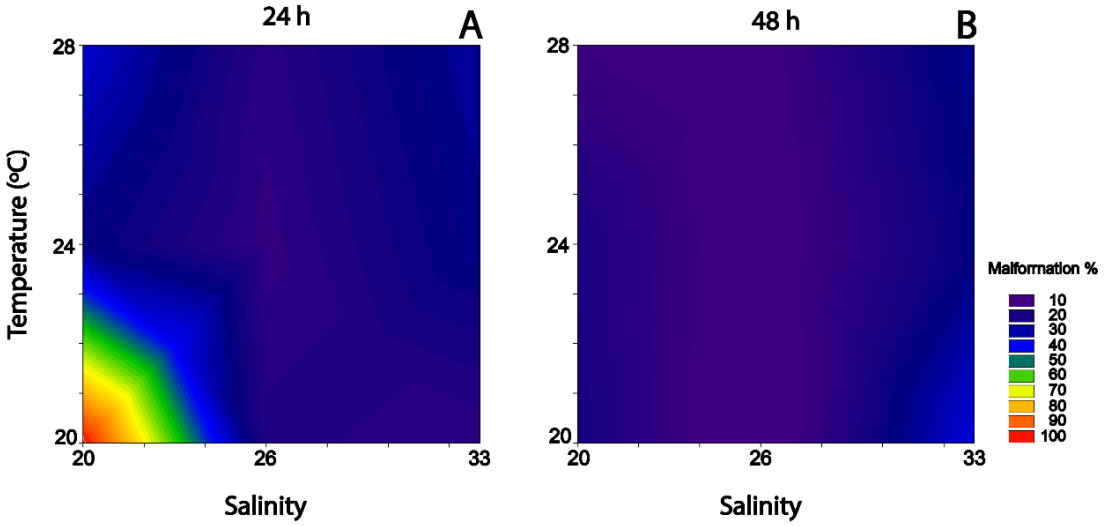

Figure 4 - *C. gigas* embryo-larvae development under varying salinity and temperature.
Contour plots representing the observed percentage of malformations at different combinations of salinity and temperature, for *C. gigas* after 24 h (A) and 48 h (B) development. Each colour represents a different category of percent frequency malformation.

Similarly to the present findings, previous studies showed low percentages of complete embryo development (D-shape) of *C. gigas* at 20 ºC and full salinity (Gamain et al., 2017) (40% D-shape larvae after 24 h post fertilization), or even complete arrest of development at salinities ranging from 15-32 at 20 ºC (100% trochophore stage at 24 h) (Moreira et al., 2018). In the present study, *Crassostrea gigas* embryos exposed to the same conditions for 48 h, showed high frequencies of developed D-shape larvae at all combinations of salinity and temperature (<35% malformed larvae), including low salinity (20) and low temperature (20 ºC) (Fig. 4B).

Taking the present findings and those from previous studies, there appears to be some degree of variability in the developing rate of *C. gigas* at suboptimal temperature (20 ºC), that are likely related to differences in development rates at suboptimal thermal regimes. Results obtained at 48 h post-fertilization, for which high frequencies of D-shape larvae were observed, demonstrated that extending the time of exposure allowed for complete embryo development at low temperature (20 ºC). These results are in accordance with those from Parker et al. (2010) that also observed high rates of embryonic development at 48 h and low temperature (20 ºC).

Differences in methodology during the embryo-larval assay, namely the origin of seawater source (His et al., 1997; Beiras and His, 2004), as well as phenotypic plasticity and genetic variability (Pace et al., 2006; Taris et al., 2006 and references therein), could explain the differences observed between our findings and some of the results reported in literature.

Influence of salinity and temperature on oysters sensitivity to As

The effects of salinity and temperature on embryos sensitivity to As, measured as the median effective concentration (EC50) at every combination of salinity and temperature tested are depicted in Table V and Figure 5.

EC50 values at standard conditions for *C. gigas* (24 ºC, 33 salinity, 24 h) was 452 µg L^{-1} As (663.5 µg L^{-1} at 48 h) (Table V), within previously reported EC50 values (920 µg L^{-1} As from Mamindy-Pajany et al. (2013); and 326 µg L^{-1} As from Martin et al. (1981)). Additionally, in a previous study (Moreira et al., 2018) As toxicity determined at standard salinity and temperature assay conditions revealed an EC50 lower than 1 µg L^{-1} As.

Among the few available data on As toxicity to bivalve embryos, a great variability is observed, in contrast to other types of pollutants (His et al., 1999). These discrepancies could be related to different reference seawater characteristics (Beiras and Albentosa, 2004), as observed in a previous study (Moreira et al., 2018), or different methodological approaches, for instance Martin et al. (1981) used different incubation temperature (20 ºC), different time of exposure (48 h) and different As form (AsO_5); other authors made no reference to measured As concentrations (Mamindy-Pajany et al., 2013), thus possibly introducing bias when determining EC50.

Table V - Embryotoxicity of As to *C. gigas* at different combinations of salinity, temperature and time of exposure.

Summary table of median effect concentrations (EC50s in µg L^{-1}) of As and relative 95% confidence intervals at different combinations of salinity and temperature, after 24 and 48 h exposures. n.c – EC50 value not calculated due to low or zero percentage of developed larvae (D-shape).

T (ºC)	Salinity	EC50 (µg As L^{-1})	
		24 h	48 h
20	20	n.c	101.1 (96.9-105.5)
	26	119.0 (114.6-123.5)	161.9 (147.2-178.0)
	33	285.5 (267.3-305.0)	530.0 (465.2-603.8)
24	20	101.9 (101.7-102.0)	80.71 (80.70-80.73)
	26	179.7 (178.6-180.7)	214.3 (206.1-222.9)
	33	451.5 (312.6-652.2)	663.5 (516.5-852.3)
28	20	103.4 (90.64-117.9)	103.3 (103.0-103.5)
	26	190.1 (180.9-199.8)	198.9 (185.5-213.3)
	33	303.7 (229.6-401.5)	493.7 (429.9-567.0)

Median effect concentrations (EC50s) of As under different salinity and temperature conditions after 24 and 48 h incubation for *C. gigas* are presented in Figure 5. Results obtained show a clear increase of EC50s with the increase of salinity, considering both 24 and 48 h larval development, considering fixed temperature levels.

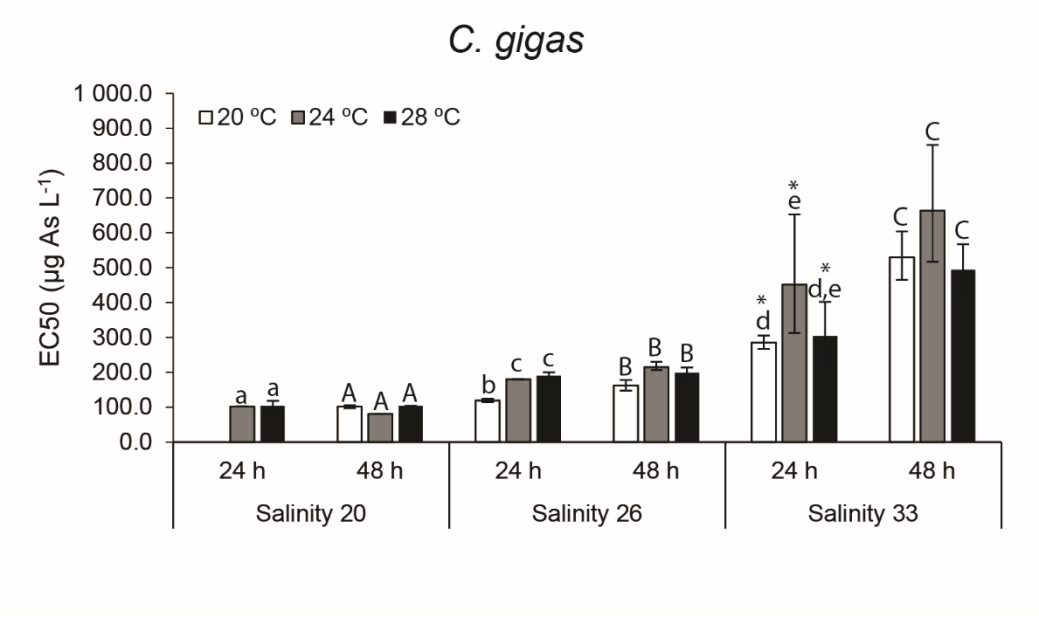

Figure 5 - Arsenic embryotoxicity under different combinations of salinity and temperature to *C. gigas*.

Median effect concentrations (EC50s) of As and relative 95% confidence intervals at different salinity and temperature conditions, after 24 and 48 h exposure. Different letters represent statistical differences between tested groups ($p \leq 0.05$): lower-case letters for comparisons between combinations of salinity and temperature conditions after 24 h; upper-case letters for comparisons between combinations of salinity and temperature conditions after 48 h; * for statistical differences between 24 and 48 h at each combination of salinity and temperature. Values are EC50 ± 95% confidence interval.

Results showing increased sensitivity of *C. gigas* to As with the decrease of salinity observed, are in line with previous studies using *C. gigas* exposed to As (Moreira et al., 2018), Ag and Cu (Coglianese, 1982), Cu and metalochlor (Gamain et al., 2016) at a range of different salinities. The increased sensitivity to As (lower EC50s) with the decrease of salinity is likely related to different osmoregulation status of oyster embryos in response to low salinty. Under hypoosmotic conditions, the ion flux between the embryos and surrounding media may increase, inducing higher uptake of soluble contaminant through active transport processes (Connel, 1989; Grosell et al., 2007). Another factor contributing to higher sensitivity to As at lower salinities, could be related to differences in As speciation with salinity variation, however we discarded such possibility, because under a similar range of salinity (and temperature) conditions, As speciation analysis showed no important differences in prevailing As species and bioavailability with varying salinity and temperature (Moreira et al., 2018).

The effect of varying temperature on *C. gigas* embryos sensitivity to As, inferred by pairwise comparisons of EC50 values among temperature levels, were variable within each salinity level and incubation period (Figure 5). At the lowest salinity (20), no significant differences were observed among EC50s obtained for all temperature levels (at both 24 and 48 h). At salinity 26, EC50 was significantly lower at 20 ºC, comparing to the remaining temperature levels, after 24 h incubation. At salinity 33 and 24 h embryo development, the highest EC50 value was observed at 24 ºC, with significant differences towards that obtained at 20 ºC (Figure 5).

These findings suggest that higher sensitivity (lower EC50s) observed at lower temperatures (20 ºC, 24 h) for all tested salinity levels likely resulted from an additive effect of delayed development induced by both As and low temperature, considering that the developing rate of oyster larvae decrease at lower temperatures (Dove & O´Conner; 2007 His et al., 1989), and that As induces a retarding effect on embryo development (Moreira et al., 2018)

Results further revealed that after 48 h no significant differences were observed among temperature levels at salinity 26 and 33 (Figure 5). These findings demonstrate that the arresting effect of As on *C. gigas* larvae development previously described is not permanent, because extending the exposure period to 48 h revealed higher frequencies of embryo-larval development completion (lower frequencies of Pre-D larvae) (Supplementary table S2), which in turn resulted in similar toxicity effects to larvae exposed at different temperature levels at 48 h, unlike results obtained at 24 h for which temperature showed a higher effect.

Comparisons between EC50 values obtained for each combination of salinity and temperature at 24 h incubation and the corresponding conditions after 48 h exposure (Figure 4), revealed no differences in EC50 values at any combination of low (20) and intermediate salinities (26) at every temperature level between 24 and 48 h exposures (Figure 5). However, significant differences were observed at all temperature levels tested at salinity 33, at which significantly higher EC50 values were observed after 48 h comparing to values obtained after 24 h. It is important to note that at salinity 20 at 20 ºC, EC50 was only possible to calculate after 48 h exposure, since after 24 h embryo development was not completed in these conditions (Figure 5A and B).

Comparisons between results obtained for *C. gigas* at 24 and 48 h further revealed a significant increase of EC50 at the highest salinity (33), and all combinations of temperature, with the increase of exposure time. These results illustrated that toxicity threshold determination can depend on time of exposure for toxicants that affect embryonic development by retarding development such as As.

3.1.1.3 Species comparison

A different range of salinity and temperature was observed for successful embryo-larval development of each species, which further reflected in the pattern of sensitivity to As observed. *C. angulata* presented a narrower range of salinity and temperature than *C. gigas* for which embryo-development successfully occurred, considering 24 and 48 h post fertilization. *C. angulata* presented better embryonic development at intermediate salinity (26) and temperatures above 20 ºC, while *C. gigas* presented high frequencies of developing embryos at all combinations of salinity and temperatures tested

Overall, results on embryo-larvae development suggest that *C. angulata* is more sensitive to As than *C. gigas*, with at least an order of magnitude lower EC50 values. Several factors could be hypothesised to have influenced these results, but the most important one could be a differentiated species-specific sensitivity to As.

Indeed, species specific tolerance to As seemed to be the main reason explaining the differentiated toxicity observed. Moreover, these results are supported by other studies, namely those that showed taxon related differences between *C. angulata* (Spanish origin) and *C. gigas* (French origin), regarding maturation trais as well as mortality of each progeny (Soletchnik et al., 2002). Also, Huvet et al., (2002) suggested that *C. gigas* could present overall better gamete quality than *C. angulata*, while studying hybrid crosses between both species. To our knowledge this is the first embryotoxicity study on *C. angulata*, and the present data add to the body of evidence that show differences in ecophysiological traits between these closely related taxa (Soletchnik et al., 2002; His et al., 1972; Goulletquer et al., 1999; Heral, 1996; Moreira et al., 2016).

Other factors such as broodstock origin or parental exposure history could also have influenced each species sensitivity to As but were unlikely given our results and the existing literature. For instance, Gamain and co-workers (2017) studied *C. gigas* from different origins (hatchery, cultivated and wild) and found no major differences in embryotoxicity of metolachlor to embryos of different parental origins. However, and in contrast with our study, progeny from hatchery oysters were more sensitive to Cu than wild and cultivated ones. Therefore, it would be unlikely that oysters (hatchery *C. gigas*, and cultivated *C. angulata*) from our study, would have presented the observed differentiated response to As, solely based on broodstock origin (hatchery and cultivated).

A recent study by Weng & Wang (2017) demonstrated maternal transfer of trace metals in *Crassostrea hongkongensis* adults to their progeny (oocytes and larvae) reflecting parental exposure from contaminated sites, with negative impacts on the most contaminated embryos development. Hence, parental transfer of trace elements to zygotes in *C. angulata* could have influenced final As toxicity, knowing that the Sado estuary is anthropogenically polluted (Costa et al., 2009). However, the results obtained in the present study on trace elements content in fertilized oocytes showed only marginal differences in trace elements concentrations between species, except for Cr and Ni (higher in *C. angulata*) and Zn (higher in *C. gigas*) (Table VI). Trace

elements content from both *C. angulata* and *C. gigas* were in the lower range of those described by Weng & Wang (2017) for *C. hongkongensis* embryos, considering the lowest reported values (lowest contaminated sites) for As, Cd, Cr, Cu, Ni and Zn (4.26 ± 0.83; 0.21 ± 0.11; 0.71 ± 0.39; 45.3 ± 21.0; 1,42 ± 1.03; 300 ± 87.8 µg g^{-1} dw, respectively). Hence, the present findings indicate that parental element transfer to embryos was not likely to have influenced final As toxicity in either species, given that we observed overall low contamination, and few differences in element concentrations in embryos between species.

Table VI - Trace elements concentrations (µg g^{-1} dry weight) determined in *C. angulata* and *C. gigas* fertilized oocytes suspensions used for embryotoxicity experiments (mean ± relative standard deviation).

	C. angulata	*C. gigas*
As	3.43±0.031	3.29±0.035
Cd	0.11±0.0046	0.10±0.0054
Cr	3.69±0.049	0.36±0.0047
Cu	7.75±0.078	7.06±0.078
Hg	0.04±0.0051	0.03±0.0031
Ni	1.91±0.026	0.76±0.012
Sn	0.46±0.0085	0.40±0.0051
Zn	56.69±0.58	78.94±0.86

3.1.2 Juveniles

Seawater acidification and Arsenic exposure

The effects of seawater acidification and As (single and combined exposures) were assessed in juvenile *C. angulata* and *C. gigas* after 28 days laboratory exposures to (As), seawater acidification (Low pH) and the combined effects of both stressors (Low pH+As) (detailed methods in section 2.4). Arsenic accumulation, biochemical markers and 2-DE proteomics were assessed in oysters soft tissue.

3.1.2.1 *Crassostrea angulata*

Influence of Seawater acidification on Arsenic accumulation

Total As (µg g^{-1} fw) accumulated in soft tissue of juvenile *C. angulata* exposed to As, Low pH and Low pH+As, are depicted in Table VII. As concentrations were significantly higher in oysters exposed to As (As; Low pH+As) than in the remaining conditions (CTL, Low pH). No significant differences were observed in As accumulation between oysters exposed to As in acidified (Low pH+As) and non-acidified (As) conditions.

Table VII – Total As (µg g^{-1} fw) accumulated in juvenile *C. angulata* exposed to CTL (control), As, Low pH and Low pH+As. Significant differences ($p \leq 0.05$) among tested conditions are represented with different letters (mean ± standard error, n=5).

	CTL	As	Low pH	Low pH+As
µg As g^{-1} fw	1.25±0.08**a**	2.88±0.1**b**	1.16±0.2**ª**	2.98±0.3**b**

The average concentration of As accumulated in juvenile *C. angulata* ranged from ca 1.0 to 3.0 µg g^{-1} fw (equivalent to ca. 5 and 15 µg g^{-1} dw) (Zhang et al., 2013). Residual As concentrations (ca. 1 µg g^{-1} fw) observed in oysters maintained in CTL, evidenced As contamination levels in the estuary where oysters were collected (Costa et al., 2009). The highest As concentrations (2.98 µg g^{-1} fw), equivalent to ca. 15 µg g^{-1} dw (Zhang et al., 2013) in oysters exposed to As, was within reported values in oysters collected in other estuarine systems e.g. up to 26.7 µg g^{-1} dw in *C. gigas* in France (Kohlmeyer et al., 2002) and 25.4 µg g^{-1} dw in *C. virginica* from the US (Valette-Silver et al., 1999).

Influence of Seawater acidification and Arsenic on oysters biochemical performance

Results on biochemical parameters studied in juvenile *C. angulata* exposed to As, Low pH and Low pH+As are depicted in Figure 6. The most prominent changes concerning the biochemical markers studied in juvenile *C. angulata* experiments, were observed in oysters exposed Low pH+As, in the form of induced SOD activity, increased GSH/GSSG, and higher LPO. Oysters exposed to Low pH presented significantly lower GSTs activity towards the remaining conditions.

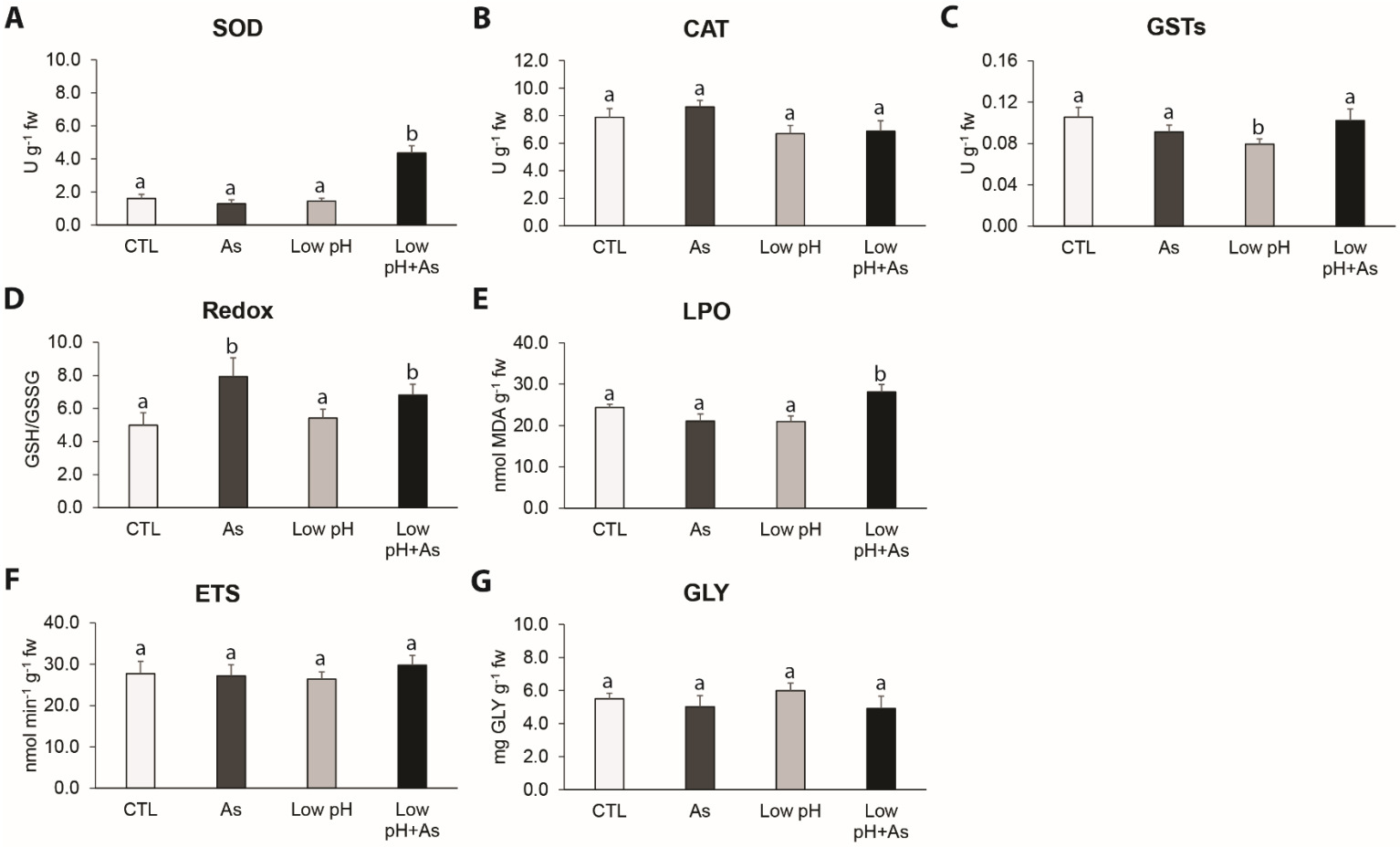

Figure 6 – Biochemical parameters studied in juvenile *C. angulata* exposed to CTL (control), As, Low pH and Low pH+As.

A: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Glutathione *S*- transferases (GSTs) activity; **D**: Reduced to oxidized glutathione ratio (Redox); **E**: Lipid peroxidation (LPO) levels; **F**: Electron transport system (ETS) activity; **G**: Glycogen (GLY) content. Significant differences ($p \leq 0.05$) between conditions are represented with different letters (Mean + standard error; n=12).

Antioxidant enzymes have been widely used as biomarkers of environmental stress in bivalve species from a wide range of stressors including the exposure to xenobiotics (e.g. Freitas et al., 2014; Regoli et al., 1997), climate change related stressors (e.g. Matoo et al., 2013; Matozzo et al., 2013), as well as to the combined effects of both xenobiotics and climate change parameters (e.g. Freitas et al., 2016a,b; Verlecar et al., 2007). Because the activity of antioxidant enzymes is often modulated in response to stress related enhanced ROS formation to prevent oxidative damage, the measurement of antioxidant enzymes (e.g. SOD and CAT) activities give indications on the oxidative challenge organisms are subjected in a given scenario (Valavanidis et al., 2006). In juvenile *C. angulata*, alterations of antioxidant enzymes activity were observed concerning SOD, that was significant higher in oysters exposed to Low pH+As compared to the remaining conditions. These results could indicate that these oysters were subjected to higher oxidative stress compared to CTL and to single exposures, and likely induced antioxidant enzymes to mitigate enhanced ROS formation.

Among non-enzymatic antioxidants, glutathione is the most abundant ROS scavenger that plays a central role in cellular redox status maintenance and oxidative stress repair (Rahman, 2007). GSH serves as electron donor in numerous reductive processes, either directly reducing oxidized molecules, or indirectly as cofactor of several antioxidant/biotransformation enzymes (e.g. GSTs, GPx). The ratio of reduced to oxidized glutathione (GSH/GSSG) gives a measure of the oxidative stress status of a given organism (Regoli & Giuliana, 2014). It is generally conceived that a shift towards an oxidized GSH/GSSG to be indicative of a prooxidant status, despite that GSSG export and glutathione reductase (GR) activity should not be neglected (Dalle-Donne et al., 2009).

The present findings, showed significantly higher GSH/GSSG in oysters exposed to As and to Low pH+As (compared to CTL and to Low pH), evidencing alterations in the redox status of oyster tissues. The increase of GSH/GSSG was due to a significant decrease in GSSG concentrations (no change in GSH concentrations were observed). At least two non-mutually exclusive hypothesis could explain the observed decrease of GSSG concentrations and concomitant increase in GSH/GSSG: i) GSSG could be excreted from the cells towards the extracellular matrix (Garcia et al., 2010; Han et al., 2006) to prevent GSSG induced protein glutathionylation under oxidative conditions (Hawkins et al., 2010; Hurd et al., 2005); and ii) a shift towards NADPH producing pathways characteristic of invertebrates stress response (Tomanek, 2014) could increase the rate of GSSG reduction by GR, thus shifting the ratio towards the reduced form (Bindoli et al., 2009). Similarly, increased NADPH, decreased GSSG and increased GSH/GSSG was observed by Yamamoto et al. (2014) in tumour cells. In either case, results on GSH/GSSG indicate a shift in the redox status, likely representing a protective mechanism towards As induced oxidative stress.

The reaction of ROS with membrane lipids is one of the most important mechanisms of cellular injury, that involves a cascade of oxidation reactions generally termed lipid peroxidation (Halliwell & Gutterige, 1999). Aldehydes such as malonaldehyde (MDA) are among the most important degradation products of the lipid peroxidation chain reaction, and therefore MDA

concentration has been used as a measure of cellular membrane peroxidation resulting from oxidative stress in aquatic organisms (Amiard-Triquet et al., 2016). The present findings showed that simultaneous exposure to both stressors (Low pH+As) induced higher LPO in juvenile *C. angulata* than single exposures, an indication of higher cellular injury induced by the presence of both stressors.

Another important result was that observed concerning GSTs activity. GSTs are important enzymes involved in cellular detoxification processes by conjugation of GSH to electrophilic centres of a variety of subtracts (Townsend & Tew, 2002). Results showing lower GSTs activity in juvenile *C. angulata* exposed to Low pH compared to the remaining conditions, suggest supressed biotransformation capacity in these oysters. These findings could be related to metabolic depression, as a mechanism of physiological adaptation to adverse environmental conditions (Guppy & Withers, 2009), also observed in bivalve molluscs exposed to seawater acidification (Lesser, 2016; Michaelidis et al., 2005). In such scenario, antioxidant suppression can be induced as an energetic trade-off mechanism to prevent energetic depletion due to limited energetic reserves (Sokolova et al., 2011), and/or due to lower ROS production rates under anaerobic respiration (Abele & Puntarulo, 2004).

Despite of this, results on the ETS activity and energetic fitness (GLY), did not suggest changes in oysters metabolic performance. Nonetheless, proteomic analysis provided other clues that are discussed in the following section.

Influence of Seawater acidification and Arsenic on oysters proteome

Proteomic analysis of juvenile *C. angulata* showed differentially expressed protein profiles among all tested conditions (Figure 7). Protein identification by MS allowed to identify several proteins with significantly altered expression levels among conditions, that play biological roles related to metabolism (ATPsynthase subunit β), cytoskeleton structure (Actin; Coronin 1-B; Severin; Gelsolin) cellular stress response (Retinal dehydrogenase; Lactoylglutathione lyase; Alpha crystallin b chain), cell signalling (progesterone-induced-blocking factor 1; piRNA biogenesis protein) proteolysis (aminopeptidase W07G4.4), cell differentiation/apoptosis (MYCBP-associated protein), cell to cell adherence related proteins (Ependymin; Tight junction protein ZO-1), transport (V-type proton ATPase subunit B), as well as two uncharacterized proteins (KIAA1109-like isoform X5; LOC105343084 isoform X1) (Figure 7 and Table VIII)

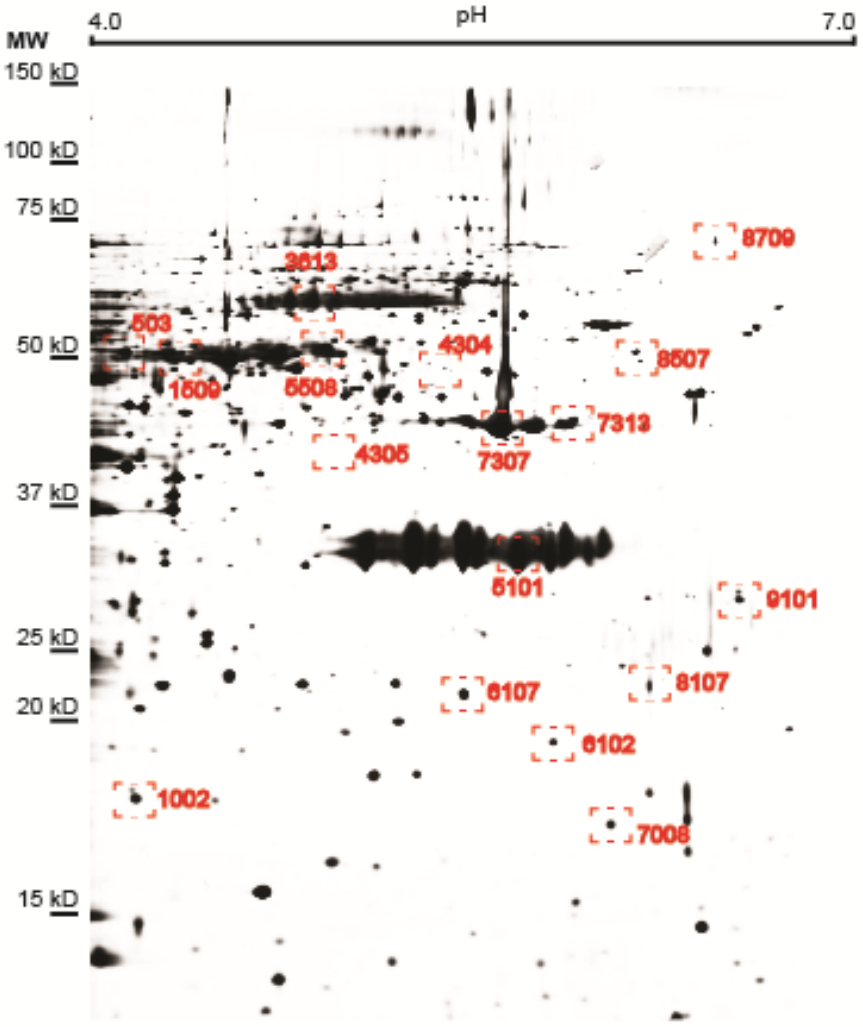

Figure 7 – 2DE protein map of identified proteins in juvenile *C. angulata* exposed to different pH levels.

Protein expression profile of juvenile *C. angulata* whole soft tissue by 2-DE. Differentially expressed proteins identified by MALDI TOF MS are highlighted in the representative gel image.

Table VIII – Differentially expressed proteins in juvenile *C. angulata* exposed to CTL (control), As, Low pH and Low pH+As.

Differentially expressed proteins identified in *Crassostrea angulata* by MALDI TOF MS. Observed molecular weight (MW) and isoelectric point (pI) are provided. Fold change of spot density between tested groups (CTL; As ; Low pH; and Low pH+As) are expressed for pairwise comparisons that showed significant differences (p≤0.05).

Spot no.	GenBank ass. no.	Protein name	Mascot score	MW (kDa)/pI	Putative biological function(s)	Fold change
503	XP_011429993.1	Putative aminopeptidase W07G4.4	64	51.8/4.3	Proteolysis	3.3 ↑ in Low pH+As vs. Low pH 7.1 ↑ in Low pH+As vs. As
1002	XP_011447841.1	MYCBP-associated protein-like isoform X5	64	18.5/4.4	Cell differentiation Apoptosis	9.1 ↑ Low pH+As vs. As
1509	XP_011436817.1	Retinal dehydrogenase 1	108	59.5/4.5	Regeneration	2.3 ↑ in Low pH vs. CTL
3613	XP_011429476.1	Coronin-1B isoform X5	118	64.0/4.9	Cytoskeleton	2.6 ↑ in Low pH+As vs. CTL
4304	EKC22157.1	Severin	108	55.1/5.2	Cytoskeleton	4.3 ↑ in As vs. CTL 4.8 ↑ in As vs. Low pH
4305	EKC30581.1	Progesterone-induced-blocking factor 1 (Pibf)	100	38.6/5.0	Immunomodulation	5.0 ↑ CTL vs. As 10.0 ↑ in Low pH+As vs. As
5101	XP_022328428.1	Uncharacterized protein KIAA1109-like isoform X5	64	34.0/5.5	Lipid metabolism	2.8 ↑ in As vs Low pH 4.2 ↑ in As vs. Low pH+As
5508	EKC36437.1	V-type proton ATPase subunit B	73	53.3/5.1	Transport	3.3 ↑ in Low pH vs. Low pH+As
6102	EKC27629.1	Alpha-crystallin B chain	111	17.3/5.6	Molecular chaperone	2.0 ↓ in As vs Low pH+As 3.0 ↓ in Low pH+As vs. CTL 4.3 ↓ in As vs Low pH 6.3 ↓ in As vs. CTL
6107	XP_011413901.1	Ependymin-related protein 1	114	22.0/5.3	Cell-matrix adhesion	4.4 ↑ in Low pH vs. Low pH+As
7008	XP_011422530.1	Lactoylglutathione lyase	118	17.8/5.7	Antioxidant defense	5.9 ↑ in Low pH+As vs. As 5.6 ↑ in CTL vs. As 5.9 ↑ in Low pH vs. As
7303	XP_011427051.1	Gelsolin-like protein 2	80	54.6/5.9	Cytoskeleton	2.6 ↑ in As vs. Low pH 4.1 ↑ in As vs. CTL
7307	NP_001295788.1	Actin	78	47.6/5.6	Cytoskeleton	4.7 ↑ in Low pH+As vs. CTL 5.9 ↑ in As vs. CTL
8107	XP_022321285.1	piRNA biogenesis protein EXD1-like	64	26.4/5.9	Cell signaling	6.7 ↓ in Low pH+As vs. CTL
8507	EKC39411.1	ATP synthase subunit beta. mitochondrial	64	65.5/5.8	Oxidative phosphorylation	2.8 ↓ in Low pH+As vs. CTL 5.2 ↓ in Low pH vs. CTL
8709	EKC34842.1	Tight junction protein ZO-1	88	91.1/6.0	Cell-Cell adherence	6.7 ↑ in CTL vs As 7.1 ↑ in Low pH vs. As 10.0 ↑ in Low pH+As vs. As
9101	XP_011448567.1	Uncharacterized protein LOC105343084 isoform X1	83	28.0/6.0	Unknown	2.5 ↑ in Low pH vs. Low pH+As

Influence of Arsenic exposure on oysters proteome

In line with results on biochemical markers, data from proteomic analysis corroborated the observed changes in redox status of juvenile *C. angulata* exposed to As, for which higher GSH/GSSG was observed. Particularly, proteomic analysis revealed a significant decrease in expression levels of Lactoylglutathione lyase (Lgl) in oysters exposed to the same condition (As) (Table VIII). Lgl is an important enzyme in the glyoxalase system, that participates in detoxification reactions of reactive α-ketoaldehydes (glycolytic by-products) using glutathione as cofactor (Regoli & Giuliana, 2014). Because Lgl regulation depends on cellular redox status (Birkenmeier et al., 2010), it is likely that the decrease of Lgl abundance indicates cellular redox imbalance induced by As exposure. Accordingly, other studies have shown a negative correlation between Lgl and pollutant exposure, including in *Macoma balthica* exposed to As (Regoli et al., 1998), and in *Saccrostrea glomerata* (Melwani et al., 2016) exposed to high impacted sites (PAHs, PCBs, TBT, Pb and Zn).

C. angulata exposed to As presented higher expression levels of cytoskeleton related proteins compared to levels observed in control conditions. These were Actin (Actin), Severin (Severin) and Gelsolin-like protein 2 (Gelsolin). Alterations in the expression levels of cytoskeletal proteins is a common response mechanism observed in marine organisms exposed to environmental stress (Tomanek, 2014), and could be indicative of cytoskeleton rearrangement to prevent oxidative damage (Dailianis et al., 2009) or to replace damaged proteins (Anderson, 2015). Accordingly, altered levels of Actin have also been reported in other oyster species (*Saccrostrea cucculata* and *Saccrostrea glomerata*) exposed to different types of contaminants (Khondee et al., 2016; Melwani et al., 2016; Muralidharan et al., 2012; Thompson et al., 2012).

Severin and Gelsolin (both assigned to the Gelsolin superfamily), play regulatory roles on Actin filament assembly and disassembly processes, and are key proteins in cytoskeleton structure maintenance and remodelling (Silacci et al., 2004). Higher abundance of these proteins were also observed in juvenile *C. angulata* exposed to As relative to control (and to Low pH), that further corroborate the role of Actin dynamics in *C. angulata* stress response to As. Similarly, other studies have described altered levels of Severin and Gelsolin-like proteins in bivalves experiencing environmental stress, such as: Severin increased expression in *C. gigas* under thermal and hypercapnic stress (Dineshram et al., 2015; Harney et al., 2016), and in *S. glomerata* exposed to highly polluted sites (Melwani et al., 2016); and increased abundance of Gelsolin-like proteins in *S. cucculata* exposed to Tributyltin (TBT) (Khondee et al., 2016), as well as in *Pecten maximus* under hypoxia (Artigaud et al., 2015). Considering these studies, results presented here suggest eminent cytoskeleton remodelling in *C. angulata* exposed to As, that could have helped to mitigate oxidative stress. For instance, Actin polymerization may lead to increased glutathionylation thus protecting the actin cytoskeleton from oxidative stress (Dailianis et al., 2009). Accordingly, cytoskleleton remodelling could help explain unaltered SOD, CAT and LPO in oysters exposed to As.

Additionally, *C. angulata* exposed to As showed significantly lower Tight junction ZO-1 protein (ZO-1) levels than in all other conditions (Table VIII). ZO-1 are scaffolding proteins integral in the link between the tight junction and the actin cytoskeleton, modulating cell to cell adherence processes. Different elements have been shown to cause tight junction restructuring and deficiency, for instance in mouse respiratory cells exposed to As (Sherwood et al., 2013) and in *Tegillarca granosa* clams exposed to Cadmium (Cd) (Bao et al., 2016). Therefore, the present data suggest that As caused impairment of cell to cell adherence process in *C. angulata*.

Adding to differences observed between As exposure and CTL, the present data showed increased expression of Uncharacterized protein KIAA1109-like (spot no. 5001, Table VIII) in *C. angulata* exposed to As compared to both acidification conditions (Low pH and Low pH+As). This protein is assigned to the Fragile site-associated protein (Fsa) domain, and is likely related to lipid storage (McKay et al., 2003; Kuo et al., 2006). Hence, increased KIAA1109-like protein in *C. angulata* exposed to As is likely related to alterations in lipid metabolism due to As as observed in other organisms (Carlson et al., 2014; Wang et al., 2015).

Influence of Seawater acidification on oysters proteome

Proteomic analysis of juvenile *C. angulata* exposed to Low pH corroborated the results obtained through biochemical makers, that suggested metabolic depression in juvenile *C. angulata* exposed to Low pH. Namely, regarding results showing lower abundance of ATP synthase subunit betta (ATP β) in oysters exposed to the same condition (Low pH) (Table VIII).

The decrease of ATP synthase subunits expression has been discussed as a means of oxidative metabolism suppression (Moya et al., 2012). Metabolic arrest is characteristic of organisms incurring extreme stress (Guppy & Withers, 1999), likely resulting from decreased aerobic scope for ATP synthesis (Sokolova et al., 2012) and a possible indication of a shift to anaerobic metabolism (Hüning et al., 2012). Similarly, other studies described ATP synthase subunit depression in marine invertebrates exposed to acidification, including coral (Moya et al., 2012), polychaete (Wäge et al., 2016) and bivalve species (Harney et al., 2016; Hüning et al., 2012). Therefore, the decrease of ATP β expression levels observed in *C. angulata* in Low pH condition most likely indicate metabolic depression in response to acidification in this species, supporting the idea that GSTs suppression was linked to metabolic arrest, as mentioned in the previous section.

Interestingly, biochemical markers revealed no alterations in metabolic potential measured by the ETS activity. This could seem contradictory, because alterations in ATP synthase subunits expression would inevitably lead to impairment of ATP synthesis through the electron transport chain (Lapaille et al., 2010; Xu et al., 2015). However, because the ETS activity assay measures INT reduction capacity primarily at complexes I and III of the electron transport chain (ETC) (Packard, 1971), and at a lower extent at extramitochondrial cytosolic, lysosomal and peroxisomal fractions (Maldonado et al., 2012), the ETS assay is insensitive to changes at respiratory chain complex V (ATP synthase). Hence, results showing downregulation of ATP β would not induce

observable effects via the ETS activity assay, and reveal higher sensitivity of the proteomic approach in this particular case.

Another protein, retinal dehydrogenase (Rdh) showed increased expression levels in *C. angulata* exposed to Low pH compared to CTL (Table VIII). Thompson et al. (2015) also observed increased Rdh in *Saccostrea glomerata* exposed to high pCO_2. Rdh catalyses the irreversible oxidation of retinal to retinoic acid (RA) (Gutierrez-Mazariegos et al., 2015). Thus, increased expression of Rdh potentially increases RA concentration in oyster tissues. The physiological effects of such alterations in invertebrates remain to be clarified although RA seems to be involved in several processes from organ formation, differentiation and regeneration (Albalat, 2009; Gutierrez-Mazariegos et al., 2015). The present data, showing upregulated Rdh suggest a protection mechanism towards acidification, and could be at least partially responsible for the unchanged antioxidant status observed.

Influence of the combined exposure to Seawater acidification and Arsenic on oysters proteome

Proteomic analysis of oysters exposed to the combination of both stressors (Low pH+As) revealed several important alterations in oysters proteome, that helped explain and corroborated the results from biochemical analysis.

Firstly, cytoskeleton modulation was upregulated in oysters exposed to Low pH+As, taken results showing enhanced Actin and Coronin abundance levels (Table VIII). Coronin is an actin filament binding protein that participates in Actin restructuring dynamics, including filament disassembly, bundling, crosslinking and several other actin-network reorganization processes (Lin et al., 2010; Rybakin & Clemen, 2005; Srivastava et al., 2015). Concomitantly, Actin was also upregulated in the same condition, supporting the effective role of cytoskeleton restructuring observed. Similarly, Thompson et al. (2015, 2016) observed simultaneous increase of both Coronin and Actin levels in *S. glomerata* exposed to acidification. In line with the results previously discussed, cytoskeleton restructuring may help mitigate oxidative stress. For instance, Actin polymerization may lead to increased glutathionylation thus protecting the actin cytoskeleton from oxidative insult (Dailianis et al., 2009 and references therein). However, under Low pH+As, cytoskeleton modulation capacity appeared not to be sufficient to prevent oxidative damage (increased LPO).

Secondly, metabolic depression via ATP β downregulation was also observed in oysters exposed to Low pH+As, despite that the decrease of ATP β was lower (2.8 fold) in Low pH+As than in Low pH single exposure (5.2 fold), an indication that metabolic adjustment was possible only to a lower extent under the combination of both stressors (Low pH+As), likely due to the effect of As on oysters oxidative stress status.

Thirdly, downregulation of piRNA biogenesis protein (piRNA) in Low pH+As (6.7-fold) compared to CTL, suggested genome reconfiguration towards environmental stress. piRNAs are involved in genome integrity maintenance via transposon regulation (Iwasaki et al., 2015; Mani & Juliano, 2014;). Transposable elements (TEs) are mobile DNA sequences that can move within

the genome, and are silenced by piRNAs (Luo & Lu, 2017). Researchers have recently proposed that stress-induced TEs mobility via piRNA downregulation allow for genome reconfiguration in response to extreme environmental stress (Ryan et al., 2016), enabling for heritable phenotypic variation (Piacentini et al., 2014). Considering this theory, and the fact that TEs are abundant in the oyster genome (Cross et al., 2014), the present data showing decreased expression of piRNA in *C. angulata* exposed to Low pH+As could imply genome reconfiguration in response to multiple stressors, a novel observation concerning oysters adaptive evolution mechanisms.

Apart from proteomic differences observed between Low pH+As and CTL, results also revealed differences in protein abundance profiles between stress conditions (Table VIII). For instance, Aminopeptidase W07G4.4 (Aminopeptidase) and MYCBP-associated protein (Mycbp) were upregulated in Low pH+As towards Low pH and/or As. On the other hand, *C. angulata* presented downregulation of V-type proton ATPase subunit B (V-ATPase b), Ependymin-related protein 1 (Epend-1) and one uncharacterized protein (spot number 9101) in Low pH+As compared to levels in Low pH single exposure (Table VIII).

Upregulation of Aminopeptidase, has also been described in *S. glomerata* oysters exposed to seawater acidification (Thompson et al., 2016), although the same Aminopeptidase was downregulated in oysters from highly contaminated sites (Melwani et al. 2016). The molecular function of Aminopeptidase suggests a primary role in protein catabolism (Brooks & Isacc, 2004) and therefore, increased levels of Aminopeptidase in *C. angulata* exposed to Low pH+As indicates higher protein turnover necessary to endure oxidative stress (Sokolova et al., 2012).

Results showing decreased expression of V-ATPase b in oysters exposed to Low pH+As compared to Low pH could be explained owing to the fact that V-ATPase b expression levels may increase in oysters exposed to OA, as observed in other oyster species (*C. gigas* and *S. glomerata*) (Dineshram et al., 2012; Thompson et al., 2015), but in turn V-ATPase b could be depressed under the combined exposure of OA and As. V-ATPases are ATP consuming ion channels that play roles in acid-base regulation (HCO_3), calcification, carbon concentration processes, as well as excessive proton excretion in marine invertebrates exposed to OA (Ivanina & Sokolova, 2015; Parker et al., 2013; Tresguerres et al., 2016). The fact that oysters exposed to Low pH+As presented significantly lower expression of V-ATPase b levels (Table VI), likely reflected the influence of As on oysters response to acidification. This could be related to the energetic costs associated to V-ATPases activity (Tresguerres et al., 2016) and the possible preferential energy allocation towards As detoxification processes in Low pH+As condition. These results highlight different modes of action of *C. angulata* towards multiple stressors.

Increased expression of Ependymin related-protein 1 (Epend) was observed in oysters exposed to Low pH compared to Low pH+As (Table VII). The physiological role of Ependymin is attributed to cell adhesion processes through cell-matrix contact formation. Generally, upregulation of these proteins reflect tissue remodelling in response to environmental stimuli as reported in *Littorina saxatilis* (Muraeva et al., 2016) and *C. gigas* (Zhao et al., 2012) under osmotic stress. Zhang et al. (2014), observed a versatile behaviour of ependymin proteins in *C. gigas* exposed to different abiotic factors. These authors described upregulation of Ependymin in

oysters exposed to thermal and osmotic stress, while the same proteins were downregulated under aerial exposure. The present results suggest that Low pH induced higher Epend to modulate cell adhesion, while in the presence of As (Low pH+As) *C. angulata* presented lower capacity to promote Epend biosynthesis with possible impacts on tissue structure modulation capacity.

Other proteins that presented altered abundance levels in single exposures (Low pH or As) did not present the same degree of alteration in oysters exposed to the combination of both stressors (e.g. Rdh, Severin, Gelsolin, Pibf, ZO-1). No alteration of Rdh levels were observed in *C. angulata* exposed to Low pH+As, in contrast with increased levels of Rdh in Low pH single exposure, could be related to inhibition of this enzymes catalytic activity by metals (Luo et al., 2014; Meng et al., 2015, Bao et al., 2016). These data show that the combined effects of Low pH and As induce a different response that the isolated exposure to Low pH regarding Rdh, with likely implications at the physiological level.

Both Severin and Gelsolin, that presented increased expression in oysters exposed to As, did not show alterations under Low pH+As, evidencing that multiple stressors induced a differentiated response capacity at the cytoskeleton level. Moreover, *C. angulata* exposed to Low pH+As presented the highest fold change of ZO-1 (10-fold lower) and Pibf abundance (10-fold higher) (Table VIII) compared to As single exposure, indicating a stronger modulation of cell to cell adherence (ZO-1), and higher immunomodulation response (Pibf) in *C. angulata* exposed to multiple stressors.

3.1.2.2 *Crassostrea gigas*

Influence of Seawater acidification on Arsenic accumulation

Results on total As (µg g^{-1} fw) accumulated in juvenile *C. gigas* soft tissue exposed to As, Low pH and Low pH+As, are depicted in Table IX. As concentrations were significantly higher in oysters exposed to As (As; Low pH+As) than in the remaining conditions (CTL, Low pH). No significant differences were observed in As accumulation between oysters exposed to As in acidified (Low pH+As) and non-acidified (As) conditions.

Table IX – Total As (µg g^{-1} fw) accumulated in juvenile *C. gigas* exposed to CTL (control), As, Low pH and Low pH+As. Significant differences ($p \leq 0.05$) among tested conditions are represented with different letters (mean ± standard error, n=5).

	CTL	As	Low pH	Low pH+As
µg As g^{-1} fw	1.03±0.1**a**	4.59±0.2**b**	1.31±0.06**a**	5.39±0.9**b**

The average concentration of As in oysters ranged from ca 1.0 to 5.4 µg g^{-1} fw (equivalent to ca. 5 and 27 µg g^{-1} dry weight (dw) (Zhang et al., 2013). The highest As concentrations (5.39 µg g^{-1} fw ca. 27 µg g^{-1} dw) accumulated in oyster tissues after exposures were equivalent to reported values in oysters collected in other estuarine systems e.g. up to 26.7 µg g^{-1} dw in *C. gigas* in France (Kohlmeyer et al., 2002) and 25.4 µg g^{-1} dw in *C. virginica* from the US (Valette-Silver et al., 1999).

Influence of Seawater acidification and Arsenic on oysters biochemical performance

Results on biochemical parameters studied in juvenile *C. gigas* exposed to As, Low pH and Low pH+As are depicted in Figure 8. Significant alterations in antioxidant (SOD and CAT) and biotransformation (GSTs) enzymes activity, cellular damage (LPO), redox status (GSH/GSSG), metabolic potential (ETS) as well as energetic reserves (GLY) were observed among tested conditions.

SOD activity was significantly lower in oysters exposed to As and Low pH compared to CTL. Oysters exposed to Low pH showed significantly higher SOD than in As exposure. Results obtained for CAT, presented a similar pattern of SOD, with the lowest activity levels observed in As exposed oysters.

Higher GSTs activities were noted in oysters exposed to As and Low pH +As, comparing to the remaining conditions, with significant differences in oysters exposed to Low pH+As compared to the remaining conditions.

GSH/GSSG and LPO were significantly higher in oysters exposed to Low pH+As compared to the remaining conditions. The ETS activity was significantly lower in oysters exposed to As comparing to the remaining conditions. The GLY content was significantly higher in oysters exposed to all stress conditions (As, Low pH and Low pH+As) compared to CTL.

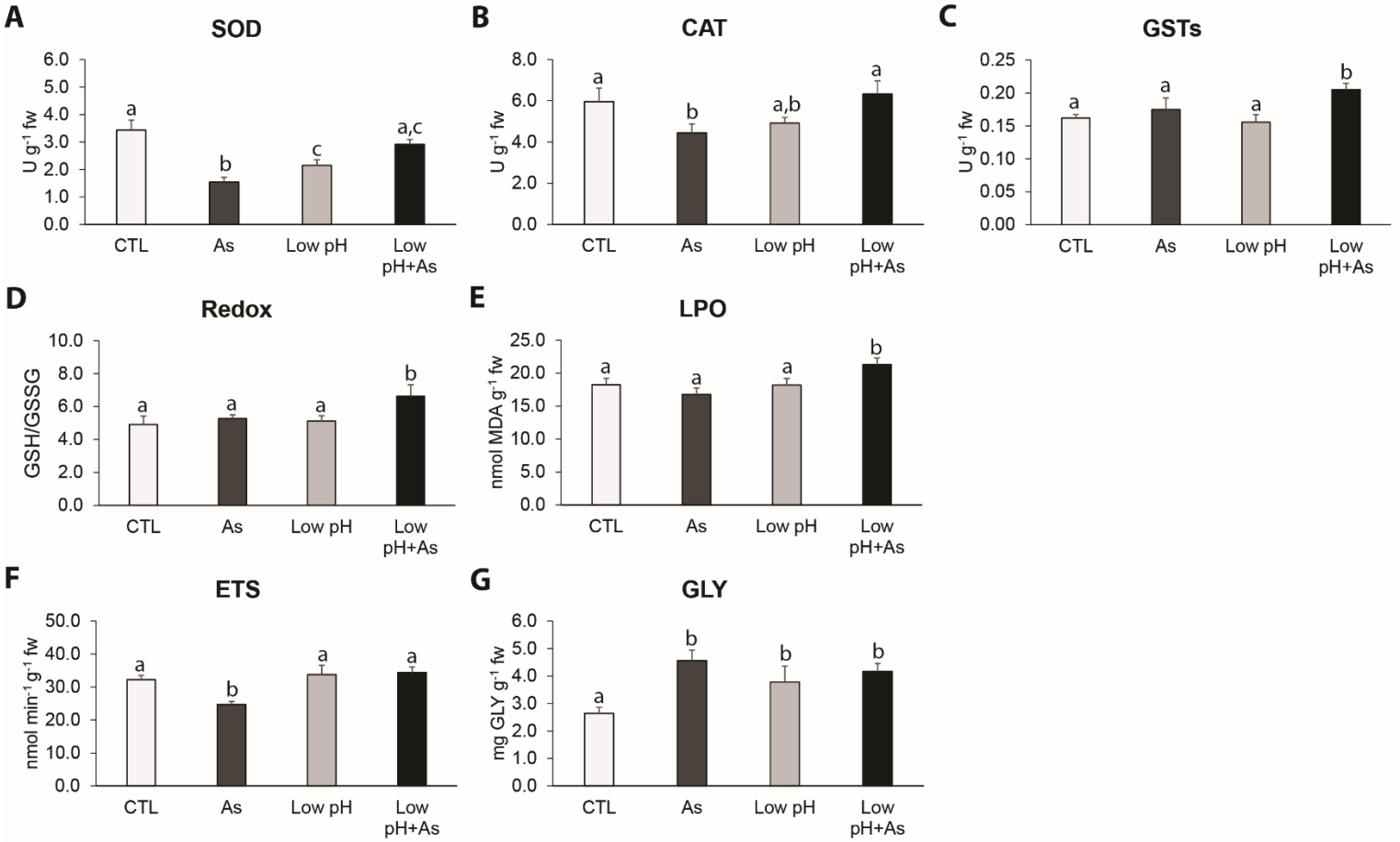

Figure 8 – Biochemical parameters studied in juvenile *C. gigas* exposed to As, Low pH, Low pH+As.

A: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Glutathione *S*- transferases (GSTs) activity; **D**: Reduced to oxidized glutathione ratio (Redox); **E**: Lipid peroxidation (LPO) levels; **F**: Electron transport system (ETS) activity; **G**: Glycogen (GLY) content. Significant differences ($p \leq 0.05$) between conditions are represented with different letters (Mean + standard error; n=12).

Several factors could explain results on decreased antioxidant enzymes (SOD and CAT) activity in juvenile *C. gigas* exposed to As, such as enzyme activity impairment, antioxidant response dynamics or lower rates of ROS production in this condition. Under oxidative stress, enzymes inhibition could result from direct interaction with ROS, or by indirect mechanisms (e.g. glycation) that prevent normal enzymatic function (Lushchak, 2007). Also, antioxidant defence systems in marine invertebrates are often dynamic, and present transient patterns of inhibition/activation depending on duration of exposure (reviewed in Regoli et al., 2011).

Interestingly, SOD and CAT activities in oysters exposed to Low pH+As were similar to values observed in CTL, and relatively higher than those observed in single exposures, evidencing a differentiated antioxidant response in this condition. The differentiated antioxidant response between oysters exposed to As in the presence or absence of seawater acidification were further elucidated by results on the ETS activity. Alterations in metabolic potential, as observed considerinfg the significant decrease of ETS activity in oysters exposed to As, further elucidate on the biochemical response of these oysters.

The ETS assay gives the maximum potential of metabolic rate of a given organism, as proxy of the electron transport flow rate through the electron transport chain (Bielen et al., 2016). Also, the ETS is the major site for ROS production in mitochondria, particularly at complexes I and III during forward electron flow in oxidative phosphorylation reactions (Murphy, 2009). Therefore, alterations in the functioning of this system may influence the overall oxidative stress status. Indeed, recent evidence suggests that complex I and III of the ETS are sites for ROS production rates regulation in marine invertebrates mitochondria, namely through changes in these proteins abundance, composition and post-translational modifications (reviewed in Tomanek, 2015). Lower ETS activity in oysters exposed to As could therefore be a mechanism induced to supress ROS production rates. This hypothesis could also explain antioxidant (SOD and CAT) suppression in the same condition. Similar, relations between ETS and antioxidants have been described in bivalves (Almeida et al., 2014). On the other hand, lower ETS activity could be indicative of mitochondria damage, knowing that As could affect cellular respiratory metabolism, given that mitochondria swelling has been described in oysters *C. gigas* exposed to As (Ettajani et al., 1996), and knowing that the activity of the ETS can be inhibited by other pollutants, as observed in *Anodonta anatina* and *Sinanodonta woodiana* mussels exposed to $ZnCl_2$ (Bielen et al., 2016). However, this should be unlikely given the results on As accumulation and results on ETS activity in oysters exposed to Low pH+As, for which no alteration in ETS was observed.

Additionally, significantly higher GSTs activity, GSH/GSSG and LPO in oysters exposed to Low pH+As, also indicated a differentiated stress response status in oysters exposed to the combination of both stressors. The GSTs family constitute a complex of enzymes specifically involved in cellular detoxification processes, through the conjugation of toxicants to glutathione or other electrophilic compounds (Townsend & Tew, 2003).

Similarly to *C. angulata*, the increase of GSH/GSSG in oysters exposed to Low pH+As, was due to a significant decrease in GSSG concentrations (no change in GSH concentrations

were observed), possibly due to GSSG excretion towards the extracellular matrix (Garcia et al., 2010; Han et al., 2006; Hawkins et al., 2010; Hurd et al., 2005); and/or a shift towards NADPH producing pathways (Tomanek, 2014) and the concomitant increase of the rate of GSSG reduction by glutathione reductase (GR), thus shifting the ratio towards the reduced form (Bindoli et al., 2009).

Similarly to *C. angulata* (previously described) *C. gigas* also presented higher LPO under the combined exposure (Low pH+As) evidencing higher cellular injury induced by the presence of both stressors. Overall, these results indicate higher oxidative stress in oysters exposed to Low pH+As, with relatively higher antioxidant capacity (SOD, CAT and GSTs) compared to single exposures, that were however insufficient to prevent increased oxidative damage (LPO). The differentiated response observed should therefore be attributed to the interactive effects of both stressors, knowing that As accumulation levels were indistinguishable between oysters exposed to As under normocapnic (As) or hypercapnic conditions (Low pH+As).

Results on GLY content, further revealed changes in oysters energetic fitness. Lower GLY content observed in oysters maintained in CTL compared to all other conditions, suggested lower physiological activity in oysters exposed to stress conditions.

Influence of Seawater acidification and Arsenic on oysters proteome

Proteomic analysis of *C. gigas* showed differentially expressed protein profiles between all tested conditions (Figure 9). MS analysis allowed to identify significantly altered proteins with biological functions related to metabolism (Enolase; Aconitate hydratase), cytoskeleton structure (Actin; Atlastine), organellar (Atlastin) and extracellular structure (Neprilysin) and cellular stress response (Heme-binding protein 2; Arginine-tRNA-protein transferase 1; Alpha crystallin a chain; Atypical serine-protein kinase ATM; Aldehyde dehydrogenase; and Retinal dehydrogenase) (Table X and Figure 9). One uncharacterized protein LOC105317411 (annotated in *C. gigas* genome) was also identified, but its molecular and biological roles remain unknown.

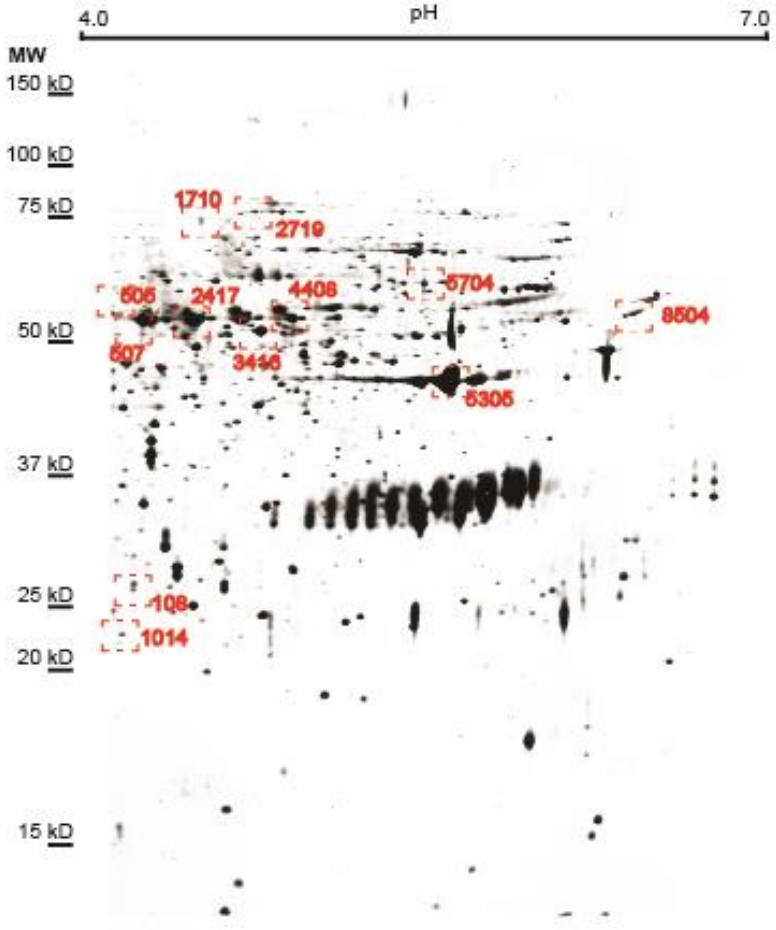

Figure 9 - 2DE protein map of identified proteins in juvenile *C. gigas* exposed to As, Low pH and Low pH+As.

Protein expression profile of juvenile *C. gigas* whole soft tissue by 2-DE. Differentially expressed proteins identified by MALDI TOF MS are highlighted in the representative gel image.

Table X – Differentially expressed proteins in juvenile *C. gigas* exposed to CTL (control), As, Low pH and Low pH+As.

Differentially expressed proteins identified in *Crassostrea angulata* by MALDI TOF MS. Observed molecular weight (MW) and isoelectric point (pI) are provided. Fold change of spot density between tested groups (CTL; As ; Low pH; and Low pH+As) are expressed for pairwise comparisons that showed significant differences ($p \leq 0.05$).

Spot no.	GenBank ass. no.	Protein name	Mascot score	MW(kDa)/pi	Putative biological function(s)	Fold change
108	EKC31868.1	Arginyl-tRNA--protein transferase 1	65	28.1/4.6	Stress signalling/apoptosis	4.5 ↓ in As vs. CTL
505	CGI_10013191	Atlastin-2-like isoform X4	64	60.9/4.4	ER Dynamics	3.1 ↑ in Low pH vs. CTL
507	XP_022316405.1	Neprilysin-2-like	64	54.6/4.5	Extracelular structure	2.4 ↑ Low pH vs. CTL
1014	XP_011448780.1	Alpha-crystallin A chain	90	23.5/4.5	Stress protein	7.1 ↓ in As vs. Low ph
1710	XP_011445349.1	Heme-binding protein 2	60	82.1/4.6	Redox balance Imune response	2.6 ↑ in As vs. CTL
2417	XP_011436817.1	Retinal dehydrogenase	129	59.5/4.5	Stress response	2.2 ↑ in Low pH vs Low pH+As
2719	XP_011411873.1	Aconitate hydratase isoform X2. cytoplasmic	90	91.8/4.7	Metabolism	2.7 ↑ in As vs Low pH
3416	XP_011436228.1	Enolase	87	55.6/4.9	Glycolitic process	2.8 ↑ in Low pH vs. Low pH+As
4408	XP_011450475.1	Aldehyde dehydrogenase. mitochondrial	94	59.5/4.8	Antioxidant	2.9 ↑ in Low pH+As vs Low pH
5305	EKC30048.1	Actin	119	45.7/5.4	Cytoskeleton	3.8 ↑ in Low pH vs. Low pH+As 4.0 ↑ in Low pH vs. As 4.1 ↑ in Low pH vs. CTL
5704	XP_019919028.1	Serine-protein kinase ATM	65	66.8/5.4	Cell cycle Stress response	2.2 ↑ in Low pH+As vs. CTL
8504	CGI_10008427	Uncharacterized LOC105317411	64	61.5/6.0	Unknown	2.9 ↓ in Low pH vs. CTL 2.3 ↓ Low pH vs. Low pH+As

Influence of Arsenic exposure on oysters proteome

Data concerning proteomic analysis of juvenile *C. gigas* exposed to As, revealed altered expression levels of proteins involved in oxidative stress repair (Heme-bp), and stress resistance (Ate-1) (Table X), that together with results on biochemical parameters provide a mechanistic view in oyster stress response mechanisms.

For instance, Heme-binding protein 2 (Heme-bp), assigned to the SOUL heme-binding superfamily, showed increased expression in *C. gigas* exposed to As. These proteins are involved in thiol/disulphide redox switches that modulate ion channel functioning, heme affinity and carbon monoxide metabolism (Ragsdale & Yi, 2011). Their biological functions have been related to oxidative stress repair, apoptosis and immune response (Fortunato et al., 2016). Other studies have described upregulated heme binding genes in marine invertebrates under environmental stress, including *C. gigas* infected by Ostreid herpesvirus-1 (He et al., 2015) and *Tigriopus japonicus* copepods exposed to Cu (Ki et al., 2009).

On the other hand, lower abundance of Arginine-tRNA-protein transferase 1 (Ate-1) was observed in *C. gigas* exposed to As in comparison to CTL (Table X). Ate-1 mediates proteolysis by catalysing protein arginylation, a process involved in actin cytoskeleton regulation (Kashina et al., 2014). Additionally, Kumar et al. (2016) recently studied Ate-1 and arginylation processes during stress response signalling towards oxidative, thermal, osmotic and metal stress. Among several findings, these authors suggested that Ate-1 depletion may increase cellular resistance to several stressors (e.g. H_2O_2, $CdCl_2$, ultra violet radiation and others) and supress apoptosis (Kumar et al., 2016). Considering these findings, decreased levels of Ate-1 in *C. gigas* exposed to As suggests increased stress resistance, that corroborate and complement results on biochemical parameters that showed no alterations in oxidative damage (unchanged LPO) despite lower antioxidant (CAT and SOD) and metabolic (ETS) capacity previously described. Interestingly, oysters exposed to the combination of Low pH+As did not present Ate-1 downregulation, but in turn presented in higher oxidative damage.

Additionally, proteomic analysis also showed higher abundance of Aconitate hydratase (Achd) in *C. gigas* exposed to As in comparison to oysters exposed to Low pH (Table X). This mitochondrial enzyme is an important player in the TCA cycle, and a potential oxidative stress biomarker because it is highly sensitive to ROS (Chercasov et al., 2007 and references therein). Khondee et al. (2016) also observed increased Achd abundance in *C. gigas* exposed to Tributyltin (TBT) and explained those results as a stimulation of the energy flow through the TCA cycle to fuel energetically demanding response mechanisms towards xenobiotic exposure. However, it is unlikely that this was the case in *C. gigas* exposed o As, given the results on lower metabolic capacity (ETS) and higher energetic reserves (GLY) observed (previous section). Other factors, such as Achd transcriptional upregulation as a compensation mechanism to counteract ROS mediated self-inactivation, as proposed for *Argopecten irradians* scallops under hypoxia (Ivanina et al., 2016); or the fact that ROS damaged Achd may form aggregates and accumulate in mitochondria (Bota et al., 2002), could explain increased abundance levels of Achd observed, in which case reflecting accumulation of damaged protein.

Infuence of Seawater acidification on oysters proteome

C. gigas exposed to Low pH presented increased expression levels of Actin, Atlastin and Neprilysin in comparison to oysters maintained in CTL conditions (Table X). These changes generally indicated cytoskeleton, organelle and extracellular matrix restructuring, with likely implications concerning the interpretation of the biochemical parameters studied.

For instance, increased expression of Actin in oysters exposed to Low pH suggested cytoskeleton restructuring, possibly helping to prevent oxidative damage (Dailianis et al., 2009), or to replace damaged protein incurring oxidative damage (Thompson et al., 2016). Actin, the predominant structural component of the cytoskeleton (Small, 1988) is among the most regulated proteins in oyster species incurring environmental stress (reviewed by Anderson et al., 2015), and has also been shown in oysters exposed to seawater acidification (Thompson et al., 2015, 2016).

Proteomic analysis further revealed induction of Atlastin in *C. gigas* exposed to Low pH compared to oysters maintained in CTL conditions (Table X). Atlastin, has a role in shaping endoplasmic reticulum (ER) tubular network, by generating ER branched structures (Barlowe, 2009) and modulating proteoliposome/membrane fusion (Farhan & Hauri, 2009). The ER is a key organelle in protein synthesis/folding, calcium storage, metabolism and many signalling processes (Görlach et al., 2006), but is also a major source of reactive oxygen species (ROS) in invertebrates experiencing environmental stress (Tomanek, 2015). Therefore, the present results suggest reconfiguration of the ER by increased abundance of Atlastin in oysters exposed to Low pH, with likely implications in all the above stated cellular processes, including modulation of ROS production rates.

Increased expression of Neprilysin was also observed in *C. gigas* exposed to Low pH comparing to CTL (Table X). In invertebrates the biological roles of Neprilysin have been related to neuro stimulation (Turner et al., 2001), regeneration capacity (Sarras et al., 2002), extracellular matrix breakdown, gelatinolytic and fibrinolytic activities (Dominguez-Perez et al., 2018). Given this, the present results suggest Neprilysin to participate in extracellular restructuring and dynamics in *C. gigas* exposed to Low pH. Overall, considering results on biochemical and proteomic markers, it seems that the above stated protein shifts were sufficient to prevent cellular damage.

Influence of the combined exposure to Seawater acidification and Arsenic on oysters proteome

In line with results on biochemical markers that suggested increased antioxidant defence, biotransformation capacity, LPO and GSH/GSSG, data from proteomic analysis of juvenile *C. gigas* exposed to Low pH+As brought new insights regarding the physiological status of these oysters.

For instance, abundance of Aldehyde dehydrogenase (Aldh) was higher in Low pH+As than in Low pH condition (Table X). Aldh is an enzyme involved in aldehyde detoxification, a biproduct of ROS interaction with polyunsaturated fatty acids (Singh et al., 2013). Hence Aldh is an important enzyme in the oxidative stress response, by mitigating deleterious effects of aldehydes formation in processes such as lipid peroxidation (Marchitti et al., 2008; Singh et al.,

2013). Several studies have demonstrated Aldh upregulation in bivalve species in response to environmental stress, including *S. cucculata* exposed to TBT (Khondee et al., 2016) and *Mytilus galloprovincialis* under thermal stress (Tomanek, 2012). Apart from aldehyde detoxification, another role of Aldh is to provide reducing equivalents (NADPH) to increase reactive oxygen species (ROS) scavenging capacity by the glutathione system (Tomanek, 2014; 2015). Therefore, the present findings showing higher Aldh levels in *C. gigas* exposed to Low pH+As in comparison to Low pH, demonstrate that the combined exposure elicited higher oxidative stress response in *C. gigas*, in accordance with data from biochemical markers.

Moreover, results also revealed differences in protein abundance profiles between stress conditions, namely Retinal dehydrogenase (Rdh) and Enolase (Table X). Results showing increased levels of Retinal dehydrogenase (Rdh) in *C. gigas* exposed to Low pH compared to Low pH+As, were similar to those observed in *C. angulata* (previously described). These findings indicate that upregulation of Rdh could be a common mechanism in oyster species exposed to acidification, also in line with results from Thompson et al. (2015), and could likely involve increased tissue regeneration capacity (Albalat, 2009; Gutierrez-Mazariegos et al., 2015). However, induction of this protein was not observed under the combined effects of Low pH+As, evidencing that the presence of both stressors induced a different response capacity, possibly because Rdh is inhibited by metals (Bao et al., 2016; Luo et al., 2014; Meng et al., 2015) and could be one of the underlying reasons explaining higher oxidative stress in this condition observed through biochemical analysis.

Juvenile *C. gigas* also presented higher expression levels of Enolase in Low pH exposure compared to Low pH+As (Table X). Other studies have described Enolase increased expression in bivalves, such as *Mytilus edulis* under thermal stress (Péden et al., 2016) and *S. cucculata* exposed to Tributyltin (TBT) (Khondee et al., 2016). Glycolysis stimulation by Enolase has been discussed as a mechanism to increase carbohydrate metabolism in bivalves experiencing energetically demanding conditions (Tomanek, 2014; Artigaud et al., 2015). Considering the concept of energy-limiting stress tolerance proposed by Sokolova et al. (2012), and the present data showing strong intra and extracellular restructuring (Actin, Atlastine and Neprilysin) as well as induced regeneration capacity (Rdh) in *C. gigas* exposed to Low pH, it is likely that Enolase was upregulated to stimulate carbohydrate metabolism as a means to fuel these energetically demanding stress response mechanisms. Significant differences of Enolase expression levels between Low pH and Low pH+As only, indicate differences in energetic modulation in oysters exposed to single and combined exposures.

It is important to note that several proteins that showed altered expression levels in single exposures (Low pH or As) did not present the same alteration in oysters exposed to the combination of both stressors. Namely Actin, Atlastine, Neprilysin, Heme-bp (Table X). Generally, unchanged expression profiles of these proteins in oysters exposed to Low pH+As, evidenced that the combined effects of different stressors induced a different proteomic response in *C. gigas*, and therefore could elucidate the results from biochemical markers analysis.

3.1.2.3 Species comparison

Different response patterns were observed regarding each species biochemical performance towards each stressor. Overall, fewer alterations in oxidative stress parameters were observed in *C. angulata* compared to *C. gigas*, with the latter presenting higher capacity to induce antioxidant and biotransformation enzymes (SOD, CAT and GSTs). Data also suggested different metabolic strategies to endure exposure conditions, with *C. angulata* presenting unaltered metabolic potential (ETS) and energetic fitness (GLY). On the contrary, *C. gigas* presented altered metabolic potential (decreased ETS activity in oysters exposed to As) and bioenergetics (increased GLY content in oysters exposed to all stress conditions). The combined effects of seawater acidification and As induced higher oxidative stress in both species, than single exposures.

The results obtained through proteomic analysis allowed for a deeper insight into the modes of action of these closely related oyster species towards the combined effects of acidification and As exposure. As a corollary, both species induced cellular remodelling in response to external stimuli, observed by altered levels cytoskeleton related proteins, namely Actin and Atlastine (*C. gigas*); Actin, Severin, Coronin and Gelsolin (*C. angulata*). However, the conditions at which each species presented such alterations differed, with *C. gigas* presenting altered levels of cytoskeleton proteins in Low pH exposures, while *C. angulata* showed most alterations under both Arsenic exposures (As and Low pH+As).

Interestingly, proteomic analysis suggested metabolic suppression in *C. angulata* (downregulation of ATP β) in both Low pH and Low pH+As exposures.

Proteomic analysis of oysters exposed to the combined exposure to Low pH+As revealed important differences in oysters response capacity compared to that observed in single exposures, corroborating the working hypothesis that multiple stressors will further challenge oyster species in the environment.

Together, analysis of biochemical performance and proteomics revealed that each species presented different mechanisms to endure tested conditions. Overall, *C. angulata* appeared to be less responsive than *C. gigas* considering the biochemical markers studied. This could either indicate a weaker response capacity, or lower sensitivity to tested conditions.

3.1.3 Adults

The effects of seawater acidification and As (single and combined exposures) were assessed in adult *C. angulata* and *C. gigas* after 28 days laboratory exposures to (As), seawater acidification (Low pH) and the combined effects of both stressors (Low pH+As). Arsenic accumulation and biochemical markers were assessed in oysters soft tissue.

3.1.3.1 *Crassostrea angulata*

Seawater acidification and Arsenic exposure

Influence of Seawater acidification on Arsenic accumulation

Total As (µg g^{-1} fw) accumulated in adult *C. angulata* exposed to As and Low pH+As, are depicted in Table XI. No significant differences were observed in As accumulation between oysters exposed to As in acidified (Low pH+As) and non-acidified (As) conditions.

Table XI- Total As (µg g^{-1} fw) accumulated in adult *C. angulata* exposed to As and Low pH+As. Significant differences ($p \leq 0.05$) among tested conditions are represented with different letters (mean ± standard error, n=3).

	As	Low pH+As
µg As g^{-1} fw	3.40±0.6**a**	3.66±0.3**a**

Average total As accumulated by adult *C. angulata* was ca. 3.5 µg g^{-1} fw (equivalent to ca. 17.5 µg g^{-1} dw) (Zhang et al., 2013), was in the low range of reported values in oysters collected the environment (e.g. up to 26.7 µg g^{-1} dw in *C. gigas* in France, Kohlmeyer et al., 2002; and 25.4 µg g^{-1} dw in *C. virginica* from the US, Valette-Silver et al., 1999). No difference in As accumulation considering acidified (Low pH+As) and non-acidified (As) conditions, indicate seawater acidification had no effect on As uptake and/or elimination processes. This is in contrast with studies on *C. virginica* exposed to Cu and Cd, that showed higher accumulation of these elements in acidified conditions (Götze et al., 2014).

Influence of Seawater acidification and Arsenic on oysters biochemical performance

Biochemical markers assessed in adult *C. angulata* exposed to As, Low pH, and Low pH+As are depicted in Figure 10.

No significant alterations were observed concerning antioxidant enzymes activities (SOD and CAT) among tested conditions. Significantly higher GSTs activity was observed in oysters exposed to As, and Low pH+As comparing to CTL.

No significant alterations were observed in either GSH content or LPO among tested conditions.

Significantly lower Carbonic anhydrase (CA) activity was observed in oysters exposed to As, Low pH and Low pH+As comparing to CTL. CA activity was significantly lower in both Low pH and Low pH+As exposures comparing to CTL and As exposure conditions.

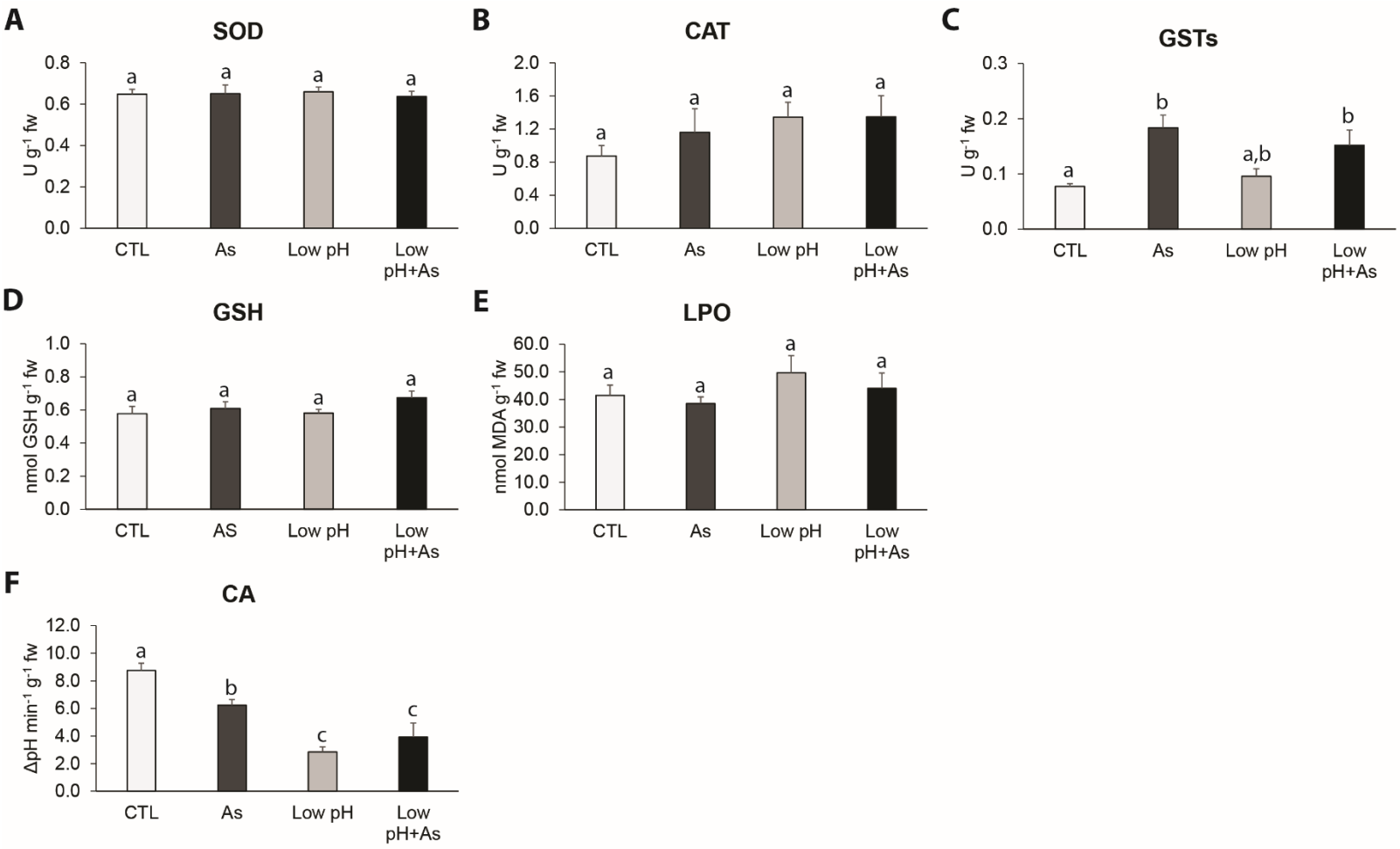

Figure 10 – Biochemical parameters studied in adult *C. angulata* exposed to CTL (control) As, Low pH and Low pH+As.

A: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Glutathione *S*-transferases (GSTs) activity; **D**: Reduced glutathione (GSH) content; **E**: Lipid peroxidation (LPO) levels; **F**: Carbonic anhydrase (CA) activity. Significant differences ($p \leq 0.05$) between conditions are represented with different letters (Mean + standard error; n=9).

Few alterations in biochemical parameters studied were observed in *C. angulata* exposed to these stressors. For instance, no alteration in either antioxidant enzymes (SOD and CAT) activity, GSH content or LPO levels were observed in adult *C. angulata*, thus suggesting that oxidative stress status was similar among tested conditions. Nonetheless, biotransformation capacity (GSTs activity) was significantly enhanced in oysters exposed to As (As and Low pH+As), compared to organisms under CTL and Low pH. Similarly, Zhang et al. (2015) found that GSTs activity was positively correlated to As biotransformation processes in *Saccostrea cucculata*. Other studies have also related GSTs activity to As detoxification steps in fish (Ventura-Lima et al., 2011).

Generally, these findings are in contrast with those observed in juvenile *C. angulata* (previously described) for which significant increases in SOD activity and LPO levels were observed in oysters exposed to Low pH+As, suggesting that juvenile *C. angulata* are more susceptible to oxidative stress than adults. Differences in antioxidant capacities between bivalve specimens of different ages can be expected to occur, although these do not always respond in the same pattern. For instance, CAT activity has been shown to decrease with age in *C. virginica* (Ivanina et al., 2008) and *M. edulis* (Viarengo et al., 1991), or to remain constant as in *M. edulis* and *Mya arenaria* (Suhkotin et al., 2002), and *Laternula elliptica* (Philipp et al., 2005). Interestingly, adults presented higher capacity to induce GSTs activity, compared to juveniles. This could be another important factor explaining differential cellular damage (LPO) in juveniles compared to adults.

Apart from oxidative stress biomarkers, results on biomineralization enzyme CA also revealed changes among conditions. CA is one of the major enzymes involved in calcification and biomineralization processes in a wide range of taxa (Le Roy et al., 2014), and has been identified as one of the most important shell formation enzymes in *C. gigas* (Zhang et al., 2012). The present findings, showing significant inhibition of this enzyme in *C. angulata* exposed to both stressors (Low pH and As), likely represents reduced biomineralization capacity, and suggest that Low pH has a more deleterious effect than As single exposure, considering CA only. Other studies have shown sensitivity of CA to pollutants. Cadmium has been shown to inhibit CA activity in *M. galloprovincialis* mussels (Lionetto et al., 2006). Skaggs & Henry (2002) demonstrated that Ag^{+}, Cd^{+}, Cu^{2+} and Zn^{+} inhibited CA activity in *Callinectes sapidus* and *Carcinus maenas* crabs.

Other authors have studied CA in oyster species, despite obtaining contrasting results. For instance, Beniash et al. (2010) observed increased CA mRNA expression in *Crassostrea virginica* exposed to hypercapnia (~3500 µatm), and suggested seawater acidification would increase CA activity. In contrast, our results indicate that low pH may in fact impact the performance of this enzyme. Dickinson et al. (2012) on the other hand, showed no variation in CA activity in *C. virginica* exposed to high pCO_2, although testing lower CO_2 concentrations than those tested here (~700-800 µatm), and therefore these discripancies could indicate that higher *p*CO2 levels could have a measurable effect. As for the combined effect of Low pH+As, no cumulative effect was noticed since between pH and pH+As, given that no significant differences were observabed considering CA activity.

Salinity and Arsenic exposure

The effects of varying salinity and As (single and combined exposures) were assessed in adult *C. angulata* and *C. gigas* after 28 days laboratory exposures to different salinities (10, 20, 30 and 40), in the presence or absence of As. Arsenic accumulation and biochemical markers were assessed in oysters soft tissue.

Influence of Salinity on Arsenic accumulation

Total As (µg g^{-1} fw) accumulated in adult *C. angulata* soft tissue exposed to As at different salinities (10, 20, 30 and 40) are depicted in Table XII. Arsenic accumulation followed a decreasing trend with the increase of salinity, with significantly higher total As concentrations observed in oysters at the lowest salinity (10) compared to oysters exposed at the highest salinity 40.

Table XII- Total As (µg g^{-1} fw) accumulated in adult *C. angulata* after 28 days laboratory exposures. Significant differences ($p \leq 0.05$) among tested conditions are represented with different letters (mean ± standard error, n=3).

Salinity	10	20	30	40
µg As g^{-1} fw	5.86±0.7**a**	5.02±0.7**a,b**	4.04±0.5**a,b**	3.39±0.09**b**

Total As concentrations in oyster tissue was highest at the lowest salinity (10) and followed a decreasing trend in concentration with the increase of salinity. The following non-mutually exclusive hypothesis could explain these results: firstly, low salinity and the inherent decrease in chloride ion concentrations, lead to decreased trace metal ion complexation, and thus enables for an increase of free trace metal ion (Bianchini & Gilles 2000; Rainbow et al., 1997). Secondly, different salinities may cause changes in elements accumulation, due to alterations of ion fluxes between organisms and the environment, consequently increasing elements uptake (Connell, 1989). Thirdly, organisms physiological behaviour can also play an important role in elements uptake, due to changed in filtration, ventilation and metabolic rates (Rainbow et al., 1997). Although neither of the above hypotheses can be excluded, data on biochemical markers presented evidence of increased metabolic rate at the lowest salinity (10) (ETS activity furtherly discussed), and could corroborate the hypothesis of the effects of physiological alterations on As uptake.

Influence of Salinity and Arsenic on oysters biochemical performance

Biochemical markers assessed in adult *C. angulata* exposed to different salinities (10, 20, 30 and 40) in the presence or absence of As are depicted in Figure 11.

Significantly higher SOD activity was observed in oysters exposed to salinities 10 and 20 compared to higher salinities (30 and 40). The lowest SOD activity was observed in oysters exposed to salinity 30 (both in the presence or absence of As). SOD activity was significantly higher in oysters exposed to salinity 10 in the presence of As compared to the respective negative control.

Catalase (CAT) presented a decreasing trend in activity with increasing salinity considering oysters exposed and unexposed to As, with significantly higher CAT activity in oysters exposed to the lowest salinity (10).

Glutathione *S*-Transferases (GSTs) activity was similar throughout tested salinities, with no significant differences among conditions, in oysters exposed and unexposed to As. At each salinity level, significantly higher GSTs activity was observed in oysters exposed to As, compared to each respective negative control (except for the lowest salinity of 10).

Considering oysters exposed to salinity only, significantly lower GSH/GSSG was observed at salinity 10, in comparison with GSH/GSSG values obtained at salinity 30. In oysters exposed to As, significantly higher values GSH/GSSG were observed in individuals exposed to salinities 20 and 30 relative to oysters exposed to salinity 40.

Lipid peroxidation (LPO) levels were significantly lower in oysters exposed to As at salinity 10, with the highest values recorded at salinity 40. In unexposed oysters, significantly lower values were observed at salinity 30, with no significant differences among the remaining conditions (10, 20 and 40). At each tested salinity, LPO was significantly higher in oysters exposed to As at the majority of tested salinity (except for salinity 10), compared to the respective negative controls.

The Electron Transport System (ETS) activity presented a decreasing trend of activity with the increase of salinity, both in the presence or absence of As, with significantly higher values at the lowest (10) compared to the highest salinity (40).

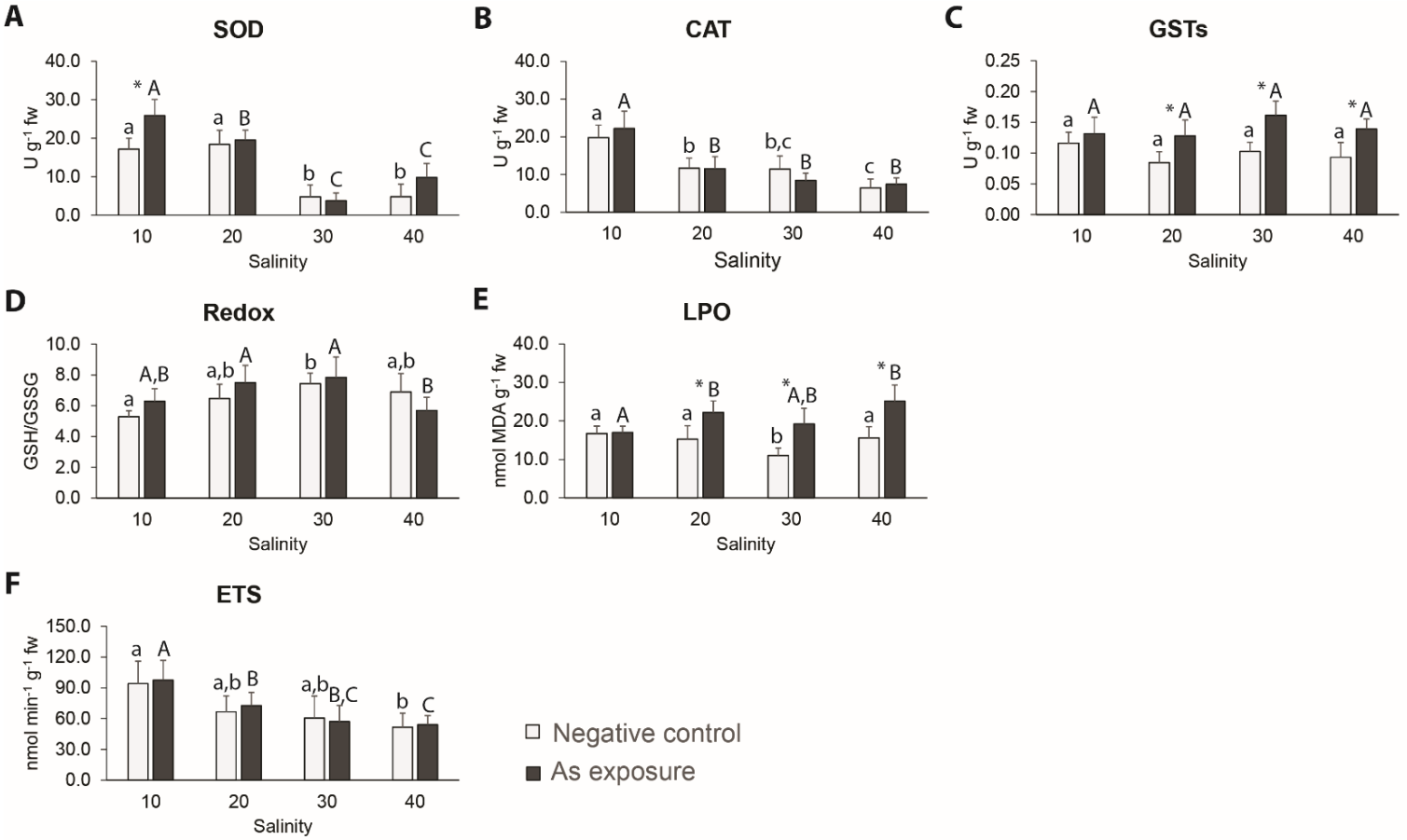

Figure 11 – Biochemical parameters studied in adult *C. angulata* exposed to salinities 10, 20, 30 and 40 in the presence or absence of As.

A: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Glutathione *S*-transferases (GSTs) activity; **D**: Reduced to oxidized glutathione ratio (Redox); **E**: Lipid peroxidation (LPO) levels; **F**: Electron transport system (ETS) activity. Significant differences ($p \leq 0.05$) between conditions are represented with different letters. Significant differences observed between oysters exposed in the presence or absence of As at each salinity are represented with an asterisk (Mean + standard error; n=9).

The most prominent changes concerning the biochemical markers studied in adult *C. angulata* were observed regarding SOD, CAT, GSTs, GSH/GSSG, LPO and ETS, reflecting the complex interaction of mechanisms of physiological adaptation that include alterations in metabolism, bioenergetics and oxidative stress (Rivera-Ingraham & Lignot, 2017).

Antioxidant enzymes SOD and CAT showed overall higher activity in oysters maintained at lower salinities (in the presence or absence of As). Higher SOD activities were observed at salinities 10 and 20 (with or without As) indicating that hypoosmotic stress mediated an antioxidant response in oysters. These findings can be explained due to higher metabolic rates (measured by ETS) observed at these salinities. Within the ETS, complexes I and III of the electron transport chain are major sites of ROS production (superoxide anions, hydrogen peroxide and hydroxyl radicals), and therefore higher metabolic rates are likely to induce higher ROS production (Guzy & Shumacker, 2006; Murphy, 2009), which in turn could have induced SOD activity. Other studies have demonstrated that hyposaline stress induced SOD mRNA expression and enzymatic activity in ark shell *Scapharca broughtonii*, accompanied by increased H_2O_2 haemolymph concentrations (An & Choi, 2010). Meng et al. (2013) identified induction of ROS transduction pathways in *C. gigas* exposed to low salinity, namely the upregulation of glutathione reductase, attributed to a response to high levels of H_2O_2, induced by hypososmotic stress. Tomanek et al. (2012) also described a possible relationship between hyposaline stress, increased energy metabolism and oxidative stress in *Mytilus trossulus.*

In oysters exposed to As, SOD activity was significantly higher compared to the respective negative control at salinity 10, probably due to higher levels of accumulated As observed in this condition. Arsenic toxicity may induce an imbalance between prooxidant and antioxidant cellular status, often leading to oxidative stress (Samuel et al., 2005), and therefore it would be expected that As could induce SOD activity. The lack of a differentiated response in SOD activity between oysters maintained at salinities 20, 30 and 40, in the presence or absence of As, may be related to lower metabolic potential observed in the same conditions, or to less effectiveness of the antioxidant defence system with increasing salinity, as described in other studies (Carregosa et al., 2014; Freitas et al., 2015).

The activity of CAT followed a similar response pattern of SOD. Activity of these enzymes are usually linked, since SOD catalyses superoxide anion into hydrogen peroxide, which in turn may be catalysed by CAT into water (Monserrat et al., 2007). In contrast, Zanette et al. (2011) did not observe alterations in CAT activity in oysters (*C. gigas*) exposed to a similar salinity gradient.

Significantly higher GSTs activity in oysters exposed to As at salinities 20, 30 and 40, are in accordance with recent studies that have implicated GSTs as important enzymes involved in As detoxification processes in oysters *C. angulata* and *C. gigas* (Moreira et al., 2016), and *Saccostrea cucculata* (Zhang et al., 2015a). Varying salinity alone did not induce significant changes in GSTs activity in the present study, neither the combination of As exposure and different salinities showed to affect GSTs activity.

Results on GSH/GSSG showed overall higher values in oysters held at intermediate salinities (20 and 30) and lower ratios in the extreme salinities tested (10 and 40) (both in the

presence or absemce of As). In the absence of As, significantly lower GSH/GSSG values were observed in oysters maintained at salinity 10 compared to oysters exposed at salinity 30. These results can be justified by generally higher prooxidant status observed in oysters kept at low salinity (10) evidenced by higher ETS, SOD and CAT activities compared to the remaining conditions. In the presence of As, significantly lower GSH/GSSG values were observed at salinity 40, compared to intermediate salinities of 20 and 30, also indicating oxidative stress in these oysters, possibly due to low activities of SOD and CAT previously described for these conditions.

Lipid peroxidation can be used as a measure of membrane damage linked to oxidative stress (de Almeida et al., 2007). In the absence of As, the lowest LPO level was observed at salinity 30, possibly due to better physiological status of oysters maintained at closer to optimum salinity. In the presence of As, significantly higher LPO levels were observed in oysters exposed to As at salinities 20, 30 and 40, compared to the respective negative controls.

Under varying environmental conditions (e.g. temperature, salinity) physiological acclimation often includes changes in the relative abundance membrane lipids, to promote adequate membrane fluidity depending on the surrounding media characteristics, a process termed homeoviscous acclimation (Nemova et al., 2013). Membrane-bound polyunsaturated fatty acids (PUFA) are among the most susceptible lipids to oxidative damage (Monserrat et al., 2007), and therefore alterations in membrane PUFA content increases membrane susceptibility to LPO. In fact, hypoosmotic stress has been demonstrated to lower PUFA content in bivalves, while the opposite trend is observed under hyperosmotic conditions (Fokina et al., 2017). It is therefore likely that oysters exposed to low salinity were less susceptible LPO, while high salinity should increase susceptibility to LPO, and could explain higher LPO observed in *C. angulata* exposed to higher salinities.

Interestingly, LPO levels between oysters exposed at salinity 10 (in the presence and absence of As) were not significantly different, despite high concentrations of accumulated As observed. At lower salinity (10), it appears that oysters presented sufficient antioxidant defence mechanisms to prevent LPO increased formation, indicating that increased SOD and CAT activities observed may have prevented ROS build up and consequent LPO accumulation. Similarly, Géret et al. (2002) also related higher antioxidant enzyme activities with decreasing LPO levels in *C. angulata.*

The electron transport system (ETS) activity can be used as a measure of metabolic rate, as it represents a proxy of the cellular respiratory potential of a given organism (García-Martin et al., 2014). The increase of the ETS activity with the decrease of salinity, was likely related to increased metabolic costs of isosmotic maintenance of osmoconforming oysters (Eierman & Hare, 2014; Meng et al., 2013). It is therefore possible that higher metabolic rate could at least partially explain higher As accumulation in oysters exposed at low salinity (10).

3.1.3.2 *Crassostrea gigas*

Seawater acidification and Arsenic exposure

Influence of Seawater acidification on Arsenic accumulation

Total As (µg g^{-1} fw) accumulated in adult *C. gigas* exposed to As and Low pH+As, are depicted in Table XIII. No significant differences were observed in As accumulation between oysters exposed to As in acidified (Low pH+As) and non-acidified (As) conditions.

Table XIII- Total As (µg g^{-1} fw) accumulated in adult *C. gigas* exposed to As and Low pH+As. Significant differences ($p \leq 0.05$) among tested conditions are represented with different letters (mean ± standard error, n=3).

	As	Low pH+As
µg As g^{-1} fw	3.25±0.2**a**	3.93±0.4**a**

Average total As accumulated by adult *C. gigas* (ca. 3.5 µg g^{-1} fw equivalent to ca. 17.5 µg g^{-1} dw, Zhang et al., 2013), was in the low range of reported values in oysters collected in the environment (e.g. up to 26.7 µg g^{-1} dw in *C. gigas* in France, Kohlmeyer et al., 2002; and 25.4 µg g^{-1} dw in *C. virginica* from the US, Valette-Silver et al., 1999). No differences in As accumulation between oysters exposed to acidified (Low pH+As) and non-acidified (As) conditions, indicate seawater acidification had no effect on As uptake and/or elimination processes.

Influence of Seawater acidification and Arsenic on oysters biochemical performance

Results obtained on the biochemical markers assessed in *C. gigas* adults exposed to As, Low pH and Low pH+As are depicted in Figure 12.

No significant differences were observed regarding SOD activity among all tested conditions. CAT activity was significantly higher in oysters exposed to Low pH and Low pH+As relative to CTL. Significantly higher GSTs activity was observed in oysters exposed to As or the combination of As with seawater acidification (As and Low pH+As) comparing to CTL.

A significant increase in reduced glutathione content (GSH) was observed in oysters exposed to As, relative to the remaining conditions. No significant differences were observed concerning LPO levels among tested conditions.

Carbonic anhydrase (CA) activity was significantly lower in oysters exposed to As relative to CTL. In oysters exposed to Low pH and Low pH+As, CA activity was significantly lower compared to both CTL and As.

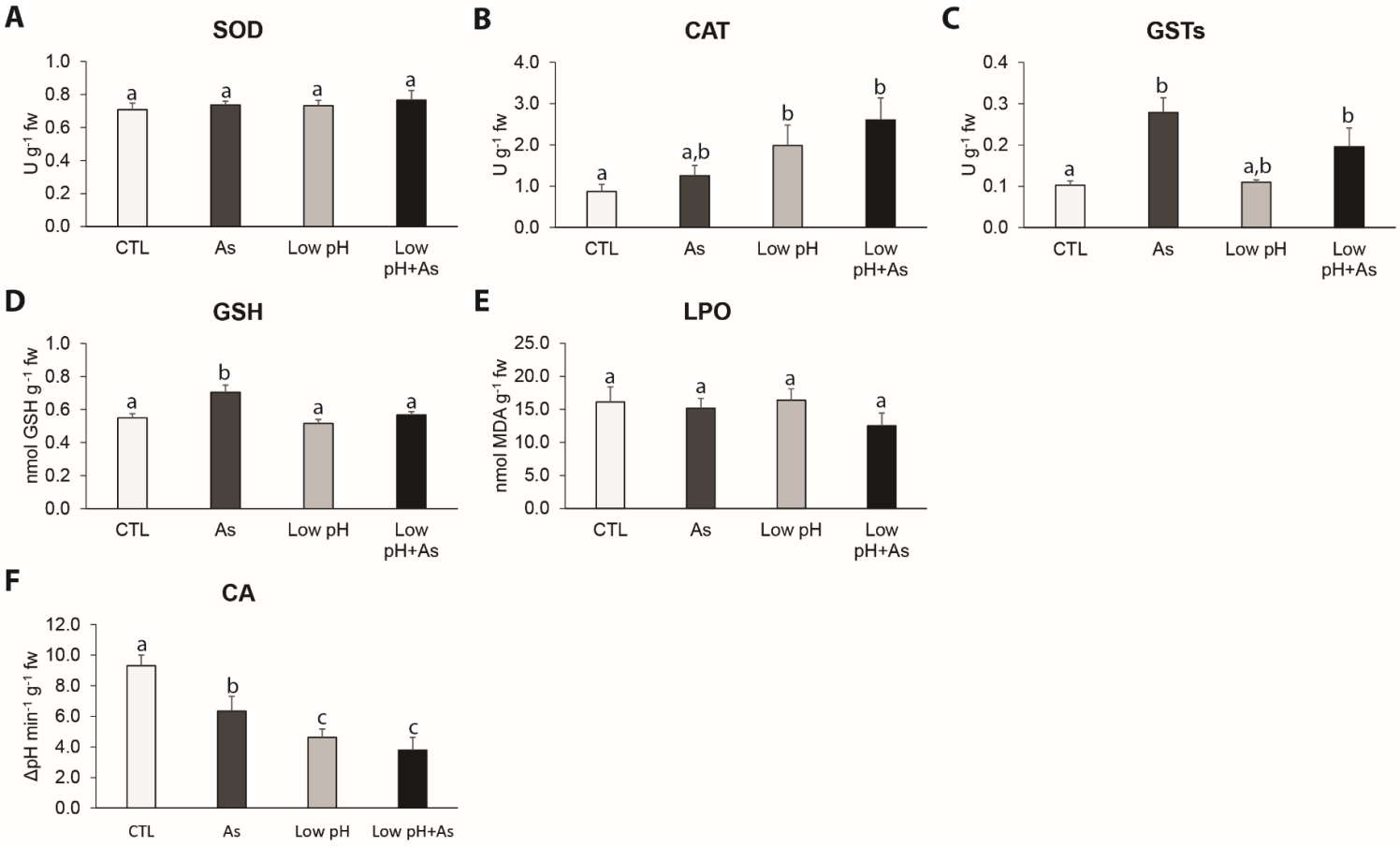

Figure 12 – Biochemical parameters studied in adult *C. gigas* exposed to As, Low pH and Low pH+As.

A: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Glutathione *S*-transferases (GSTs) activity; **D**: Reduced glutathione (GSH) content; **E**: Lipid peroxidation (LPO) levels; **F**: Carbonic anhydrase (CA) activity. Significant differences ($p \leq 0.05$) between conditions are represented with different letters (Mean + standard error; n=9).

The most prominent changes concerning the biochemical markers studied in adult *C. gigas* exposed to seawater acidification and As, were observed regarding CAT, GSTs, GSH and CA.

Higher CAT activity in oysters exposed to Low pH relative to CTL (including the combined exposure of Low pH+As) suggests enhanced antioxidant capacity towards seawater acidification. Similarly, Matozzo et al. (2013) evaluated the effects of seawater acidification on the oxidative stress response of the clam *Chamelea gallina* and the mussel *M. galloprovinciales*, revealing significant increases in CAT activity (pH 7.4).The increase of GSH in *C. gigas* exposed to As can be explained as a means of cells to either eliminate ROS induced by As, or to indirectly participate on As biotransformation processes. These hypothesis are supported by studies that have implicated GSH as an important player on As detoxification, by either enzymatic (Erickson et al., 2011; Kala et al., 2004; Shiomi et al., 1996) or non-enzymatic (Kobayashi et al., 2005; Thomas et al., 2001) processes. Moreover Bagnyukova et al. (2007) showed an increase of GSH concentrations in goldfish *Carassius auratus* exposed to As, leading authors to propose that increased GSH synthesis can be an effective mechanism to withstand As exposure. The capability to synthesize more GSH would also allow for more efficient GSTs activity, by providing more substrate for conjugation with GSTs.

Interestingly, the oxidative stress status of adult *C.* gigas showed an overall different pattern than juveniles (previously discussed). Generally, adults presented higher capacity to induce antioxidant enzymes (CAT; GSTs), GSH synthesis, possibly influencing the lower propensity for LPO observed than juvenile oysters.

Results on CA activity in *C. gigas* were consistent with those observed in *C. angulata,* reinforcing the present findings showing that seawater acidification may affect these species biomineralization capacity.

Salinity and Arsenic exposure

Influence of Salinity and Arsenic on oysters biochemical performance

Results on As quantification in adult *C. gigas* tissues (µg g^{-1} fw) exposed to As at different salinities are depicted in Table XIV. Results showed that As accumulation was inversely related to ambient salinity. The highest As concentrations were observed in oysters exposed to As at the lowest salinity (10), with significant differences towards all other conditions. Intermediate levels of As accumulation were observed at salinity 20, with significant differences compared to all other conditions. The lowest As concentrations were observed at the higher salinities (30 and 40).

Table XIV- Total As (µg g^{-1} fw) accumulated in adult *C. gigas* after 28 days laboratory exposures. Significant differences ($p \leq 0.05$) among tested conditions are represented with different letters (mean ± standard error, n=3).

Salinity	10	20	30	40
µg As g^{-1} fw	6.72±0.2**a**	4.42±0.4**b**	1.32±0.3**c**	2.19±0.7**c**

The pattern of As accumulation at different salinity levels in adult *C. gigas* was similar to that observed in *C. angulata*. Generally, As content was highest at the lowest salinity (10) and followed a decreasing trend in concentration with the increase of salinity. Similarly to results discussed for *C. angulata*, the As accumulation pattern observed could be related to the interaction of several factors: i) higher availability of free metal ions at lower salinities (Bianchini & Gilles 2000; Rainbow et al., 1997); ii) alterations of ion flux between the organisms and the environment (Connell, 1989); iii) physiological alterations, such as increased filtration rates induced by hypoosmotic stress, could reflect on higher As accumulation (Rainbow et al., 1997).

Influence of Salinity and Arsenic on oysters biochemical performance

Biochemical markers assessed in adult *C. gigas* exposed to As at different salinities are depicted in Figure 13.

Oysters unexposed to As presented significantly higher SOD activity at the lowest salinity (10) compared to the remaining conditions. In oysters exposed to As, SOD activity was significantly higher at salinities 10 and 20 compared to salinities 30 and 40. Significantly higher SOD activity was observed in oysters exposed to As at salinity 20 compared to the respective negative control.

CAT activity was significantly higher at salinities 30 and 40, compared to the lowest salinity levels (10 and 20) (negative controls). Oysters exposed to As at different salinities presented no significant alterations in CAT activity.

GSTs activity was significantly higher at the lowest salinity (10) relative to the remaining conditions in oysters exposed and unexposed to As. However, GSTs activity was significantly higher in oysters exposed to As at salinities 10 and 30 compared to the respective negative controls.

LPO was significantly lower at salinities 10 and 30 compared that observed at salinities 20 and 40 in oysters exposed or unexposed to As. Oysters exposed to As at salinity 20 presented significantly lower LPO than the respective negative control. On the contrary, oysters exposed to As at salinity 40 presented higher LPO than the respective negative control.

GSH/GSSG was significantly higher in oysters maintained at salinities 10 and 30 compared to oysters maintained at salinities 20 and 40. In oysters exposed to As at different salinities, significantly higher GSH/GSSG was observed at the lowest salinity (10) relative to the remaining conditions. Significantly higher GSH/GSSG in oysters exposed to As at salinity 10, relative to the respective negative control.

In the absence of As, the ETS activity was significantly higher in oysters maintained at the lowest salinity (10) compared to the remaining conditions. Similarly, in oysters exposed to As at different salinities, the highest ETS activity was observed at the lowest salinity (10), with significant differences towards the highest salinity (40).

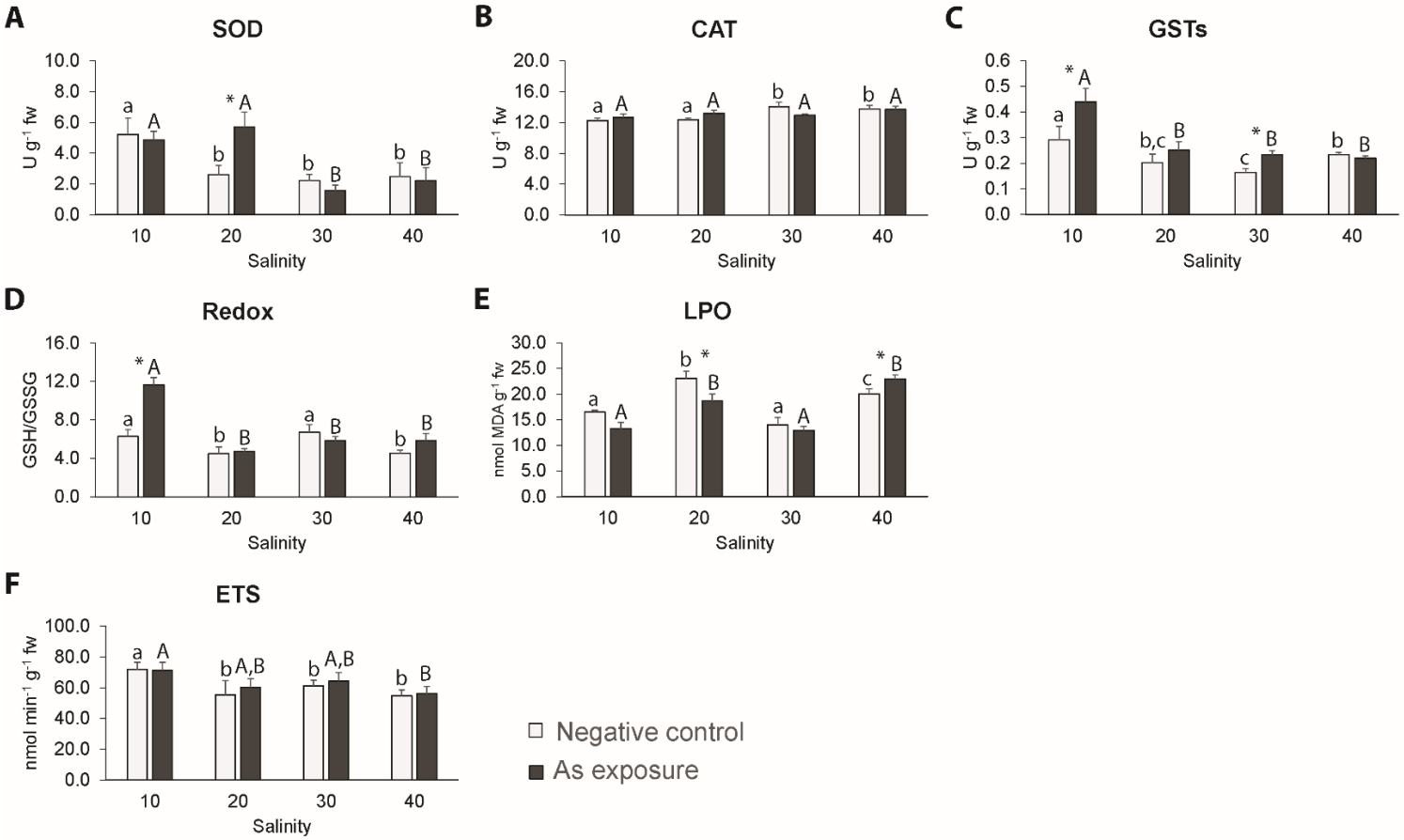

Figure 13 – Biochemical parameters studied in adult *C. gigas* exposed to salinities 10, 20, 30 and 40 in the presence or absence of As.

A: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Glutathione *S*-transferases (GSTs) activity; **D**: Reduced to oxidized glutathione ratio (Redox); **E**: Lipid peroxidation (LPO) levels; **F**: Electron transport system (ETS) activity. Significant differences ($p \leq 0.05$) between conditions are represented with different letters. Significant differences observed between oysters exposed in the presence or absence of As at each salinity are represented with an asterisk (Mean + standard error; n=9).

The most prominent changes concerning the biochemical markers studied in adult *C. gigas*, were observed regarding SOD, GSTs, GSH/GSSG, LPO and ETS. These findings refleted mechanisms of physiological adaptation that included alterations in metabolism, bioenergetics and oxidative stress (Rivera-Ingraham & Lignot, 2017).

Significant biochemical alterations were observed at the lowest salinity (10), in which oysters presented enhanced activities of SOD, GSTs and ETS, as well as lower GLY content regardless of the presence of As, that overall indicate an oxidative stress response to hypoosmotic stress. Additionally, results also showed the influence of As on biomarker response, namely considering the observations of higher GSTs and GSH/GSSG in oysters exposed to As at salinity 10 compared to the respective negative control.

Significantly enhanced SOD activity in oysters exposed to the lowest salinity (10) without As could be related to higher ROS production rates associated to increased metabolism related to physiological change under hypoosmotic stress previously described (Rivera-Ingraham & Lignot, 2017). In agreement with the results obtained and discussed for *C. angulata* (previously discussed), the hypothesis of increased metabolism are supported by results showing higher ETS activity and lower GLY content in these oysters.

Results showing higher GSTs activity at the lowest salinity in the absence of As could indicate the involvement of GSTs as antioxidant, knowing that some GST isoforms may also catalyse the reduction of H_2O_2 to water (Regoli & Giuliana, 2014). Zanette and co-workers (2011), found a similar trend of decreasing GSTs activity in *C. gigas* with the increase of salinity (9, 15, 25 and 35) despite not significantly, but after 10 days of exposure only. These authors further demonstrated that salinity influenced GSTs activity in the presence of xenobiotics, namely the lack of induced biotransformation capacity at salinities other than the optimum (9, 15 and 35). Interestingly, the present findings showed GSTs activity to be induced in the presence of As only at salinities 10 and 30. It is possible that at the lowest salinity GSTs were induced to catalyse H_2O_2 produced from enhanced SOD activity, while at salinity (30) the optimum physiological status allowed for GSTs induction.

Results on GSH/GSSG further revealed alterations in oysters redox status. The significant increase of GSH/GSSG in oysters exposed to As at salinity 10 was due to lower GSSG observed in the same condition, indicative of deregulation of the glutathione system, possibly due to GSSG excretion to prevent protein glutathionylation previously described. In oysters exposed to different salinities in the absence As, significantly lower GSH/GSSG was observed at salinities 20 and 40, that resulted from a shift of the glutathione system towards the oxidized form (lower GSH and higher GSSG), evidencing the involvement of GSH in oxidative stress repair mechanisms in these conditions.

Increased LPO observed in oysters exposed to As at salinities 20 and 40 could be partially explained by alterations in oysters antioxidant capacity in these conditions. For instance, the absence of GSTs induction in the same conditions could have enabled for LPO increase. Zanette et al. (2011) related the lack of GSTs induction with increased MDA levels in *C. gigas* exposed to diesel at different salinities.

However, the antioxidant capacity could not explain results showing higher LPO in oysters maintained at the highest salinity (40) in the absence of As. These findings could rather be explained by alterations in the composition of the lipid bilayer that influences membranes susceptibility to oxidative damage. Under varying environmental conditions (e.g. temperature, salinity) physiological acclimation often includes changes in the relative abundance membrane lipids, to promote adequate membrane fluidity depending on the surrounding media characteristics, a process termed homeoviscous acclimation (Nemova et al., 2013). Membrane-bound polyunsaturated fatty acids (PUFA) are among the most susceptible lipids to oxidative damage (Monserrat et al., 2007), and therefore alterations in membrane PUFA content increases membrane susceptibility to LPO. In fact, hypoosmotic stress has been demonstrated to induce lower PUFA content in bivalves, while the opposite trend is observed under hyperosmotic conditions (Fokina et al., 2017). It is therefore likely that oysters exposed to low salinity were less susceptible LPO, while high salinity should increase susceptibility to lipid peroxidation reactions, and therefore could explain higher LPO observed in oysters exposed to high salinity (40).

3.1.3.3 Species comparison

Seawater acidification and Arsenic exposure

Results demonstrated that adult *C. angulata* and *C. gigas* were sensitive to As, to Low pH, and Low pH+As. Both species showed low oxidative stress response, despite significant enhancement of GSTs activity in oysters exposed to As, that may have helped prevent alterations of lipid peroxidation levels. However, biomineralization enzyme CA was significantly inhibited by As, and even higher inhibition of CA was observed in low pH exposures. These results bring new insights on the negative effects of high CO_2 on oysters calcification capacity. The combined effect of both stressors proved to have a negative effect on both species response to As, namely concerning GSTs activity. Overall *C. gigas* presented higher stress response capacity (SOD, CAT and GSH) than *C. angulata*, suggesting that the latter species was either less sensitive to the tested conditions or presented a lower capacity to respond to tested scenarios.

Salinity and Arsenic exposure

Results from exposures to different salinity levels showed that both species presented alterations in oxidative stress status and metabolic performance. Salinity also affected As accumulation dynamics, with oysters exposed to lower salinities tending to accumulate higher As concentrations. Overall adult specimens of both species showed similar stress response patterns to salinity and As exposures, presenting higher antioxidant capacity (SOD) and metabolic potential (ETS) at lower salinities (10 and 20). However, differences were observed considering the influence of salinity on each species biotransformation capacity (GSTs), with *C. angulata* presenting induced GSTs at a wider range of salinities (20, 30 and 40) compared to *C. gigas* (10 and 30). Moreover, *C. angulata* presented higher capacity to maintain redox balance at the range of salinities tested, and lower degree of alterations in LPO levels among conditions.

3.2 *Crassostrea brasiliana* and *Crassostrea gigas* (Brazil)

The effects of different seawater acidification (pH 7.8, pH 7.4 and pH 7.0) and temperature (24, 28 and 32 ºC) levels were assessed in juvenile and adult *C. brasiliana* and *C. gigas* after 28 days laboratory exposures. Biochemical markers were assessed in oyster soft tissue in order to compare species response capacity to these environmental stressors.

3.2.1 Juveniles

3.2.1.1 *Crassostrea brasiliana*

Seawater acidification

The effects of different levels of seawater acidification on juvenile *C. brasiliana* assessed on the basis of several biochemical markers are depicted in Figure 14. Results showed unaltered antioxidant enzymes activity (SOD and CAT) and Redox status among tested conditions. Alterations in lipid peroxidation (LPO) levels were observed, with oysters exposed to the highest acidification level (pH 7.0) presenting significantly lower LPO compared to the remaining conditions. Oysters exposed to both acidification levels (pH 7.4 and pH 7.0) presented significantly lower ETS activity compared to oysters in control (pH 7.8). Glycogen (GLY) content was significantly lower at the intermediate acidification level (pH 7.4) compared to the remaining conditions.

The most prominent changes concerning the biochemical markers studied in juvenile *C. brasiliana* exposed to different acidification levels, included changes in membrane damage (LPO), metabolic potential (ETS) and energetic fitness (GLY) among conditions.

The decrease of metabolic potential (ETS) with the increase of acidification, observed (lower ETS activity in oysters exposed to pH 7.4 and 7.0 compared to CTL), indicate a down regulation of metabolic capacity. Other studies have described metabolic depression in marine invertebrates exposed to high CO_2 concentrations (e.g. Michaelidis et al. 2005; Pörtner et al, 1998; Reipschläger & Pörtner, 1996), which can be indicative of organisms experiencing envirormental stress (Guppy & Withers, 1999; Lannig et al., 2010; Parker et al., 2013). Metabolic depression in response to hypercapnia can imply shifts in preferential metabolic pathways (Pörtner et al., 2005), as observed in *C. gigas* through shotgun sequencing (Timmins-Shiffman et al., 2014). In the present study, *C. brasiliana* appeared to have achieved a new state in metabolic respiration, with lower potential aerobic capacity after four weeks of exposure to seawater acidification conditions, possibly to reconfigure energetic balance. Similarly, the ETS activity has also been shown to decrease in *Scrobicularia plana* clams (*p*CO2 >5000 μatm), and authors suggested these results could be related to metabolic depression to maintain energetic fitness (Freitas et al., 2016a).

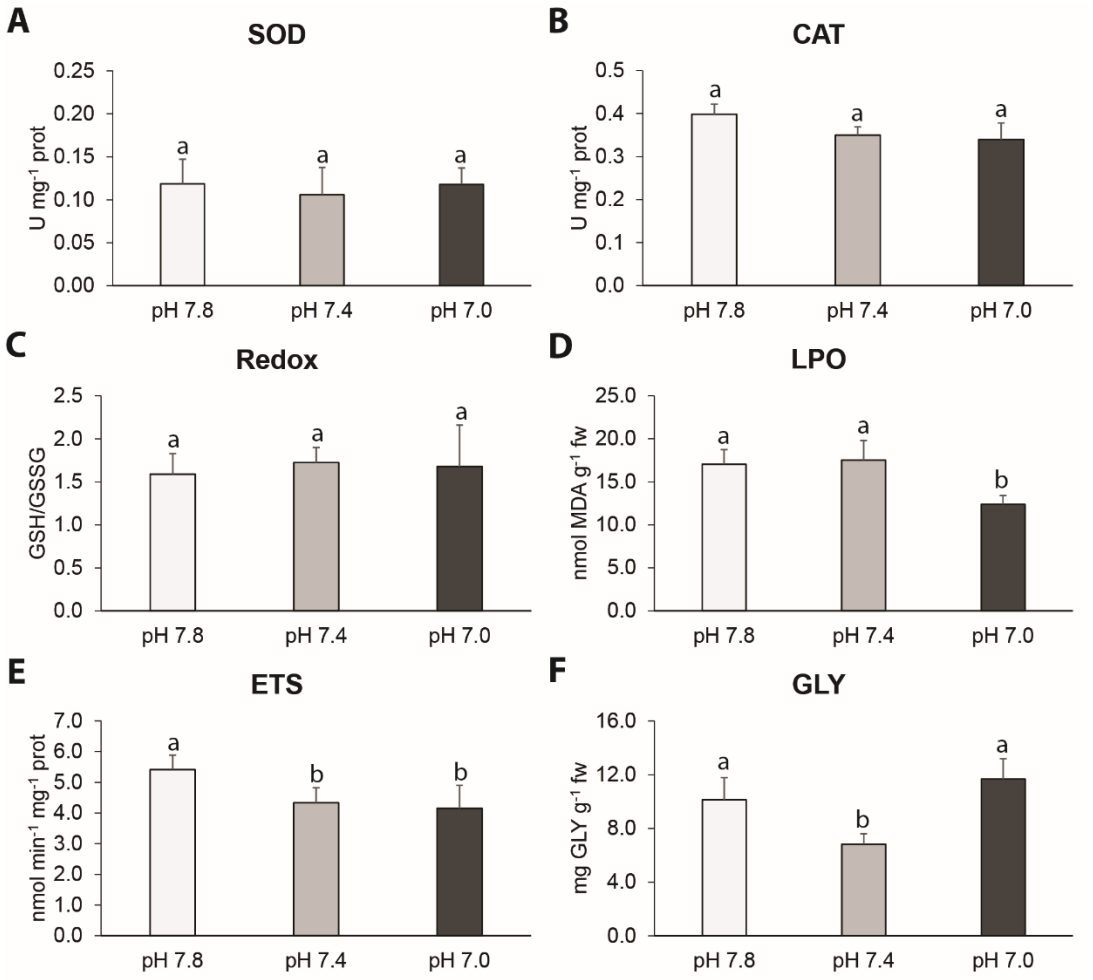

Figure 14 – Biochemical parameters in juvenile *C. brasiliana* exposed to seawater acidification. **A**: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Reduced to oxidized glutathione ratio (Redox); **D**: Lipid peroxidation (LPO) levels; **E**: Electron transport system (ETS) activity; and **F**: Glycogen (GLY) content. Significant differences ($p \leq 0.05$) between conditions are represented with different letters (mean + standard error; n=24).

The relatively low antioxidant response observed, as well as the decrease of metabolic potential (ETS) with the increase of acidification observed in *C. brasiliana*, could indicate rearrangement of metabolic pathways towards lower ROS production (Tomanek, 2015). Under extreme environmental conditions, facultative anaerobes such as oysters, may switch to anaerobic metabolism to extend energetic resources until favourable environmental conditions are restored (Sokolova et al., 2012). This mechanism also allows for a decrease of ROS production rates (Abele et al., 2007; Anestis et al., 2007; Pörtner et al., 2010), and has been shown in *M. edulis* under hypoxia (Rivera-Inhagram et al., 2013). Considering this, the present data suggest that *C. brasiliana* developed a depressed metabolic status, preventing excessive ROS production through alterations on the electron transport chain functioning, as well as maintaining energetic balance.

Lower LPO observed in juvenile *C. brasiliana* at the highest acidification level (pH 7.0), corroborates the hypothesis that oysters were depressing metabolism, also in accordance with results obtained regarding the ETS activity and unchanged antioxidant capacity (SOD, CAT and GSH/GSSG). Accordingly, Rivera-Ingraham et al. (2013) showed decreased ROS production in *M. edulis*, mussels exposed to anoxia, accompanied by no change in oxidative damage parameters (MDA and protein carbonylation).

At the intermediate acidification level (pH 7.4) juvenile *C. brasiliana* presented significantly lower GLY content, indicative of oysters enduring energetic burden in response to stress (Sokolova & Lannig, 2008). Energetic reserves expenditure has also been demonstrated in juvenile *C. virginica* exposed to acidification (ca. 800 µatm pCO_2) (Dickinson et al., 2012). At the highest acidification level (pH 7.0) however, high GLY content in juvenile *C. brasiliana* (similar to values in control conditions (pH 7.8), indicate these oysters were under an arrested metabolic state, a mechanism employed to conserve energy reported for other mollusc species (Gazeau et al., 2013; Michaelidis et al., 2005). The rate of carbohydrate catabolism in facultative anaerobes such as oysters is reduced during transition to the *pessimum* range of tolerance to environmental stressors (Sokolova et al., 2012), which could explain similar GLY content observed between low (pH 7.8) and high hypercapnia (pH 7.0). Similarly, *M. galloprovincialis* mussels presented low energetic expenditure (high GLY content) when exposed to acidification (Freitas et al., 2017). However, metabolic depression is only a time limited mechanism to endure extreme stress (Sokolova et al., 2012), and therefore the impacts of extended exposure to acidification would likely represent a deleterious scenario for these oysters.

Thermal stress

The effects of different temperature levels on juvenile *C. brasiliana* assessed on the basis of several biochemical markers are depicted in Figure 15.

The highest SOD activity was observed in juvenile *C. brasiliana* exposed to the lowest temperature (24 ºC), with significant differences towards 28 and 32 ºC. Moreover, SOD activity in oysters exposed to 28 ºC was significantly lower compared to that observed at 32 ºC.

Significantly higher LPO was observed in oysters exposed to control temperature (24 ºC) compared to the upper temperature levels tested (24 and 28 ºC).

The highest GSH/GSSG was observed in oysters maintained at 28 ºC, the lowest values observed at 32 ºC, and an intermediate value was observed at 24 ºC.

The ETS activity was significantly lower at the highest temperature (32 ºC) relative to oysters maintained in control conditions. GLY content was significantly lower in oysters exposed to the highest temperature tested compared to that observed in the remaining conditions.

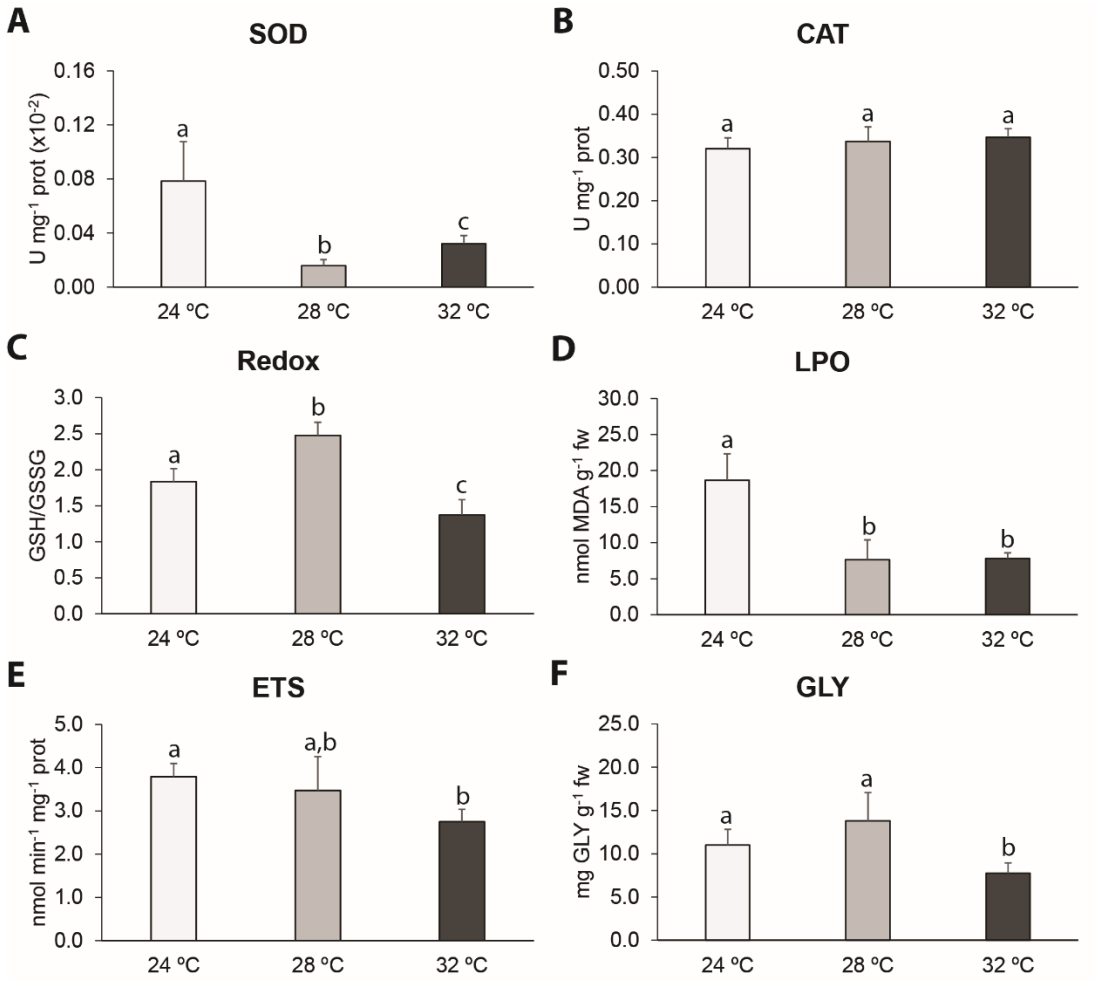

Figure 15 – Biochemical parameters studied in juvenile *C. brasiliana* exposed to thermal stress. **A**: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) actvity; **C**: Reduced to oxidized glutathione ratio (Redox); **D**: Lipid peroxidation (LPO) levels; **E**: Electron transport system (ETS) activity; and **F**: Glycogen (GLY) content. Significant differences ($p \leq 0.05$) between conditions are represented with different letters (mean + standard error; n=12).

The most prominent changes considering the biochemical markers studied in juvenile *C. brasiliana* exposed to different temperature levels, included changes in antioxidant activity (SOD), redox balance (GSH/GSSG), cellular damage (LPO), metabolic potential (ETS) and glycogen (GLY) content.

Adaptation to thermal variations can cause shifts in the antioxidant defence status of marine bivalve species (Abele et al., 2002; Dimitriadis et al., 2012; Lockwood et al., 2010; Tomanek & Zuzow, 2010), that can be influenced by changes in metabolism and membrane phospholipid structure that may alter ROS production rates and sources (Pörtner, 2010; Sappal et al., 2015). SOD is one of the most important antioxidant enzyme that catalyses free radicals, and has been shown to reflect ambient temperature in bivalves such as *Mytilus edulis* (Lesser et al., 2010), *Perna viridis* (Verlecar et al., 2007), and *Scapharca broughtonii* (An & Choi, 2010).

Juvenile *C. brasiliana* presented significantly different SOD activities among all acclimation temperatures, with the highest activity values observed at the lowest temperature (24 ºC) and the

lowest SOD activities at 28 ºC. Higher SOD activity in juvenile *C. brasiliana* maintained at 24 ºC could reflect higher proportions of polyunsaturated fatty acids (PUFA) in membranes, characteristic of homeoviscous adaptation to lower temperatures (Crockett, 2008). PUFA are highly susceptible to oxidation, and readily form lipid peroxyl radicals (Lira et al., 2013). Hence, higher SOD activity at 24 ºC could be induced as a protective mechanism to prevent excessive PUFA oxidation in *C. brasiliana*.

The highest GSH/GSSG observed in juveniles at 28 ºC resulted from relatively higher GSH concentrations observed (data not shown), resulting from increased GSH synthesis in response to increasing temperature (increased total glutathione levels). At 32 ºC, GSH/GSSG in juvenile oysters was the lowest, as a result of a shift towards the oxidized form (GSSG), an indication of the involvement of glutathione in the oxidative stress response (Lesser, 2006).

Interestingly, the highest LPO levels in juvenile *C. brasiliana* were observed in juveniles at the lowest temperature (24 ºC), which may have resulted from higher PUFA proportions at lower temperatures and thus higher susceptibility to ROS. Accordingly, higher levels of PUFA have been observed in *C. rhizophorae* (here identified as *C. brasiliana*) during winter (24 ºC) (Lira et al., 2013; Martino & Cruz, 2004).

The decrease of ETS activity in juveniles at the highest temperature (32 ºC) was likely related to reduced physiological fitness observed at the same condition, indicated by lower GLY content (furtherly discussed). Elevated temperatures may induce physiological stress due to increased metabolic activity and result in reduced fitness (Hering et al., 2010). It is likely that juvenile oysters maintained at 32 ºC were shifting into a moderate stress status, with the associated expenditure of energetic reserves (GLY) and lower GSH/GSSG, indicating an oxidative status (Sokolova et al., 2012). Juvenile *C. brasiliana* may have induced a decrease of active ETS enzyme concentrations at this temperature as an energetic trade-off mechanism.

Lower GLY content in juvenile *C. brasiliana* at 32 ºC, could result from a shift to a moderate stress status (Sokolova et al., 2012), and therefore may indicate the upper thermal tolerance limit of these oysters to be close to the highest temperature tested.

3.2.1.2 *Crassostrea gigas* (Brazil)

Seawater acidification

The effects of seawater acidification levels on juvenile *C. gigas* assessed on the basis of several biochemical markers are depicted in Figure 16.

Antioxidant enzymes activity (SOD and CAT) were significantly higher in oysters maintained in control conditions (pH 7.8), compared to increased acidification (pH 7.4 and pH 7.0).

Significantly higher LPO was observed at the intermediate acidification level (pH 7.4), while Redox status was significantly higher at the highest acidification level (pH 7.0), compared to the remaining conditions.

The ETS activity was significantly higher at the intermediate acidification level (pH 7.4) relative to control (pH 7.8). No alterations in glycogen (GLY) content were observed among tested conditions.

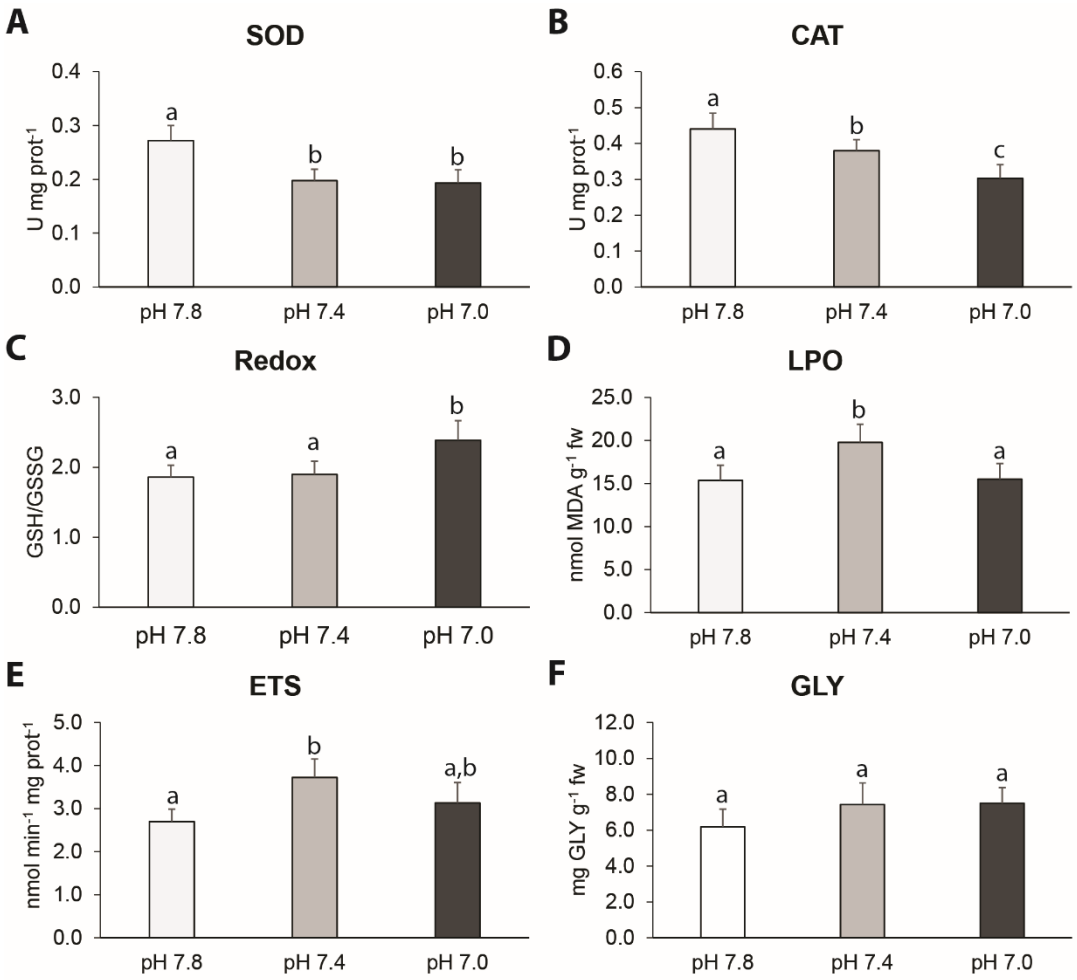

Figure 16 – Biochemical parameters in juvenile *C. gigas* exposed to seawater acidification.

A: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Reduced to oxidized glutathione ratio (Redox); **D**: Lipid peroxidation (LPO) levels; **E**: Electron transport system (ETS) activity; and **F**: Glycogen (GLY) content. Significant differences ($p \leq 0.05$) between conditions are represented with different letters (mean + standard error; n=12).

The most prominent changes concerning the biochemical markers studied in juvenile *C. gigas* exposed to seawater acidification levels, included changes in antioxidants activity (SOD and CAT), redox balance (GSH/GSSG), cellular damage (LPO) and metabolic potential (ETS) among conditions.

These findings can be integrated and discussed in light of the concept of energy-limited tolerance to stress (Sokolova, 2013; Sokolova et al., 2012). According to this concept, the stress status of a given organism exposed to increasing levels of environmental stress may be generally classified in two main categories: moderate or extreme stress. These are mainly defined by the organisms metabolic strategy (compensation or conservation), that in turn depend on organisms energetic fitness, aerobic scope, and energetic trade-offs between cellular maintenance costs, growth and reproduction.

The antioxidant capacity of juvenile *C. gigas* exposed to increasing acidification, characterized by depressed antioxidant enzymes (SOD, CAT), could be related to the preferential use of other antioxidants, namely glutathione (GSH), an important non-enzymatic antioxidant scavenger that is a key participant in processes of ROS neutralization (Rahman, 2007). The present data suggest that juvenile *C. gigas* shifted towards the preferential use of GSH as primary detoxification mechanism, despite presenting differentiated capacities. The significant decrease of both reduced (GSH), and oxidized (GSSG) glutathione content observed in juvenile *C. gigas* with the increase of acidification (data not presented), that resulted in lower tGSH levels and higher GSH/GSSG at the highest acidification level, indicate that glutathione was being involved in detoxification mechanisms in response to acidification, as reported in other bivalve species under hypercapnic or hypoxic conditions (Khan & Ringwood 2016; Nardi et al., 2017). These findings could explain results showing lower SOD and CAT activities in juvenile *C. gigas* at both acidification levels (pH 7.4; pH 7.0), that together indicate a metabolic shift towards glutathione mediated ROS-quenching pathways, as observed in Mytilid species exposed to heat stress (Tomanek, 2014). The increase of GSH/GSSG observed in juveniles at the highest acidification level (pH 7.0) further indicate oysters were actively transporting glutathione in its oxidised form (GSSG) out of the organism, also reflecting in a lower tGSH content (data not shown). Under oxidative conditions, excessive GSSG can react with thiol groups of proteins, a process known as glutathionylation, leading to alterations of protein functioning (Hawkins et al., 2010; Hurd et al., 2005). The loss of cellular GSH/GSSG redox control makes glutathionylation a deleterious event (Ghezzi & Di Simplicio, 2009), hence GSSG can be exported from the cell to the extracellular matrix (Garcia et al., 2010; Han et al., 2006). Given this, these findings suggest that the antioxidant capacity of juvenile *C. gigas* at the highest acidification level (pH 7.0) was exceeded, with excess glutathione oxidation, and GSSG excretion resulting in lower total glutathione content, as seen in other bivalve species experiencing oxidative stress (Hannam, 2010; Peña-Llopis et al., 2002; Regolli et al., 1999). It is possible that juveniles presented a preferential use of GSH as the main antioxidant defence in detriment of antioxidant enzymes (SOD and CAT), because it is energetically less costly (Pannunzio & Storey, 1988), the capacity to replenish tGSH levels showed to be insufficient at the highest acidification level (pH 7.0).

Juvenile *C. gigas* were susceptible to membrane damage, with an observed increase of LPO in oysters exposed to the intermediate acidification level (pH 7.4), which could have resulted from significantly lower SOD and CAT activities previously discussed. At the highest acidification level (pH 7.0), LPO in juvenile *C. gigas* was similar to that observed in oysters maintained in control (pH 7.8), possibly as a result of glutathione mediated ROS quenching capacity previously described. However, tGSH depletion associated to excessive GSSG may be a precursor of increased LPO (Ringwood et al., 1999). Therefore, this mechanism is likely to become time limited for juvenile *C. gigas*.

Enhanced ETS activity observed in juvenile *C. gigas* at the intermediate acidification level (pH 7.4), indicate the development of increased metabolic potential in response to intermediate acidification in these oysters. These results are in line with those described for other ectothermic marine metazoans exposed to acidification. Strobel et al. (2013) observed increased aerobic capacity (higher activities of citrate synthase and cytochrome oxidase enzymes) in red muscle of *Notothenia rossii* fish exposed to acidification, and suggested that this could either be a mechanism to sustain elevated costs of acid-base balance regulation, or a compensation mechanism for alterations in mitochondria metabolism. Similarly, Harms et al. (2014) observed upregulation of ETS related genes in *Hyas araneus* crab exposed to >900 µatm *p*CO2, and their results were justified as a mechanism to compensate for increased energetic costs of acid-base maintenance in acidification exposed animals. Also, the ETS activity in *C. gigas* has been shown to increase in conditions of hypoxia (Le Moullac et al., 2007; Samain & McCombie, 2008), and could be a common response mechanism triggered by these stressors, because hypoxia and acidification/hypercapnia often occur simultaneously in the environment, namely during eutrophication (Willson & Burnett, 2000).

Interestingly, the same was not observed at the highest acidification level (pH 7.0). Instead, oysters showed intermediate ETS activity compared to the remaining conditions. It is likely that oysters exposed to higher levels of hypercapnia induced metabolic arrest, indicative of a transition towards the *pessimum* range of aerobic scope, consistent with unaltered energetic reserves (GLY) (Sokolova et al., 2012).

Thermal stress

The effects of different temperature levels on juvenile *C. gigas* assessed on the basis of several biochemical markers are depicted in Figure 17. Juveniles presented high mortality rates at 32 ºC and were not considered for biochemical analysis.

Significantly higher SOD activity was observed in juvenile *C. gigas* at 28 ºC compared to that observed under control temperature (24 ºC). No alterations in CAT activity was observed between oysters exposed to the temperature levels tested.

No significant alterations were observed regarding redox status (GSH/GSSG) among tested temperatures. LPO levels were significantly lower in juvenile *C. gigas* exposed to 28 ºC compared to results obtained in oysters maintained at control temperature (24 ºC). No significant alterations were observed regarding the ETS activity among tested temperatures. Significantly lower GLY content was observed in oysters exposed to the highest temperature.

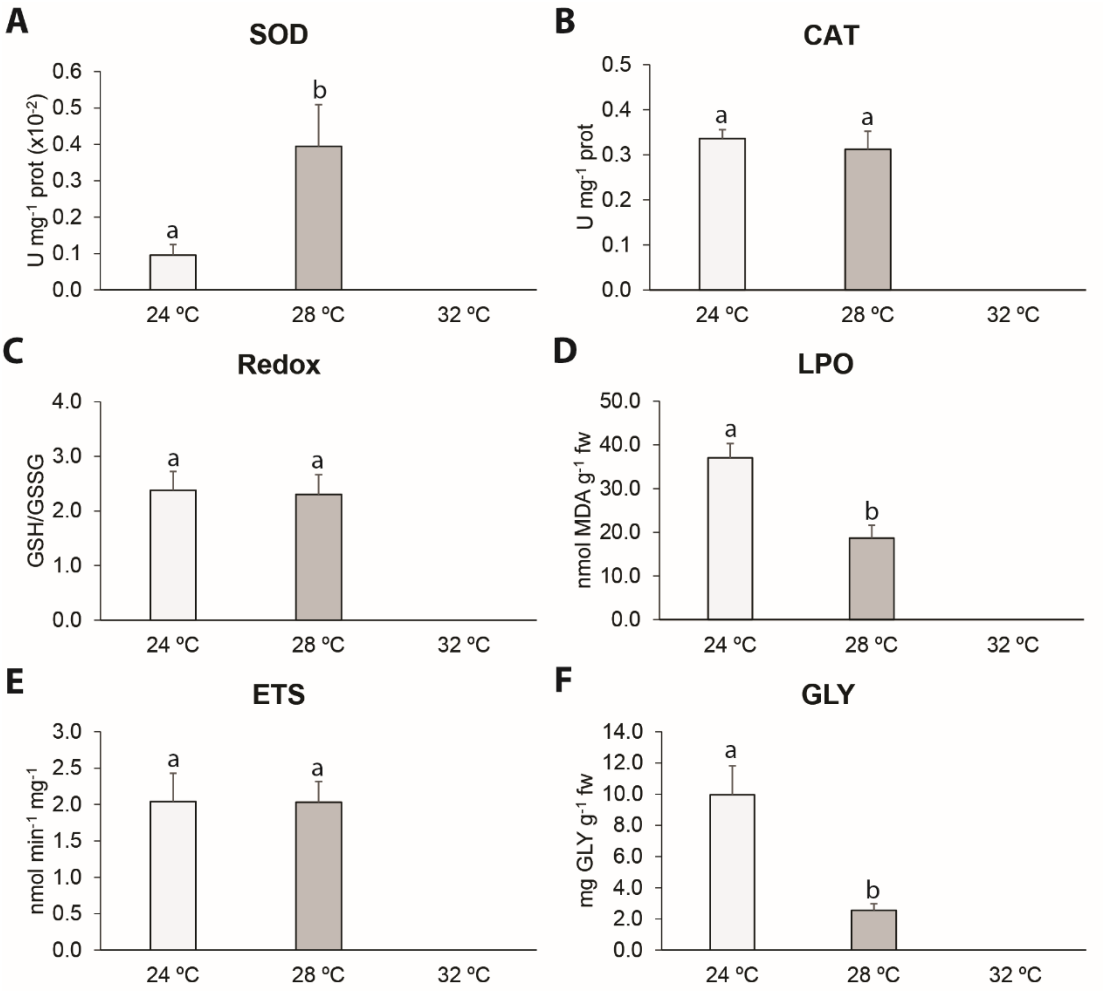

Figure 17 – Biochemical parameters studied in juvenile *C. gigas* exposed to thermal stress.

A: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Reduced to oxidized glutathione ratio (Redox); **D**: Lipid peroxidation (LPO) levels; **E**: Electron transport system (ETS) activity; and **F**: Glycogen (GLY) content. Significant differences ($p \leq 0.05$) between conditions are represented with different letters (mean + standard error; n=12)

Due to high mortality (67%) observed for *C. gigas* juveniles exposed to the highest temperature (32 ºC), oysters that survived at 32 ºC were excluded from analysis in order prevent a biased effect of selecting the most resistant individuals. The most prominent changes in juvenile *C. gigas* exposed to thermal stress were observed concerning SOD activity, LPO levels and GLY content.

Higher SOD activity observed in juvenile *C. gigas* maintained at 28 ºC, could relate to species metabolic adjustment. Metabolic adaptation during transitions from metabolically active states to arrested metabolic states in response to stress, organisms may experience what can be defined as moderate stress (Sokolova et al., 2012). At this physiological status, organisms develop several mechanisms to mitigate the impacts of a given stressor. These are energetically costly, and can include increased metabolic rates and upregulation of cellular protection mechanisms, such as antioxidant enzyme superoxide dismutase (Sokolova et al., 2012; Tomanek, 2014). Accordingly, lower GLY in juvenile *C. gigas* at 28 ºC, indicate a mismatch between energy supply and demand, a characteristic of a shift to a moderate stress status (Sokolova & Lannig, 2008; Sokolova et al., 2012). Additionally, increased SOD activity shows upregulation of antioxidant defences towards suboptimal temperature.

It is possible that the increase of energy demand to cope with high temperature and consequent GLY depletion, likely led to high mortality at 32 ºC in *C. gigas* juveniles. Similarly, Flores-Vergara et al. (2004) observed increased mortality in *C. gigas* spat reared up to 6 weeks at 32 ºC, and also showed progressive energetic depletion measured as carbohydrate content with increasing temperatures (23, 26, 29 and 32 ºC).

Higher LPO levels observed in juvenile *C. gigas* at the lowest temperature (24 ºC), was explained by the interaction of several factors, namely membrane structure, metabolic adjustment and antioxidant capacity. Temperature variations induce membrane restructuring in biological systems, a common response mechanism known to ectotherms (Crockett, 2008). The most important of these mechanisms involve changes of phospholipids unsaturation levels, with organisms commonly present higher proportions of membrane polyunsaturated fatty acids (PUFA), when either acclimated or acclimatized to lower temperatures (Hazel & Williams, 1990). In turn, the saturation state of biological membranes is likely to influence organisms susceptibility to LPO (Crockett, 2008; Halliwell & Gutteridge, 1999), since higher proportions of PUFA increase the propensity of membranes to peroxidation reactions (de Zwart et al., 1999). Higher LPO levels in juvenile *C. gigas* at 24 ºC, could therefore be a result of higher PUFA proportions in membranes. Studies on *C. gigas* from the environment have correlated higher PUFA levels with lower temperatures in adults (Dagorn et al., 2016; Pazos et al., 1996), as well as in spat under laboratory conditions (23-32 ºC) (Flores-Vergara et al., 2004). Increased LPO in juveniles at the lowest temperature could therefore be partially explained by membranes fatty acid composition, and has also been hypothesised for oysters collected in the environment (Zanette et al., 2006).

3.2.1.3 Species comparison

Seawater acidification

Different response patterns were observed in juvenile *C. brasiliana* and *C. gigas* exposed to seawater acidification. In fact, an opposite trend was demonstrated regarding metabolic potential modulation between both species, assessed by the ETS activity, with *C. gigas* presenting increased metabolic capacity (ETS) with the increase of acidification, while *C. brasiliana* presented depressed metabolic potential under the same conditions.

The decrease of antioxidant capacity observed in *C. gigas* juveniles exposed to seawater acidification, demonstrated by lower antioxidant enzymes SOD and CAT activity, increased LPO, as well as impairment of the redox balance (GSH/GSSG) observed, indicated the onset of oxidative stress in these oysters. In contrast, *C. brasiliana* showed unaltered antioxidant capacity, and lower LPO at the highest acidification level (pH 7.0), consistent with the results suggesting metabolic depression (ETS, GLY).

Given these results it seems that *C. brasiliana* juveniles were more tolerant to seawater acidification than *C. gigas*, despite the fact that metabolic depression is only a time limited mechanism to endure extreme stress.

Thermal stress

Different response patterns were observed in juvenile *C. brasiliana* and *C. gigas* exposed to increasing temperature. High mortality observed in *C. gigas* juveniles exposed to the highest temperature (32 ºC) indicates the thermal tolerance window was exceeded in this species considering the juvenile life stage. Moreover, results showed enhanced antioxidant capacity in *C. gigas* exposed to 28 ºC (SOD), possibly related to lower cellular damage (LPO) observed, despite with significant energetic depletion (GLY).

An opposite trend was observed regarding *C. brasiliana* juveniles stress response to temperature rise, during which enzymatic antioxidant capacity (SOD) was depressed, while GSH mediated ROS quenching capacity was enhanced. This stress response strategy was accompanied by metabolic depression (ETS) and lower cellular damage (LPO) with the increase of ambient temperature, despite that energetic burden was observed at 32 ºC (GLY).

Overall, these findings indicate that *C. brasiliana* juveniles were more tolerant to temperature rise than *C. gigas*, with the latter species presenting a lower maximum thermal tolerance range. Nonetheless, given that metabolic depression is only a time limited mechanism to endure abiotic stress, the physiological fitness and survival of *C. brasiliana* juveniles may depend on the duration of episodes of thermal stress, especially if environmental temperatures exceed 32 ºC.

3.2.2 Adults

Hypercapnic stress

3.2.2.1 *Crassostrea brasiliana*

The effects of different seawater acidification levels on adult *C. brasiliana* assessed on the basis of several biochemical markers are depicted in Figure 18.

SOD activity was significantly higher in adult *C. brasiliana* exposed to the intermediate acidification level (pH 7.4) towards that observed in control (pH 7.8). No alterations in either CAT activity, GSH/GSSG, or LPO levels were observed among tested acidification levels. Significantly lower ETS activity was observed at both acidification levels (pH 7.4 and pH 7.0) compared to control (pH 7.8). Glycogen (GLY) content tended to be lower at the highest acidification levels (pH 7.4 and pH 7.0) compared to control (pH 7.8), despite not statistically significant.

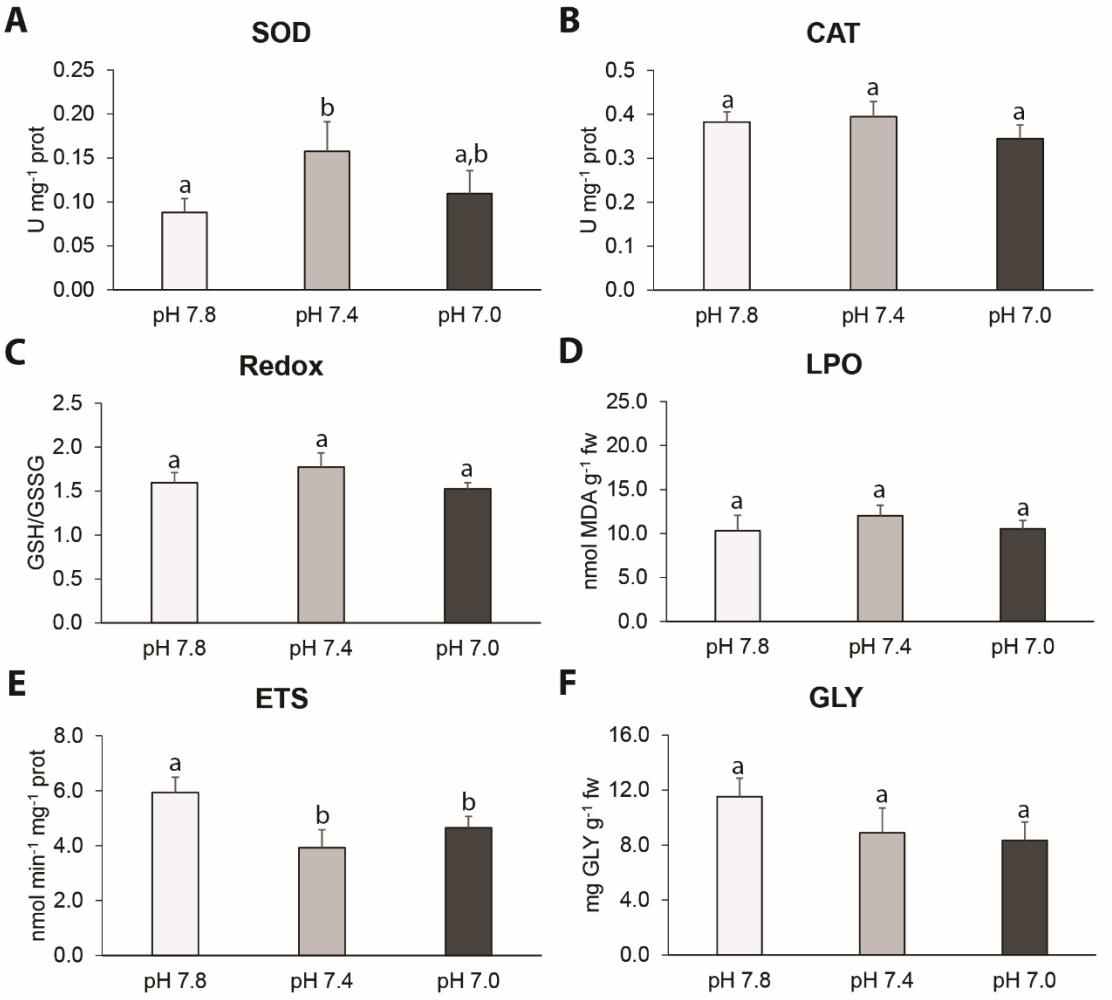

Figure 18 – Biochemical parameters in adult *C. brasiliana* exposed to seawater acidification.

A: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Reduced to oxidized glutathione ratio (Redox); **D**: Lipid peroxidation (LPO) levels; **E**: Electron transport system (ETS) activity; and **F**: Glycogen (GLY) content. Significant differences ($p \leq 0.05$) between conditions are represented with different letters (mean + standard error; n=12)

The most prominent changes concerning the biochemical markers studied in adult *C. brasiliana* exposed to different acidification levels, included changes in antioxidant activity (SOD) and metabolic potential (ETS) among tested conditions.

Overall, adult *C. brasiliana* presented a similar response pattern to that observed in juvenile specimens. Like juveniles, adult *C. brasiliana* presented decreased metabolic potential (ETS) with the increase of hypercapnia, that indicated metabolic depression in the presence of high CO_2 concentrations, in line with results from other studies on bivalve molluscs (Michaelidis et al., 2005; Pörtner et al., 1998; Reipschläger & Pörtner, 1996), that are generally indicative of organisms experiencing environmental stress (Guppy & Withers, 1999; Lannig et al., 2010; Parker et al., 2013).

Nonetheless, and unlike juveniles, adult *C. brasiliana* presented increased SOD activity with the increase of acidification, unaltered GLY content and LPO. These results suggest that adult oyster were more efficient in maintaining energetic fitness than juveniles, possibly because younger organisms generally require higher metabolic demand (Hawkins, 1995).

Thermal stress

The effects of different temperature levels on adult *C. brasiliana* assessed on the basis of several biochemical markers are depicted in Figure 19.

No significant alterations were observed concerning SOD activity among tested conditions, despite an apparent decreasing trend in SOD activity with the increase of temperature. Significantly higher CAT activity was observed at both increased temperature scenarios (28 and 32 ºC) compared to control temperature (24 ºC). The highest GSH/GSSG was observed in oysters maintained at 32 ºC, the lowest values observed at 28 ºC, and an intermediate value was observed at 24 ºC. Results showed no alterations in either LPO, ETS activity or GLY content among temperature levels.

Increased CAT activity in adult *C. brasiliana* exposed to 28 and 32 ºC relative to the lowest temperature, indicated that these oysters induced higher antioxidant capacity towards hydrogen peroxide at higher temperatures. Moreover, results showing higher GSH/GSSG in oysters exposed to 32 ºC compared to 28 ºC, were influenced by a significant increase of GSH concentrations (data not shown), indicating oysters were actively synthesising GSH at 32 ºC in response to thermal stress.

Taking into consideration the biomarkers studied, it appears that adult *C. brasiliana* was fairly insensitive to the tested temperatures, and was able to maintain overall physiological fitness (unchanged LPO, ETS and GLY among conditions). Interestingly, these findings are in contrast with those observed in juvenile specimens that showed higher responsiveness to thermal stress.

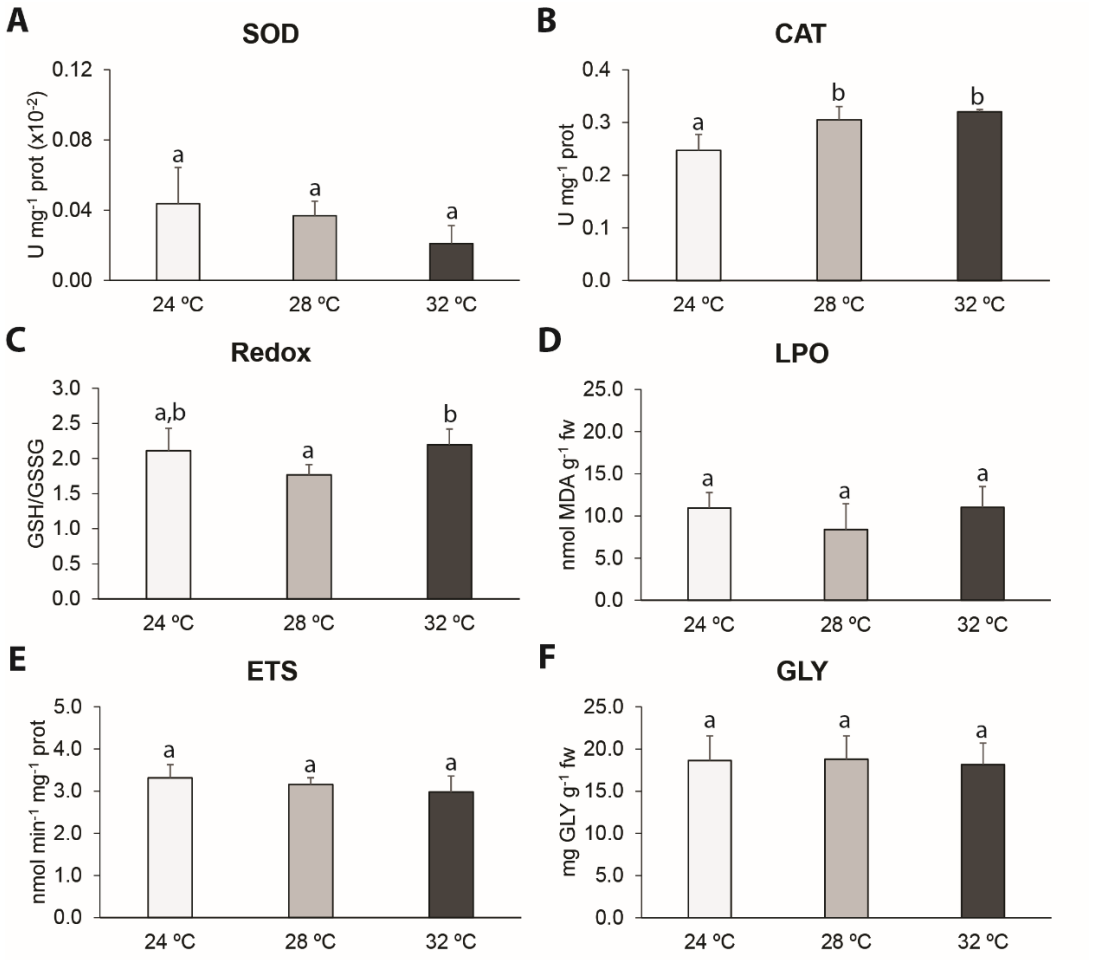

Figure 19 – Biochemical parameters studied in adult *C. brasiliana* exposed to thermal stress. **A**: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Reduced to oxidized glutathione ratio (Redox); **D**: Lipid peroxidation (LPO) levels; **E**: Electron transport system (ETS) activity; and **F**: Glycogen (GLY) content. Significant differences ($p \leq 0.05$) between conditions are represented with different letters (mean + standard error; n=6)

Differences in antioxidant capacities between bivalve individuals of different age can be expected to occur, although these do not always respond in the same pattern. For instance, CAT activity has shown to decrease with age in *C. virginica* (Ivanina et al., 2008) and *M. edulis* (Viarengo et al., 1991), or to remain constant as in *M. edulis* and *Mya arenaria* (Suhkotin et al., 2002), and *L. elliptica* (Philipp et al., 2005). These are in contrast to our findings for *C. brasiliana*, although they were obtained for wider age ranges, different species and tissues than those from the present study. Nonetheless, the mechanisms responsible for these patterns are still under debate (Ivanina et al., 2008), and the present findings further illustrate that different temperatures of acclimation can yield different antioxidant response (CAT and SOD) among different life stages.

Additionally, and unlike juveniles, adult *C. brasiliana* presented unchanged metabolic potential (ETS) and energetic reserves (GLY) among temperatures. Unchanged metabolic potential in adults, could derive from the fact that respiratory enzymes tend to remain fully active within the organisms thermal tolerance range (Simčič et al., 2014; Yurista, 1999), and knowing

that the tested temperatures were similar to the natural temperature range described for the species geographical distribution (20 to 32 ºC) (Menzel, 1991). However, the different response pattern observed in juveniles, was likely related to differences in energetic efficiency, knowing that juvenile bivalves generally present higher metabolic activity that adults (Sukhotin & Pörtner, 2001). It is therefore possible that energetic reserves could suffice to maintain ETS concentrations equal among temperatures in adults, whereas in juveniles this balance was impaired. Indeed, results on oysters energetic fitness (GLY) corroborated this hypothesis, for which juveniles presented higher energetic burden (decreased GLY at the highest temperature), while adults showed no alterations in the same parameter. Unaltered GLY content in adults with the increase of temperature suggests that the thermal range did not induce higher energetic burden, or that the glycolytic flux was down-regulated (Storey, 1998).

3.2.2.2 *Crassostrea gigas* (Brazil)

Seawater acidification

The effects of seawater acidification on adult *C. gigas* assessed on the basis of several biochemical markers are depicted in Figure 20.

Significantly lower SOD activity was observed in adult *C. gigas* exposed to the intermediate acidification level (pH 7.4) compared to the remaining conditions. Similarly, significantly lower CAT activity was observed in oysters exposed to the intermediate acidification level (pH 7.4), with significant differences towards the highest acidification level (pH 7.0). No alterations were observed regarding Redox status, nor LPO levels among tested conditions.

The ETS activity was significantly higher at the highest acidification level (pH 7.0) compared to control (pH 7.8). A significant decrease in GLY content was observed between intermediate (pH 7.4) and high (pH 7.0) acidification levels.

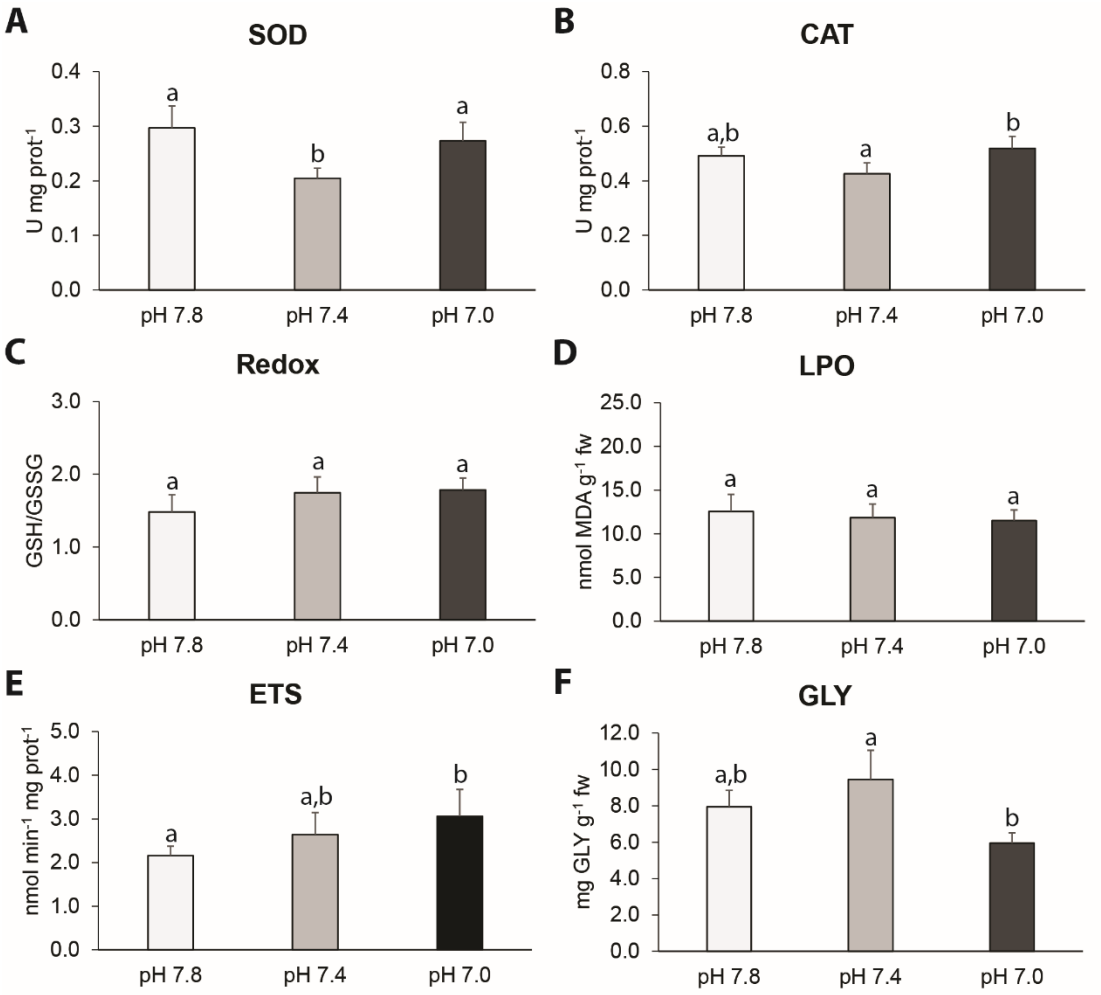

Figure 20 – Biochemical parameters in adult *C. gigas* exposed to seawater acidification.

A: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Reduced to oxidized glutathione ratio (Redox); **D**: Lipid peroxidation (LPO) levels; **E**: Electron transport system (ETS) activity; and **F**: Glycogen (GLY) content. Significant differences ($p \leq 0.05$) between conditions are represented with different letters (mean + standard error; n=12)

The most prominent changes concerning the biochemical markers studied in juvenile *C. gigas* exposed to different acidification levels, were observed at the highest acidification level (pH 7.0), and included changes in antioxidant activity (CAT), metabolic potential (ETS) and energetic fitness (GLY) among conditions.

Increased ETS activity observed in adult *C. gigas* at the highest acidification level (pH 7.0), indicate the development of increased metabolic potential in response to extreme hypercapnia in these oysters, and are in line with recent studies on other ectothermic marine metazoans exposed to acidification (Harms et al., 2014; Strobel et al., 2013). It is important to note that adults presented increased metabolic rate at a higher acidification level than juveniles, which could indicate a differentiated metabolic response capacity of adults towards tested acidification levels.

Induced CAT activity at the highest acidification level (pH 7.0), coincided with significantly higher metabolic potential (ETS), and could have been triggered to mitigate the negative effects of higher ROS production occurring from the mitochondria electron transport chain (Gibbin et al., 2017; Harms et al., 2014).

Although no alterations were noted in GSH/GSSG in adult *C. gigas*, results also showed higher total glutathione (tGSH) concentrations in oyster tissues (data not shown). The increase of tGSH, by *de novo* synthesis of GSH is a common mechanism that may allow for higher antioxidant capacity in these oysters (Trevisan et al., 2014). These results were different from those observed in juvenile *C. gigas* (previously discussed) that evidenced GSH/GSSG alterations and even decreased tGSH replenishment capacity in oysters exposed to the highest acidification level (pH 7.0). Together these data suggest higher capacity to maintain redox balance in adult oysters. Similarly, Philipp et al. (2008) observed a more pronounced decrease of glutathione in young *Aequipecten opercularis* scallops than adults after swimming bursts, and concluded that younger animals were less effective on homeostatic regulation.

In line with this, unchanged LPO in adult *C. gigas* among acidification levels, suggest that cellular or physiological mechanisms could have been employed to prevent membrane oxidative damage. Indeed, higher CAT activities at the highest acidification level, as well as an increase of the glutathione pool (tGSH), together may have prevented LPO increased formation.

Results obtained for *C. gigas* indicate higher energetic expenditure in adult oysters exposed to the highest acidification level (pH 7.0) (lower GLY content). These results are important considering that the energetic status of bivalves can reflect the level of environmental stress (Storey, 1998). Also, the decrease of GLY content was consistent with results obtained regarding higher metabolic costs of increased ETS and antioxidant enzymes activities observed in the same condition (CAT, tGSH). In contrast, Timmins-Shiffman and co-authors (2014) found no change in GLY content of *C. gigas* exposed to acidification, although testing lower pCO_2 concentrations (2,800 μatm) than in the present study. Hence, our results suggest that higher levels of pCO_2 (10,000 μatm) may further challenge adult *C. gigas* energetic fitness.

Together, results obtained concerning GLY, ETS and antioxidant capacity in *C. gigas* indicate acidification induced a transition to a moderate stress status, according to the concept of

energy-limited stress tolerance (Sokolova et al. 2012), for which an increase of metabolic capacity and energetic turnover occurs as a compensation mechanism for homeostatic maintenance and damage repair in response to a given stressor.

Thermal stress

The effects of different temperature levels on adult *C. gigas* assessed on the basis of several biochemical markers are depicted in Figure 21.

No significant alterations were observed concidering SOD and CAT activities in oysters among tested conditions. Redox status (GSH/GSSG) and LPO levels were significantly lower at the highest temperature level (32 ºC). The ETS activity was significantly higher in oysters exposed to 28 ºC compared to control (24 ºC). Lower glycogen (GLY) content was observed in oysters exposed to higher temperatures (28 and 32 ºC) compared to those exposed at 24 ºC, despite differences were not significant.

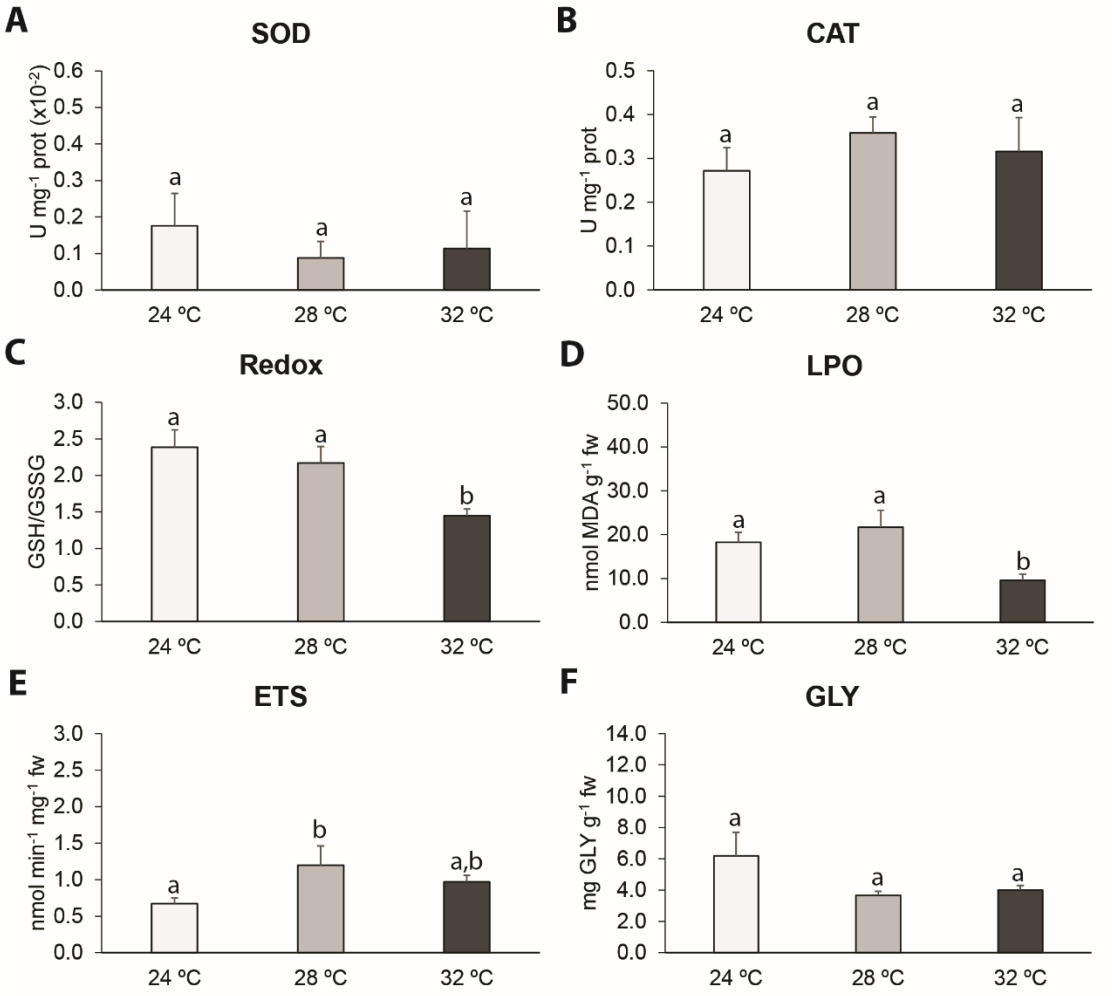

Figure 21 – Biochemical parameters studied in adult *C. gigas* exposed to thermal stress.

A: Superoxide dismutase (SOD) activity; **B**: Catalase (CAT) activity; **C**: Reduced to oxidized glutathione ratio (Redox); **D**: Lipid peroxidation (LPO) levels; **E**: Electron transport system (ETS) activity; and **F**: Glycogen (GLY) content. Significant differences ($p \leq 0.05$) between conditions are represented with different letters (mean + standard error; n=6)

The most prominent changes in adult *C. gigas* exposed to thermal stress were observed concerning GSH/GSSG, LPO levels and ETS activity.

Significantly lower GSH/GSSG in adults at 32 ºC comparing to individuals exposed to 28 ºC, indicate a prooxidant status at this temperature level. Lannig et al. (2006) also observed lower GSH/GSSG in *Crassostrea virginica* with increasing temperature, as well as a limited capacity of this species to synthesise GSH at high temperatures (up to 28 ºC).

Alterations in LPO levels in adult *C. gigas* can be explained by a number of mechanisms and their interactions, such as membrane structure, metabolic adjustment and antioxidant capacity. Unlike juveniles, LPO levels in adult *C. gigas* did not show lower LPO at the lowest temperature (24 ºC), instead adults showed lower LPO at the highest temperature (32 ºC). These results suggest an age dependent function of LPO formation. Accordingly, Ivanina et al. (2008) observed higher rates of fluorescent ageing pigments (the end product of LPO) formation in younger individuals of *C. virginica*. These differences could be related to differentiated metabolic rates between age groups (Ivanina et al., 2008; Sukhotin & Pörtner, 2001). Higher metabolic activity in juveniles could render higher ROS as a function of size, or because older organisms can present depressed metabolic rates (Sukhotin & Portner, 2001). The decrease of LPO at 32 ºC in adult oysters could be explained by the onset of anaerobiosis at the pessimum range of thermal tolerance (Sokolova et al., 2012). Successful facultative anaerobes such as oysters can alternate between aerobic and anaerobic metabolism in response to conditions of extreme stress thus minimizing ROS production rates (Anestis et al., 2007; Pörtner, 2010; Tomanek, 2014). Rivera-Ingraham et al. (2013), showed lower ROS production in *Mytillus edulis* under hypoxic conditions, evidencing that intertidal molluscs possess mechanisms that allow for anaerobic respiration with low deleterious free radical production. Several adaptive mechanisms are known to mitigate ROS formation in marine facultative anaerobes (Abele et al., 2007). During anaerobic respiration such mechanisms are likely to become active and could explain lower LPO levels in adults comparing to the lower temperatures where aerobic metabolism is probably more prevalent. Further investigation on aerobic and anaerobic respiration is required to understand these mechanisms.

The increase of ETS activity in adults indicate an increase of active ETS enzyme concentrations in response to the intermediate temperature (28 ºC). It is generally accepted that in the absence of other limiting factors, increasing temperature induces higher metabolic rates (Angilletta, 2009; Pörtner et al., 2006). Le Moullac et al. (2007) observed increased ETS activity with increasing temperature in adult *C. gigas*, although testing a temperature range of 12, 15 and 20 ºC. Assuming these authors results, and those observed in the present study (increased ETS activity from 24 to 28 ºC), overall the findings support that adult *C. gigas* can increase the efficiency and/or concentrations of active ETS units in response to increasing temperature up to a certain threshold. Increasing the number of rate limiting enzymes to sustain aerobic metabolism (as proposed here for ETS) has been shown in mussel *Modiolus modiolus*, although during acclimation to cold temperatures, namely aerobic enzymes citrate synthase and cytochrome oxidase (Lesser & Kruse, 2004). Other examples of such adaptation mechanisms include studies

on the copepod *Acartia tonsa*, that showed increased activity of ETS with the increase of temperature from 17 to 21 or 29 ºC, leading the author to conclude that higher concentrations of rate limiting enzymes were present at higher temperatures (Båmstedt, 1980). The ETS of zebra mussel *Dreissena polymorpha* in the environment also presented different activity levels depending on season and site location, with higher activities observed during late spring (Fanslow et al., 2001). No further increase of ETS activity at the highest temperature could be indicative of a transition into an arrested state of metabolic potential, in line with the concept of energy limited stress tolerance (Sokolova, 2013). No change in GLY content could further corroborate the hypothesis of metabolic conservation strategy. Indeed, bivalve molluscs may present decreased glycolytic flux and ATP consumption, as a means of GLY reserves maintenance to extend survival during periods of stress (Brooks & Storey, 1997; Storey, 1998)

3.2.2.3 Species comparison

Seawater acidification

The present data show marked differences in each species response pattern to this environmental stressor, in accordance with other studies assessing comparative performances between other closely related bivalve species enduring abiotic stress, namely Mytilid (Tomanek, 2014) and Venerid (Velez et al., 2016) congeners. Similarly to juveniles, an opposite trend was demonstrated regarding metabolic potential between both species, assessed by the ETS activity, with *C. gigas* presenting increased metabolic capacity (ETS) in acidification exposures.

Adult *C. gigas* presented higher capacity to induce antioxidant enzymes activity than juveniles, namely at the highest acidification level, demonstrated by the increase of antioxidant enzymes SOD and CAT activities, as well as changes in non-enzymatic ROS scavenger GSH oxidation form and concentration, indicate that these conditions induced a prooxidant status. *C. gigas* presented GSH as preferential antioxidant to cope with acidification induced oxidative stress, with observable effects on the total glutathione pool (tGSH) and GSH/GSSG, most evident in juvenile specimens at the highest acidification level. The antioxidant capacity of *C. gigas* may have mitigated increased cellular damage (LPO), except in juveniles held at the intermediate acidification level. Nonetheless, data on GSH mediated antioxidant response suggest that this mechanism is time limited.

In contrast, *C. brasiliana* presented a decrease of metabolic potential, noted by lower ETS activity with the increase of hypercapnia. These results suggest metabolic depression to withstand acidification in this species, and were further supported by low antioxidant capacity, no change or even decrease (juveniles at pH 7.0) of LPO, indicating reduced aerobic scope to sustain energetic fitness under seawater acidification.

Thermal stress

Generally, the present findings suggest that *C. brasiliana* presented a higher capacity to utilize GSH as an antioxidant than *C. gigas* (higher total glutathione pool, data not shown). The use of GSH as an antioxidant can be energetically less costly than to induce antioxidant enzymes (Pannunzio & Storey, 1998), and could therefore present advantages for energetic fitness. The preferential use of GSH observed in *C. brasiliana* juveniles may be related to the induction of metabolic pathways towards lower ROS production (NADPH oriented) and higher GSH quenching capacity) described for Mytillid species (Tomanek, 2014).

The present study illustrates the complexity of the biochemical response of two oyster species to different temperature levels, and reflects different species strategies to endure the tested thermal regimes. Overall, juveniles from both species showed to be more responsive to thermal regime than adults, presenting higher mortality, higher susceptibility to cellular damage, stimulated antioxidant response and energetic depletion, with results suggesting higher upper thermal tolerance in the native species. On the contrary, adult oysters showed to be less responsive to thermal stress. Comparisons between species revealed that thermal stress induced greater alterations in *C. gigas*, with overall higher SOD activity comparing to *C. brasiliana*, also indicating greater energetic costs in response to increasing temperature in *C. gigas* comparing to *C. brasiliana*. These results suggest that energetic metabolism of *C. brasiliana* was more efficient than that of *C. gigas* under the tested thermal window, possibly because this species is adapted to lower latitudes, and thus to warmer thermal regimes. Despite high mortality of juveniles, the introduced species presented mechanisms that allowed adults to be resilient to thermal stress, at temperatures higher than those predicted for *C. gigas* in south America (Carrasco & Barón, 2009). This study brings new insights on the biochemical mechanisms involved in the response of two important oyster species to different thermal regimes, and highlights the importance of studying different life stages in order to better understand the ecological impacts of environmental stressors. Results obtained indicate that *C. gigas* holds mechanisms that may enable it to survive at higher temperatures. The question remains if these mechanisms would be sufficient on the long term for the introduced species to survive such thermal regime. If adults are able to survive and spawn, increased resistance of offspring (Parker et al., 2012) could enable for competition in a changing environment.

4 CONCLUDING REMARKS

Experiments conducted during the development of the present thesis allowed to infer on different oyster species performance towards several climate change related stressors, with the aim to answer the following questions: i) How do different oyster species respond to climate change related phenomena? ii) How does the combination of climate change and pollutant exposure influence oyster species performance? iii) Do different life stages present different susceptibilities to these stressors?

Data obtained during this investigation allowed to answer the above stated questions, bringing new insights into stress response signatures that characterize species tolerance capacities in the perspective of global change. The present findings thus contribute to better understand the molecular and physiological mechanisms that define different oyster species capacities to endure and compete in a changing environment. Ultimately, and in line with the goals of experimental biology, this study presents evidence on species capacities and limits to adaptation, important for policy makers and management entities (Sutherland et al., 2004).

The complexity of the stress response signatures of *Crassostrea* species were evident, and were interpreted considering the interplay between subcellular stress response mechanisms, oysters energetic and physiological fitness, metabolic capacity, homeoviscous adaptation and aerobic scope, in light of the existing literature. Indeed, different stress response strategies and performances were observed between species, and life stages. The combination of climate change and pollutant exposure also revealed to influence oysters performance.

4.1 *Crassostrea angulata* and *Crassostrea gigas* (Portugal)

4.1.1 Embryo-larvae

Differences in each species embryo-development capacity were evident, concerning the tolerance range to salinity and temperature, which further reflected in the pattern of sensitivity to As observed. *C. angulata* presented a narrower range of salinity and temperature than *C. gigas* for which embryo-development successfully occurred, considering 24 and 48 h post fertilization. *C. angulata* presented better embryonic development at intermediate salinity (26) and temperatures above 20 ºC, while *C. gigas* presented high frequencies of developing embryos at all combinations of salinity and temperatures tested. Overall, these results suggest that early ontogeny of *C. angulata* may be limited to a narrower range of abiotic factors (salinity and temperature) compared to *C. gigas*, with possible implications at the population level. Considering that early life stages generally constitute the most susceptible stage of oysters life cycle, the thermohaline differences observed for embryo development may dictate species competitive advantages towards one another under the projected scenarios of climate driven alterations of temperature and salinity regimes in estuarine systems worldwide. Hence, *C. angulata* may be more susceptible to environmental change than *C. gigas*.

Comparisons of As toxicity between species (measured as EC50 values) showed that *C. angulata* was at least 10 times more sensitive to As than *C. gigas* and these differences were

likely species related. These findings suggest that the survival of *C. angulata* strongly depends on a narrower range of abiotic factors compared to its closely related congener, and therefore the future of this species populations may be endangered considering the future projections on climate change and pollution worldwide.

4.1.2 Juveniles

Different response patterns were observed regarding each species biochemical performance towards each stressor. Overall, fewer alterations in oxidative stress parameters were observed in *C. angulata* compared to *C. gigas*, with the latter presenting higher capacity to induce antioxidant and biotransformation enzymes (SOD, CAT and GSTs). Data also suggested different metabolic strategies to endure exposure conditions, with *C. angulata* presenting unaltered metabolic potential (ETS) and energetic fitness (GLY). On the contrary, *C. gigas* presented altered metabolic potential and bioenergetics (GLY). The combined effects of seawater acidification and As induced higher oxidative stress in both species (LPO) than single exposures.

The results obtained through proteomic analysis allowed for a deeper insight into the modes of action of these closely related oyster species towards the combined effects of acidification and As exposure. As a corollary, both species induced cellular remodelling in response to external stimuli, observed by altered levels cytoskeleton related proteins, namely Actin and Atlastine (*C. gigas*); Actin, Severin, Coronin and Gelsolin (*C. angulata*). However, the conditions at which each species presented such alterations differed, with *C. gigas* presenting altered cytoskeleton proteins in Low pH exposures, while *C. angulata* showed most alterations under both Arsenic exposures (As and Low pH+As). Interestingly, proteomic analysis revealed metabolic suppression in *C. angulata* (downregulation of ATP β) in both Low pH and Low pH+As exposures.

Protein changes observed in oysters exposed to the combined exposure to Low pH+As revealed important differences in oysters response capacity compared to that observed in single exposures, corroborating the working hypothesis that multiple stressors will further challenge oyster species in the environment. Together, analysis of biochemical performance and proteomics revealed that each species presented different mechanisms to endure tested conditions. Overall, *C. angulata* appeared to be less responsive than *C. gigas* considering the biochemical markers studied. This could either indicate a weaker response capacity, or lower sensitivity to tested conditions.

Overall, data on protein changes towards Low pH+As evidenced the induction of different modes of action by each species. Interestingly, decreased abundance of piRNA in *C. angulata* suggested genome reconfiguration in response to Low pH+As (as opposed to *C. gigas*), possibly induced as an adaptive mechanism towards extreme stress. These data add to recent findings by Li et al. (2017) that showed higher phenotypic plasticity of *C. angulata* compared to *C. gigas* towards environmental change and suggested higher adaptive potential of the former species in a changing environment.

4.1.3 Adults

Overall results demonstrated that adult *C. angulata* and *C. gigas* were less responsive to tested conditions than juveniles considering seawater acidification experiments (Low pH, As, and Low pH+As). Both species showed low oxidative stress response towards tested conditions, despite significant enhancement of GSTs activity in oysters exposed to As, that may have helped prevent alterations of lipid peroxidation levels. Interestingly, biomineralization capacity (CA) was significantly impaired by both Low pH and As exposures. The combined exposure to both stressors proved to have a negative effect on both species response capacity to As, namely regarding GSTs activity. Overall *C. gigas* presented higher stress response capacity (SOD, CAT and GSH) than *C. angulata*, suggesting that the latter species was either less sensitive to the tested conditions or presented a lower capacity to respond to the tested scenarios.

Results from exposures to different salinity levels showed that both species presented alterations in oxidative stress status and metabolic performance. Salinity also affected As accumulation dynamics, with oysters exposed to lower salinities tending to accumulate higher As concentrations. Overall adult specimens of both species showed similar stress response patterns to salinity and As exposures, presenting higher antioxidant capacity (SOD) and metabolic potential (ETS) at lower salinities (10 and 20). However, differences were observed considering the influence of salinity on each species biotransformation capacity (GSTs), with *C. angulata* presenting induced GSTs at a wider range of salinities (20, 30 and 40) compared to *C. gigas* (10 and 30). Moreover, *C. angulata* presented higher capacity to maintain redox balance at the range of salinities tested, and lower degree of alterations in LPO levels among conditions.

4.2 *Crassostrea brasiliana* and *Crassostrea gigas* (Brazil)

4.2.1 Juveniles and adults

Seawater acidification

Results obtained concerning both species stress response to acidification, highlighted different strategies to cope with increased pCO_2, and bring new insights on species tolerance capacity and differentiated response mechanisms. The time duration of environmental hypercapnia in estuarine systems may be of upmost importance, since oyster response mechanisms to high environmental pCO_2 suggested to be time limited. According to the energy-limited tolerance concept (Sokolova et al., 2012) the present data indicate that the mangrove oyster (*C. brasiliana*) transitioned into the *pessimum* tolerance range when exposed to acidification, as a conservation mechanism to endure extreme stress, while response patterns observed for the pacific oyster (*C. gigas*) reflected a moderate stress status, which could generally imply a wider range of tolerance towards acidification than *C. brasiliana*, as well as a longer and more sustainable energetic balance. The differentiated response pattern observed could have further implications at the population level, and influence species competitive advantages towards each other in the event of ocean acidification. In a scenario of coexistence of the two species in the same geographical areas, it appears that *C. gigas* may be more resilient than the native species (*C. brasiliana*) to environmental hypercapnia, with ecological repercussions that are hard to predict. Therefore, efforts should be made to prevent the spread of the non-native species into pristine environments where *C. brasiliana* still thrives.

Thermal stress

The present study illustrates the complexity of the biochemical response of two oyster species to different temperature levels, and reflects different species strategies to endure the tested thermal window. Overall, juveniles of both species showed to be more responsive to thermal stress than adults, presenting higher mortality, higher susceptibility to cellular damage, stimulated antioxidant response and energetic depletion, with results suggesting higher upper thermal tolerance in the native species (*C. brasiliana*). On the contrary, adult oysters showed to be less responsive to thermal stress. Comparison between species revealed that thermal stress induced greater alterations in *C. gigas*, with overall higher SOD activity comparing to *C. brasiliana*, also indicating greater energetic costs in response to increasing temperature in *C. gigas* comparing to *C. brasiliana*. These results suggest that energetic metabolism of *C. brasiliana* was more efficient than that of *C. gigas* considering the tested thermal window. Despite of high mortality of juvenile *C. gigas,* adult oysters developed mechanisms that enabled to cope thermal stress, at temperatures higher than those predicted for *C. gigas* in south America (Carrasco & Barón, 2009). This study brings new insights on the biochemical mechanisms involved in the response of two important oyster species to different thermal regimes, and highlights the importance of studying different life stages in order to better understand the ecological impacts of

environmental stressors. Results obtained indicate that *C. gigas* holds mechanisms that may enable it to survive at higher temperatures. The question remains if these mechanisms would be sufficient on the long term for the introduced species to survive such thermal regime. If adult are able to survive and spawn, increased resistance of offspring (Parker et al., 2012) could enable for competition in a changing environment.

5 SUPPLEMENTARY DATA

Supplementary table S1 – Mean percentage of *C. angulata* embryos presenting delayed development (Pre-D) at different combinations of salinity, temperature and As concentrations, after 24 and 48-hour exposures. Conditions for which 100 % embryos were classified as Pre-D are highlighted in dark grey.

			µg L^{-1} of As							
24 h	Salinity	Temperature (ºC)	0	30	60	120	240	480	960	1920
	20	20	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		24	75.3	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		28	75.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	26	20	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		24	4.0	24.0	95.7	100.0	100.0	100.0	100.0	100.0
		28	8.0	19.0	93.0	99.3	100.0	100.0	100.0	100.0
	33	20	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		24	4.3	5.0	97.7	98.0	99.3	100.0	100.0	100.0
		28	5.0	5.3	62.0	88.7	99.3	100.0	100.0	100.0
48 h	Salinity	Temperature (ºC)								
	20	20	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		24	35.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		28	62.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	26	20	10.3	48.3	100.0	100.0	100.0	100.0	100.0	100.0
		24	0.3	3.0	93.3	100.0	100.0	100.0	100.0	100.0
		28	4.7	6.3	87.0	15.0	100.0	100.0	100.0	100.0
	33	20	97.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		24	1.3	1.5	43.0	90.3	99.0	100.0	100.0	100.0
		28	39.0	76.7	63.0	33.7	1.0	0.3	100.0	100.0

Supplementary table S2 – Mean percentage of *C. gigas* embryos presenting delayed development (Pre-D) at different combinations of salinity, temperature and As concentrations, after 24 and 48-h exposures. Conditions for which 100 % embryos were classified as Pre-D are highlighted in dark grey.

			µg L^{-1} of As							
24 h	Salinity	Temperature (ºC)	0	30	60	120	240	480	960	1920
	20	20	99.3	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		24	20.7	6.7	88.0	100.0	100.0	100.0	100.0	100.0
		28	14.7	23.0	25.3	12.0	0.3	100.0	100.0	100.0
	26	20	6.3	1.7	2.3	28.0	89.7	99.7	100.0	100.0
		24	1.0	1.3	22.7	79.7	99.3	100.0	100.0	100.0
		28	5.0	3.7	4.3	18.7	79.0	99.3	100.0	100.0
	33	20	10.0	12.0	4.3	6.3	17.3	40.7	77.7	96.0
		24	5.3	1.7	1.3	2.3	4.3	20.0	84.7	83.0
		28	4.0	1.3	2.7	2.7	4.3	41.0	81.0	92.7
48 h	Salinity	Temperature (ºC)								
	20	20	4,3	4,0	14,3	60,0	100,0	100,0	100,0	100,0
		24	11,0	8,0	27,0	99,0	100,0	100,0	100,0	100,0
		28	1,3	3,0	11,3	73,0	100,0	100,0	100,0	100,0
	26	20	0,3	3,3	1,3	14,3	53,0	99,7	100,0	100,0
		24	1,5	2,7	0,7	7,7	67,0	96,3	100,0	100,0
		28	6,0	8,0	14,7	21,3	13,7	0,7	100,0	100,0
	33	20	7,3	0,3	1,3	2,3	0,3	42,7	86,7	96,0
		24	0,0	0,3	0,7	0,0	0,0	20,0	58,7	74,0
		28	25,3	21,0	30,0	29,7	27,7	35,3	23,3	5,3

6 REFERENCES

Abele, D., Heise, K., Pörtner, H.O., Puntarulo, S., 2002. Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam Mya arenaria. Journal of Experimental Biology. 205, 1831–1841.

Abele, D., Philipp, E., Gonzalez, P., Puntarulo, S., 2007. Marine invertebrate mitochondria and oxidative stress. Frontiers in Bioscience 12, 933–946.

Albalat, R., 2009. The retinoic acid machinery in invertebrates: Ancestral elements and vertebrate innovations. Molecular and Cellular Endocrinology 313, 23–35. https://doi.org/10.1016/j.mce.2009.08.029

Almeida, Â., Calisto, V., Esteves, V.I., Schneider, R.J., Soares, A.M.V.M., Figueira, E., Freitas, R., 2014. Presence of the pharmaceutical drug carbamazepine in coastal systems: Effects on bivalves. Aquatic Toxicology 156, 74–87. https://doi.org/10.1016/j.aquatox.2014.08.002

Amaral, V., Cabral, H.N., Bishop, M.J., 2012. Moderate acidification affects growth but not survival of 6-month-old oysters. Aquatic ecology 46, 119–127.

Amaral, V.S.D., Simone, L.R.L., 2014. Revision of genus Crassostrea (Bivalvia: Ostreidae) of Brazil. Journal of the Marine Biological Association of the United Kingdom 94, 811–836. https://doi.org/10.1017/S0025315414000058

Amemiya, I., 1926. Notes on Experiments on the Early Developmental Stages of the Portuguese, American and English Native Oysters, with Special Reference to the Effect of Varying Salinity. Journal of the Marine Biological Association of the United Kingdom (New Series) 14, 161–175.

Amiard-Triquet, C., Amiard, J.-C., Rainbow, P.S., 2016. Ecological Biomarkers: Indicators of Ecotoxicological Effects. CRC Press.

Anderson, K., Taylor, D.A., Thompson, E.L., Melwani, A.R., Nair, S.V., Raftos, D.A., 2015. Meta-Analysis of Studies Using Suppression Subtractive Hybridization and Microarrays to Investigate the Effects of Environmental Stress on Gene Transcription in Oysters. PLOS ONE 10, e0118839. https://doi.org/10.1371/journal.pone.0118839

Anderson, M., Gorley, R.N., Clarke, R.K., 2008. Permanova+ for Primer: Guide to Software and Statisticl Methods.

Anestis, A., Lazou, A., Pörtner, H.O., Michaelidis, B., 2007. Behavioral, metabolic, and molecular stress responses of marine bivalve Mytilus galloprovincialis during long-term acclimation at increasing ambient temperature. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 293, R911–R921.

Angilletta, M.J., 2009. Thermal Adaptation: A Theoretical and Empirical Synthesis. OUP Oxford.

An, M.I., Choi, C.Y., 2010. Activity of antioxidant enzymes and physiological responses in ark shell, Scapharca broughtonii, exposed to thermal and osmotic stress: effects on hemolymph and biochemical parameters. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 155, 34–42.

Annamaria, V.G., Chiara, L., Alessandra, A.N., Alvise, B., His, E., Francesco, G.P., 2005. Mytilus galloprovincialis as bioindicator in embryotoxicity testing to evaluate sediment quality in the lagoon of Venice (Italy). Chemistry and Ecology 21, 455–463. https://doi.org/10.1080/02757540500438516

Antonov, J.I., Levitus, S., Boyer, T.P., n.d. Steric sea level variations during 1957–1994: Importance of salinity. Journal of Geophysical Research: Oceans 107, SRF 14–1–SRF 14–8. https://doi.org/10.1029/2001JC000964

Aposhian, H., Zakharyan, R.A., Avram, M.D., Sampayo-Reyes, A., Wollenberg, M.L., 2004. A review of the enzymology of arsenic metabolism and a new potential role of hydrogen peroxide in the detoxication of the trivalent arsenic species. Toxicology and Applied Pharmacology, Arsenic in Biology and Medicine 198, 327–335. https://doi.org/10.1016/j.taap.2003.10.027

Applebaum, S.L., Pan, T.-C.F., Hedgecock, D., Manahan, D.T., 2014. Separating the Nature and Nurture of the Allocation of Energy in Response to Global Change. Integrative and Comparative Biology 54, 284–295. https://doi.org/10.1093/icb/icu062

Aretxabaleta, A.L., Smith, K.W., Kalra, T.S., 2017. Regime Changes in Global Sea Surface Salinity Trend. Journal of Marine Science and Engineering 5, 57. https://doi.org/10.3390/jmse5040057

Artigaud, S., Lacroix, C., Richard, J., Flye-Sainte-Marie, J., Bargelloni, L., Pichereau, V., 2015. Proteomic responses to hypoxia at different temperatures in the great scallop (Pecten maximus). PeerJ 3, e871. https://doi.org/10.7717/peerj.871

ASTM (2004). Standard guide for conducting static acute toxicity tests starting with embryos of four species of saltwater bivalve molluscs E724. West Conshohocken: American Society for Testing and Materials.

Bagnyukova, T.V., Luzhna, L.I., Pogribny, I.P., Lushchak, V.I., 2007. Oxidative stress and antioxidant defenses in goldfish liver in response to short-term exposure to arsenite. Environmental and Molecular Mutagenesis. 48, 658–665. https://doi.org/10.1002/em.20328

Båmstedt, U., 1980. ETS activity as an estimator of respiratory rate of zooplankton populations. The significance of variations in environmental factors. Journal of Experimental Marine Biology and Ecology 42, 267–283. https://doi.org/10.1016/0022-0981(80)90181-1

Bao, Y., Liu, X., Zhang, W., Cao, J., Li, W., Li, C., Lin, Z., 2016. Identification of a regulation network in response to cadmium toxicity using blood clam *Tegillarca granosa* as model. Scientific Reports 6, 35704. https://doi.org/10.1038/srep35704

Barlowe, C., 2009. Atlasin GTPases Shape Up ER Networks. Developmental Cell 17, 157–158. https://doi.org/10.1016/j.devcel.2009.07.019

Batista, F.M., López-Sanmartín, M., Grade, A., Morgado, I., Valente, M., Navas, J.I., Power, D.M., Ruano, F., 2015. Sequence variation in ostreid herpesvirus 1 microvar isolates detected in dying and asymptomatic Crassostrea angulata adults in the Iberian Peninsula: Insights into viral origin and spread. Aquaculture 435, 43–51. https://doi.org/10.1016/j.aquaculture.2014.09.016

Bayne, B.L., 2017. Biology of Oysters. Academic Press.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry 44, 276–287. https://doi.org/10.1016/0003-2697(71)90370-8

Beck, M.W., Brumbaugh, R.D., Airoldi, L., Carranza, A., Coen, L., Crawford, C., Defeo, O., J Edgar, G., Hancock, B., Kay, M., Lenihan, H., Luckenbach, M., L Toropova, C., Zhang, G., 2009. Shellfish Reefs at Risk: A Global Analysis of Problems and Solutions.

Beck, M.W., Brumbaugh, R.D., Airoldi, L., Carranza, A., Coen, L.D., Crawford, C., Defeo, O., Edgar, G.J., Hancock, B., Kay, M.C., Lenihan, H.S., Luckenbach, M.W., Toropova, C.L., Zhang, G., Guo, X., 2011. Oyster Reefs at Risk and Recommendations for Conservation, Restoration, and Management. BioScience 61, 107–116. https://doi.org/10.1525/bio.2011.61.2.5

Beiras, R., Albentosa, M., 2004. Inhibition of embryo development of the commercial bivalves Ruditapes decussatus and Mytilus galloprovincialis by trace metals; implications for the implementation of seawater quality criteria. Aquaculture 230, 205–213. https://doi.org/10.1016/S0044-8486(03)00432-0

Beiras, R., His, E., 1994. Effects of dissolved mercury on embryogenesis, survival, growth and metamorphosis of Crassostrea gigas oyster larvae. Marine Ecology Progress Series 113, 95–103. https://doi.org/10.3354/meps113095

Beniash, E., Ivanina, A., Lieb, N.S., Kurochkin, I., Sokolova, I.M., 2010. Elevated level of carbon dioxide affects metabolism and shell formation in oysters Crassostrea virginica. Marine Ecology Progress Series 419, 95–108.

Berridge, M.V., Herst, P.M., Tan, A.S., 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnology annual review 11, 127–152.

Beukema, J.J., 1997. Caloric values of marine invertebrates with an emphasis on the soft parts of marine bivalves. Oceanography and Marine Biology: An Annual Review.

Bianchini, A., Gilles, R., 2000. Is the digestive tract an important access route for mercury in the Chinese crab Eriocheir sinensis (Crustacea, Decapoda)? Bulletin of environmental contamination and toxicology 64, 412–417.

Bielen, A., Bošnjak, I., Sepčić, K., Jaklič, M., Cvitanić, M., Lušić, J., Lajtner, J., Simčič, T., Hudina, S., 2016. Differences in tolerance to anthropogenic stress between invasive and native bivalves. Science of The Total Environment 543, 449–459.

Bindoli, A., Fukuto, J.M., Forman, H.J., 2008. Thiol Chemistry in Peroxidase Catalysis and Redox Signaling. Antioxid Redox Signal 10, 1549–1564. https://doi.org/10.1089/ars.2008.2063

Birkenmeier, G., Stegemann, C., Hoffmann, R., Günther, R., Huse, K., Birkemeyer, C., 2010. Posttranslational Modification of Human Glyoxalase 1 Indicates Redox-Dependent Regulation. PLOS ONE 5, e10399. https://doi.org/10.1371/journal.pone.0010399

Bota, D.A., Van Remmen, H., Davies, K.J.A., 2002. Modulation of Lon protease activity and aconitase turnover during aging and oxidative stress. FEBS Letters 532, 103–106. https://doi.org/10.1016/S0014-5793(02)03638-4

Bouchon-Brandely, M., 1882. On the sexuality of the common oyster (Ostrea edulis) and that of the Portuguese oyster (O. angulata). Artificial fecundation of the Portuguese oyster. Annals and Magazine of Natural History 10, 328–330. https://doi.org/10.1080/00222938209459717

Boukadida, K., Banni, M., Gourves, P.-Y., Cachot, J., 2016. High sensitivity of embryo-larval stage of the Mediterranean mussel, Mytilus galloprovincialis to metal pollution in combination with temperature increase. Marine Environmental Research 122, 59–66. https://doi.org/10.1016/j.marenvres.2016.09.007

Boyer, T.P., Levitus, S., Antonov, J.I., Locarnini, R.A., Garcia, H.E., n.d. Linear trends in salinity for the World Ocean, 1955–1998. Geophysical Research Letters 32. https://doi.org/10.1029/2004GL021791

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72, 248–254.

Brander, K.M., 2007. Global fish production and climate change. PNAS 104, 19709–19714. https://doi.org/10.1073/pnas.0702059104

Brierley, A.S., Kingsford, M.J., 2009. Impacts of Climate Change on Marine Organisms and Ecosystems. Current Biology 19, R602–R614. https://doi.org/10.1016/j.cub.2009.05.046

Bromirski, P.D., Flick, R.E., Cayan, D.R., 2003. Storminess Variability along the California Coast: 1858–2000. J. Climate 16, 982–993. https://doi.org/10.1175/1520-0442(2003)016<0982:SVATCC>2.0.CO;2

Brooks, D.R., Isaac, R.E., 2004. Nematode Aminopeptidases, in: Aminopeptidases in Biology and Disease, Proteases in Biology and Disease. Springer, Boston, MA, pp. 309–329.

Brooks, S.P.J., Storey, K.B., 1997. Glycolytic controls in estivation and anoxia: A comparison of metabolic arrest in land and marine molluscs. Comparative Biochemistry and Physiology Part A: Physiology 118, 1103–1114. https://doi.org/10.1016/S0300-9629(97)00237-5

Buege, J.A., Aust, S.D., 1978. [30] Microsomal lipid peroxidation. Methods in Enzymology, Biomembranes - Part C: Biological Oxidations 52, 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6

Buestel, D., Ropert, M., Prou, J., Goulletquer, P., 2009. History, status, and future of oyster culture in France. Journal of Shellfish Research 28, 813–820.

Byrne, M., 2012. Global change ecotoxicology: Identification of early life history bottlenecks in marine invertebrates, variable species responses and variable experimental approaches. Marine Environmental Research, Emerging and persistent impacts on Marine Organisms: Detection methods and action mechanisms 76, 3–15. https://doi.org/10.1016/j.marenvres.2011.10.004

Byrne, M., 2011. Impact of ocean warming and ocean acidification on marine invertebrate life history stages, in: Oceanography and Marine Biology, Oceanography and Marine Biology - An Annual Review. CRC Press.

Cai, W.-J., 2011. Estuarine and coastal ocean carbon paradox: CO2 sinks or sites of terrestrial carbon incineration? Ann Rev Mar Sci 3, 123–145. https://doi.org/10.1146/annurev-marine-120709-142723

Calabrese, A., Collier, R.S., Nelson, D.A., MacInnes, J.R., 1973. The toxicity of heavy metals to embryos of the American oyster Crassostrea virginica. Marine Biology 18, 162–166. https://doi.org/10.1007/BF00367984

Caldeira, K., Wickett, M.E., 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. Journal of Geophysical Research: Oceans 110.

Carballeira, C., Martín-Díaz, L., DelValls, T.A., 2011. Influence of salinity on fertilization and larval development toxicity tests with two species of sea urchin. Marine Environmental Research 72, 196–203. https://doi.org/10.1016/j.marenvres.2011.08.008

Carlson, P., Van Beneden, R.J., 2014. Arsenic exposure alters expression of cell cycle and lipid metabolism genes in the liver of adult zebrafish (Danio rerio). Aquatic Toxicology, Proceedings from the 17th International Symposium on Pollutant Responses in Marine Organisms (PRIMO17) 153, 66–72. https://doi.org/10.1016/j.aquatox.2013.10.006

Carrasco, M.F., Barón, P.J., 2009. Analysis of the potential geographic range of the Pacific oyster Crassostrea gigas (Thunberg, 1793) based on surface seawater temperature satellite data and climate charts: the coast of South America as a study case. Biological Invasions 12, 2597–2607. https://doi.org/10.1007/s10530-009-9668-0

Carregosa, V., Velez, C., Soares, A.M.V.M., Figueira, E., Freitas, R., 2014. Physiological and biochemical responses of three Veneridae clams exposed to salinity changes. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 177–178, 1–9. https://doi.org/10.1016/j.cbpb.2014.08.001

Cherkasov, A.A., Overton, R.A., Sokolov, E.P., Sokolova, I.M., 2007. Temperature-dependent effects of cadmium and purine nucleotides on mitochondrial aconitase from a marine ectotherm, Crassostrea virginica: a role of temperature in oxidative stress and allosteric enzyme regulation. Journal of Experimental Biology 210, 46–55. https://doi.org/10.1242/jeb.02589

Cheung, W.W.L., Lam, V.W.Y., Sarmiento, J.L., Kearney, K., Watson, R., Pauly, D., 2009. Projecting global marine biodiversity impacts under climate change scenarios. Fish and Fisheries 10, 235–251. https://doi.org/10.1111/j.1467-2979.2008.00315.x

Coen, L.D., Brumbaugh, R.D., Bushek, D., Grizzle, R., Luckenbach, M.W., Posey, M.H., Powers, S.P., Tolley, S.G., 2007. Ecosystem services related to oyster restoration. Marine Ecology Progress Series 341, 303–307. https://doi.org/10.3354/meps341303

Coglianese, M.P., 1982. The effects of salinity on copper and silver toxicity to embryos of the Pacific Oyster. Archives of Environmental Contamination and Toxicology. 11, 297–303. https://doi.org/10.1007/BF01055206

Comps, M., 1988. Epizootic diseases of oysters associated with viral infections. American Fisheries Society Special Publication 18, 23–37.

Connell, D.W., 1989. Bioaccumulation of xenobiotic compounds. CRC Press.

Coppola, F., Almeida, Â., Henriques, B., Soares, A.M.V.M., Figueira, E., Pereira, E., Freitas, R., 2018. Biochemical responses and accumulation patterns of Mytilus galloprovincialis exposed to thermal stress and Arsenic contamination. Ecotoxicology and Environmental Safety. 147, 954–962. https://doi.org/10.1016/j.ecoenv.2017.09.051

Costa, P.M., Lobo, J., Caeiro, S., Martins, M., Ferreira, A.M., Caetano, M., Vale, C., DelValls, T.Á., Costa, M.H., 2008. Genotoxic damage in Solea senegalensis exposed to sediments from the Sado Estuary (Portugal): Effects of metallic and organic contaminants. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 654, 29–37. https://doi.org/10.1016/j.mrgentox.2008.04.007

Cranfield, H.J., Michael, K.P., Doonan, I.J., 1999. Changes in the distribution of epifaunal reefs and oysters during 130 years of dredging for oysters in Foveaux Strait, southern New Zealand. Aquatic Conservation: Marine and Freshwater Ecosystems. 9, 461–483. https://doi.org/10.1002/(SICI)1099-0755(199909/10)9:5<461::AID-AQC353>3.0.CO;2-Z

Crockett, E.L., 2008. The cold but not hard fats in ectotherms: consequences of lipid restructuring on susceptibility of biological membranes to peroxidation, a review. Journal of Comparative Physiology B. 178, 795–809. https://doi.org/10.1007/s00360-008-0275-7

Cross, I., Merlo, M.A., Rodríguez, M.E., Portela-Bens, S., Rebordinos, L., 2014. Adaptation to abiotic stress in the oyster Crassostrea angulata relays on genetic polymorphisms. Fish & Shellfish Immunology 41, 618–624. https://doi.org/10.1016/j.fsi.2014.10.011

Dailianis, S., Patetsini, E., Kaloyianni, M., 2009. The role of signalling molecules on actin glutathionylation and protein carbonylation induced by cadmium in haemocytes of mussel Mytilus galloprovincialis (Lmk). Journal of Experimental Biology 212, 3612–3620. https://doi.org/10.1242/jeb.030817

Dalle-Donne, I., Rossi, R., Colombo, G., Giustarini, D., Milzani, A., 2009. Protein S-glutathionylation: a regulatory device from bacteria to humans. Trends in Biochemical Sciences. 34, 85–96. https://doi.org/10.1016/j.tibs.2008.11.002

David, E., Tanguy, A., Pichavant, K., Moraga, D., 2005. Response of the Pacific oyster Crassostrea gigas to hypoxia exposure under experimental conditions. FEBS Journal 272, 5635–5652. https://doi.org/10.1111/j.1742-4658.2005.04960.x

Davies, K.J.A., 2000. Oxidative Stress, Antioxidant Defenses, and Damage Removal, Repair, and Replacement Systems. IUBMB Life 50, 279–289. https://doi.org/10.1080/713803728

de Almeida, E.A., Bainy, A.C.D., de Melo Loureiro, A.P., Martinez, G.R., Miyamoto, S., Onuki, J., Barbosa, L.F., Garcia, C.C.M., Prado, F.M., Ronsein, G.E., others, 2007. Oxidative stress in Perna perna and other bivalves as indicators of environmental stress in the Brazilian marine environment: antioxidants, lipid peroxidation and DNA damage. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 146, 588–600.

De Coen, W.M., Janssen, C.R., 1997. The use of biomarkers in Daphnia magna toxicity testing. IV. Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. Journal of Aquatic Ecosystem Stress and Recovery 6, 43–55.

Dekshenieks, M.M., Hofmann, E.E., Klinck, J.M., Powell, E.N., 2000. Quantifying the effects of environmental change on an oyster population: A modeling study. Estuaries 23, 593. https://doi.org/10.2307/1352887

Deslous-Paoli, J.-M., Héral, M., 1988. Biochemical composition and energy value of Crassostrea gigas (Thunberg) cultured in the bay of Marennes-Oléron. Aquatic Living Resources 1, 239–249.

de Zwart, L.L., Meerman, J.H., Commandeur, J.N., Vermeulen, N.P., 1999. Biomarkers of free radical damage applications in experimental animals and in humans. Free Radical Biology and Medicine. 26, 202–226.

Dickinson, G.H., Ivanina, A.V., Matoo, O.B., Pörtner, H.O., Lannig, G., Bock, C., Beniash, E., Sokolova, I.M., 2012. Interactive effects of salinity and elevated CO2 levels on juvenile eastern oysters, Crassostrea virginica. Journal of Experimental Biology. 215, 29–43. https://doi.org/10.1242/jeb.061481

Dickson, A.G., 1990. Standard potential of the reaction: AgCl(s) + 12H2(g) = Ag(s) + HCl(aq), and and the standard acidity constant of the ion HSO4− in synthetic sea water from 273.15 to 318.15 K. The Journal of Chemical Thermodynamics 22, 113–127. https://doi.org/10.1016/0021-9614(90)90074-Z

Dickson, A.G., Millero, F.J., 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Research Part A. Oceanographic Research Papers 34, 1733–1743. https://doi.org/10.1016/0198-0149(87)90021-5

Diederich, S., Nehls, G., van Beusekom, J.E.E., Reise, K., 2005. Introduced Pacific oysters (Crassostrea gigas) in the northern Wadden Sea: invasion accelerated by warm summers? Helgoland Marine Research 59, 97–106. https://doi.org/10.1007/s10152-004-0195-1

Dimitriadis, V.K., Gougoula, C., Anestis, A., Pörtner, H.O., Michaelidis, B., 2012. Monitoring the biochemical and cellular responses of marine bivalves during thermal stress by using biomarkers. Marine Environmental Research 73, 70–77. https://doi.org/10.1016/j.marenvres.2011.11.004

Dineshram, R., Q., Q., Sharma, R., Chandramouli, K., Yalamanchili, H.K., Chu, I., Thiyagarajan, V., 2015. Comparative and quantitative proteomics reveal the adaptive strategies of oyster larvae to ocean acidification. Proteomics 15, 4120–4134. https://doi.org/10.1002/pmic.201500198

Domínguez-Pérez, D., Campos, A., Alexei Rodríguez, A., Turkina, M.V., Ribeiro, T., Osorio, H., Vasconcelos, V., Antunes, A., 2018. Proteomic Analyses of the Unexplored Sea Anemone Bunodactis verrucosa. Marine Drugs 16. https://doi.org/10.3390/md16020042

Doney, S.C., Ruckelshaus, M., Emmett Duffy, J., Barry, J.P., Chan, F., English, C.A., Galindo, H.M., Grebmeier, J.M., Hollowed, A.B., Knowlton, N., Polovina, J., Rabalais, N.N., Sydeman, W.J., Talley, L.D., 2012. Climate Change Impacts on Marine Ecosystems. Annual Review of Marine Science 4, 11–37. https://doi.org/10.1146/annurev-marine-041911-111611

Dove, M.C., O'connor, W.A., 2007. Salinity and temperature tolerance of sydney rock oysters saccostrea glomerata during early ontogeny. Journal of Shellfish Research 26, 939–947. https://doi.org/10.2983/0730-8000(2007)26[939:SATTOS]2.0.CO;2

Duarte, G., Calderon, E.N., Pereira, C.M., Marangoni, L.F.B., Santos, H.F., Peixoto, R.S., Bianchini, A., Castro, C.B., 2015. A novel marine mesocosm facility to study global warming, water quality, and ocean acidification. Ecology and Evolution. 5, 4555–4566. https://doi.org/10.1002/ece3.1670

Eierman, L.E., Hare, M.P., 2014. Transcriptomic analysis of candidate osmoregulatory genes in the eastern oyster Crassostrea virginica. BMC Genomics 15. https://doi.org/10.1186/1471-2164-15-503

Ereira, T., Coelho, J.P., Duarte, A.C., Pardal, M.A., Pereira, M.E., 2015. Size-Dependent Arsenic Accumulation in Scrobicularia plana in a Temperate Coastal Lagoon (Ria de Aveiro, Portugal). Water Air Soil Pollut 226, 1–7. https://doi.org/10.1007/s11270-015-2484-5

Erickson, R.J., Mount, D.R., Highland, T.L., Russell Hockett, J., Jenson, C.T., 2011. The relative importance of waterborne and dietborne arsenic exposure on survival and growth of juvenile rainbow trout. Aquatic Toxicology. 104, 108–115. https://doi.org/10.1016/j.aquatox.2011.04.003

Ettajani, H., Amiard-Triquet, C., Jeantet, A.Y., Amiard, J.C., 1996. Fate and effects of soluble or sediment-bound arsenic in oysters (Crassostrea gigas Thun.). Archives of environmental contamination and toxicology 31, 38–46.

Fabbri, R., Montagna, M., Balbi, T., Raffo, E., Palumbo, F., Canesi, L., 2014. Adaptation of the bivalve embryotoxicity assay for the high throughput screening of emerging contaminants in Mytilus galloprovincialis. Mar. Environ. Res. 99, 1–8. https://doi.org/10.1016/j.marenvres.2014.05.007

Fabioux, C., Huvet, A., Lapegue, S., Heurtebise, S., Boudry, P., 2002. Past and present geographical distribution of populations of Portuguese (Crassostrea angulata) and Pacific (C. gigas) oysters along the European and north African Atlantic coats. Haliotis 31, 33–44.

Fabry, V.J., Seibel, B.A., Feely, R.A., Orr, J.C., 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. ICES Journal of Marine Sciences. 65, 414–432. https://doi.org/10.1093/icesjms/fsn048

Food and Agriculture Organization (FAO) , 2015. World aquaculture production of fish, crustaceans, molluscs, etc., by principal species in 2013. Available at: fao.org/fishery/docs/STAT/summary/a-6.pdf

Fanslow, D.L., Nalepa, T.F., Johengen, T.H., 2001. Seasonal changes in the respiratory electron transport system (ETS) and respiration of the zebra mussel, Dreissena polymorpha in Saginaw Bay, Lake Huron. Hydrobiologia 448, 61–70. https://doi.org/10.1023/A:1017582119098

Farhan, H., Hauri, H.-P., 2009. Membrane Biogenesis: Networking at the ER with Atlastin. Current Biology 19, R906–R908. https://doi.org/10.1016/j.cub.2009.08.029

Fattorini, D., Notti, A., Regoli, F., 2006. Characterization of arsenic content in marine organisms from temperate, tropical, and polar environments. Chemistry and Ecology 22, 405–414. https://doi.org/10.1080/02757540600917328

Fattorini, D., Regoli, F., 2004. Arsenic speciation in tissues of the Mediterranean polychaete Sabella spallanzanii. Environmental Toxicology and Chemistry. 23, 1881–1887.

Feely, R.A., Alin, S.R., Newton, J., Sabine, C.L., Warner, M., Devol, A., Krembs, C., Maloy, C., 2010. The combined effects of ocean acidification, mixing, and respiration on pH and carbonate saturation in an urbanized estuary. Estuarine, Coastal and Shelf Science 88, 442–449. https://doi.org/10.1016/j.ecss.2010.05.004

Flores-Vergara, C., Cordero-Esquivel, B., Cerón-Ortiz, A.N., Arredondo-Vega, B.O., 2004. Combined effects of temperature and diet on growth and biochemical composition of the Pacific oyster Crassostrea gigas (Thunberg) spat. Aquaculture Research 35, 1131–1140. https://doi.org/10.1111/j.1365-2109.2004.01136.x

Fodrie, F.J., Rodriguez, A.B., Gittman, R.K., Grabowski, J.H., Lindquist, N.L., Peterson, C.H., Piehler, M.F., Ridge, J.T., 2017. Oyster reefs as carbon sources and sinks. Procedings of the Royal Society B. 284, 20170891. https://doi.org/10.1098/rspb.2017.0891

Foighil, D.Ó., Gaffney, P.M., Wilbur, A.E., Hilbish, T.J., 1998. Mitochondrial cytochrome oxidase I gene sequences support an Asian origin for the Portuguese oyster Crassostrea angulata. Marine Biology 131, 497–503. https://doi.org/10.1007/s002270050341

Fokina, N.N., Nemova, T.R.R. and N.N., 2017. Lipid Composition Modifications in the Blue Mussels (Mytilus edulis L.) from the White Sea. Organismal and Molecular Malacology. https://doi.org/10.5772/67811\.

Fortunato, A.E., Sordino, P., Andreakis, N., 2016. Evolution of the SOUL Heme-Binding Protein Superfamily Across Eukarya. Journal of Molecular Evolution. 82, 279–290. https://doi.org/10.1007/s00239-016-9745-9

Freitas, R., Almeida, Â., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2016a. The impacts of pharmaceutical drugs under ocean acidification: New data on single and combined long-term effects of carbamazepine on Scrobicularia plana. Science of The Total Environment 541, 977–985. https://doi.org/10.1016/j.scitotenv.2015.09.138

Freitas, R., De Marchi, L., Bastos, M., Moreira, A., Velez, C., Chiesa, S., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2017. Effects of seawater acidification and salinity alterations on metabolic, osmoregulation and oxidative stress markers in Mytilus galloprovincialis. Ecological Indicators 79, 54–62. https://doi.org/10.1016/j.ecolind.2017.04.003

Freitas, R., Martins, R., Antunes, S., Velez, C., Moreira, A., Cardoso, P., Pires, A., Soares, A.M., Figueira, E., 2014. Venerupis decussata under environmentally relevant lead concentrations: bioconcentration, tolerance, and biochemical alterations. Environmental toxicology and chemistry 33, 2786–2794.

Freitas, R., Pires, A., Quintino, V., Rodrigues, A.M., Figueira, E., 2012. Subcellular partitioning of elements and availability for trophic transfer: Comparison between the Bivalve Cerastoderma edule and the Polychaete Diopatra neapolitana. Estuarine, Coastal and Shelf Science 99, 21–30. https://doi.org/10.1016/j.ecss.2011.11.039

Freitas, R., Salamanca, L., Velez, C., Wrona, F.J., Soares, A.M.V.M., Figueira, E., 2016b. Multiple stressors in estuarine waters: Effects of arsenic and salinity on Ruditapes philippinarum. Science of The Total Environment 541, 1106–1114. https://doi.org/10.1016/j.scitotenv.2015.09.149

Fuhrmann, M., Delisle, L., Petton, B., Corporeau, C., Pernet, F., 2018. Metabolism of the Pacific oyster, Crassostrea gigas, is influenced by salinity and modulates survival to the Ostreid herpesvirus OsHV-1. Biology Open 7, bio028134. https://doi.org/10.1242/bio.028134

Gagnaire, B., Frouin, H., Moreau, K., Thomas-Guyon, H., Renault, T., 2006. Effects of temperature and salinity on haemocyte activities of the Pacific oyster, Crassostrea gigas (Thunberg). Fish & Shellfish Immunology 20, 536–547. https://doi.org/10.1016/j.fsi.2005.07.003

Galvão, M.S.N., Pereira, O.M., Hilsdorf, A.W.S., 2013. Molecular identification and distribution of mangrove oysters (Crassostrea) in an estuarine ecosystem in Southeast Brazil: implications for aquaculture and fisheries management. Aquacic Research. 44, 1589–1601. https://doi.org/10.1111/j.1365-2109.2012.03166.x

Gamain, P., Gonzalez, P., Cachot, J., Clérandeau, C., Mazzella, N., Gourves, P.Y., Morin, B., 2017. Combined effects of temperature and copper and S-metolachlor on embryo-larval development of the Pacific oyster, Crassostrea gigas. Marine pollution bulletin 115, 201–210.

Gamain, P., Gonzalez, P., Cachot, J., Pardon, P., Tapie, N., Gourves, P.Y., Budzinski, H., Morin, B., 2016. Combined effects of pollutants and salinity on embryo-larval development of the Pacific oyster, Crassostrea gigas. Marine Environmental Research 113, 31–38. https://doi.org/10.1016/j.marenvres.2015.11.002

Garcia, J., Han, D., Sancheti, H., Yap, L.-P., Kaplowitz, N., Cadenas, E., 2010. Regulation of Mitochondrial Glutathione Redox Status and Protein Glutathionylation by Respiratory Substrates. Journal of Biological Chemistry. 285, 39646–39654. https://doi.org/10.1074/jbc.M110.164160

García-Esquivel, Z., Bricelj, V.M., González-Gómez, M.A., 2001. Physiological basis for energy demands and early postlarval mortality in the Pacific oyster, Crassostrea gigas. Journal of Experimental Marine Biology and Ecology 263, 77–103.

Garrabou, J., Coma, R., Bensoussan, N., Bally, M., Chevaldonné, P., Cigliano, M., Diaz, D., Harmelin, J.G., Gambi, M.C., Kersting, D.K., Ledoux, J.B., Lejeusne, C., Linares, C., Marschal, C., Pérez, T., Ribes, M., Romano, J.C., Serrano, E., Teixido, N., Torrents, O., Zabala, M., Zuberer, F., Cerrano, C., 2009. Mass mortality in Northwestern Mediterranean rocky benthic communities: effects of the 2003 heat wave. Global Change Biology 15, 1090–1103. https://doi.org/10.1111/j.1365-2486.2008.01823.x

Gazeau, F., Parker, L.M., Comeau, S., Gattuso, J.-P., O'Connor, W.A., Martin, S., Pörtner, H.-O., Ross, P.M., 2013. Impacts of ocean acidification on marine shelled molluscs. Marine Biology. 160, 2207–2245. https://doi.org/10.1007/s00227-013-2219-3

Geffard, O., Budzinski, H., Augagneur, S., Seaman, M.N.L., His, E., 2001. Assessment of sediment contamination by spermiotoxicity and embryotoxicity bioassays with sea urchins (Paracentrotus lividus) and oysters (Crassostrea gigas). Environmental Toxicology and Chemistry 20, 1605–1611. https://doi.org/10.1002/etc.5620200727

Géret, F., Jouan, A., Turpin, V., Bebianno, M.J., Cosson, R.P., 2002. Influence of metal exposure on metallothionein synthesis and lipid peroxidation in two bivalve mollusks: the oyster (Crassostrea gigas) and the mussel (Mytilus edulis). Aquatic Living Resources 15, 61–66. https://doi.org/10.1016/S0990-7440(01)01147-0

Ghezzi, P., Di Simplicio, P., 2009. Protein Glutathiolation, in: Junioressor, J.-P.C.J., Winyardessor, P.G. (Eds.), Redox Signaling and Regulation in Biology and Medicine. Wiley-VCH Verlag GmbH & Co. KGaA, pp. 123–141.

Gibbin, E.M., Chakravarti, L.J., Jarrold, M.D., Christen, F., Turpin, V., N'Siala, G.M., Blier, P.U., Calosi, P., 2017. Can multi-generational exposure to ocean warming and acidification lead to the adaptation of life history and physiology in a marine metazoan? Journal of Experimental Biology 220, 551–563. https://doi.org/10.1242/jeb.149989

Gomes, C., Silva, F.C., Lopes, G.R., Melo, C.M.R., 2014. The reproductive cycle of the oyster Crassostrea gasar. Brazilian Journal of Biology 74, 967–976.

Görlach, A., Klappa, P., Kietzmann, T., 2006. The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. Antioxiddants and Redox Signaling. 8, 1391–1418. https://doi.org/10.1089/ars.2006.8.1391

Gosling, E., 2008. Bivalve Molluscs: Biology, Ecology and Culture. John Wiley & Sons.

Götze, S., Matoo, O.B., Beniash, E., Saborowski, R., Sokolova, I.M., 2014. Interactive effects of CO2 and trace metals on the proteasome activity and cellular stress response of marine bivalves Crassostrea virginica and Mercenaria mercenaria. Aquatic Toxicology 149, 65–82. https://doi.org/10.1016/j.aquatox.2014.01.027

Grabowski, J.H., Brumbaugh, R.D., Conrad, R.F., Keeler, A.G., Opaluch, J.J., Peterson, C.H., Piehler, M.F., Powers, S.P., Smyth, A.R., 2012. Economic Valuation of Ecosystem Services Provided by Oyster Reefs. BioScience 62, 900–909. https://doi.org/10.1525/bio.2012.62.10.10

Grade, A., Chairi, H., Lallias, D., Power, D.M., Ruano, F., Leitão, A., Drago, T., King, J.W., Boudry, P., Batista, F.M., 2016. New insights about the introduction of the Portuguese oyster, Crassostrea angulata, into the North East Atlantic from Asia based on a highly polymorphic mitochondrial region. Aquatic Living Resources. 29, 404. https://doi.org/10.1051/alr/2016035

Gran, G., 1952. Determination of the equivalence point in potentiometric titrations. Part II. Analyst 77, 661–671. https://doi.org/10.1039/AN9527700661

Greco, L., Pellerin, J., Capri, E., Garnerot, F., Louis, S., Fournier, M., Sacchi, A., Fusi, M., Lapointe, D., Couture, P., 2011. Physiological effects of temperature and a herbicide mixture on the soft-shell clam Mya arenaria (Mollusca, Bivalvia). Environ. Toxicol. Chem. 30, 132–141. https://doi.org/10.1002/etc.359

Grosell, M., Blanchard, J., Brix, K.V., Gerdes, R., 2007. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. Aquatic toxicology 84, 162–172.

Guo, X., 2009. Use and exchange of genetic resources in molluscan aquaculture. Reviews in Aquaculture 1, 251–259. https://doi.org/10.1111/j.1753-5131.2009.01014.x

Guppy, M., Withers, P., 1999. Metabolic depression in animals: physiological perspectives and biochemical generalizations. Biological Reviews of the Cambridge Philosophical Society. 74, 1–40.

Gutierrez-Mazariegos, J., Schubert, M., Laudet, V., 2014. Evolution of retinoic acid receptors and retinoic acid signaling, in: The Biochemistry of Retinoic Acid Receptors I: Structure, Activation, and Function at the Molecular Level. Springer, pp. 55–73.

Guzy, R.D., Schumacker, P.T., 2006. Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. Experimental physiology 91, 807–819.

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.

Halliwell, B., Gutteridge, J.M.C., 2011. Free radicals in biology and medicine. NY: Oxford University Press.–1999.–968 p.

Hamer, B., Jakšić, Ž., Pavičić-Hamer, D., Perić, L., Medaković, D., Ivanković, D., Pavičić, J., Zilberberg, C., Schröder, H.C., Müller, W.E., others, 2008. Effect of hypoosmotic stress by low salinity acclimation of Mediterranean mussels Mytilus galloprovincialis on biological parameters used for pollution assessment. Aquatic Toxicology 89, 137–151.

Han, D., Hanawa, N., Saberi, B., Kaplowitz, N., 2006. Mechanisms of Liver Injury. III. Role of glutathione redox status in liver injury. American Journal of Physiology - Gastrointestinal and Liver Physiology 291, G1–G7. https://doi.org/10.1152/ajpgi.00001.2006

Hannam, M.L., Bamber, S.D., Moody, A.J., Galloway, T.S., Jones, M.B., 2010. Immunotoxicity and oxidative stress in the Arctic scallop Chlamys islandica: effects of acute oil exposure. Ecotoxicology and Environmental Safety. 73, 1440–1448. https://doi.org/10.1016/j.ecoenv.2010.06.012

Hansen, J., Kharecha, P., Sato, M., Masson-Delmotte, V., Ackerman, F., Beerling, D.J., Hearty, P.J., Hoegh-Guldberg, O., Hsu, S.-L., Parmesan, C., Rockstrom, J., Rohling, E.J., Sachs, J., Smith, P., Steffen, K., Susteren, L.V., Schuckmann, K. von, Zachos, J.C., 2013. Assessing "Dangerous Climate Change": Required Reduction of Carbon Emissions to Protect Young People, Future Generations and Nature. PLOS ONE 8, e81648. https://doi.org/10.1371/journal.pone.0081648

Harley, C.D.G., Randall Hughes, A., Hultgren, K.M., Miner, B.G., Sorte, C.J.B., Thornber, C.S., Rodriguez, L.F., Tomanek, L., Williams, S.L., 2006. The impacts of climate change in coastal marine systems. Ecology Letters 9, 228–241. https://doi.org/10.1111/j.1461-0248.2005.00871.x

Harms, L., Frickenhaus, S., Schiffer, M., Mark, F.C., Storch, D., Held, C., Pörtner, H.-O., Lucassen, M., 2014. Gene expression profiling in gills of the great spider crab Hyas araneus in response to ocean acidification and warming. BMC Genomics 15, 789. https://doi.org/10.1186/1471-2164-15-789

Harney, E., Artigaud, S., Le Souchu, P., Miner, P., Corporeau, C., Essid, H., Pichereau, V., Nunes, F.L.D., 2016. Non-additive effects of ocean acidification in combination with warming on the larval proteome of the Pacific oyster, Crassostrea gigas. Journal of Proteomics, Proteomics in Evolutionary Ecology 135, 151–161. https://doi.org/10.1016/j.jprot.2015.12.001

Hawkins, A.J.S., 1995. Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam Mya arenaria. Journal of Thermal Biology, Effects of Rising Temperature on the Ecology and Physiology of Aquatic Organisms 20, 23–33. https://doi.org/10.1016/0306-4565(94)00023-C

Hawkins, B.J., Irrinki, K.M., Mallilankaraman, K., Lien, Y.-C., Wang, Y., Bhanumathy, C.D., Subbiah, R., Ritchie, M.F., Soboloff, J., Baba, Y., Kurosaki, T., Joseph, S.K., Gill, D.L., Madesh, M., 2010. S-glutathionylation activates STIM1 and alters mitochondrial homeostasis. The Journal of Cell Biology jcb.201004152. https://doi.org/10.1083/jcb.201004152

Hazel, J.R., 1995. Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? Annual Review of Physiology. 57, 19–42. https://doi.org/10.1146/annurev.ph.57.030195.000315

Hazel, J.R., Williams, E.E., 1990. The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. Progress in Lipid Research. 29, 167–227.

Heral, M., 1989. Traditional oyster culture in France. In: Barnabe Aquaculture 342–387.

Hering, D., Haidekker, A., Schmidt-Kloiber, A., Barker, T., Buisson, L., Graf, W., Grenouillet, G., Lorenz, A., Sandin, L., Stendera, S., 2010. Monitoring the Responses of Freshwater Ecosystems to Climate Change, in: Kernan, rtin, Battarbee, R.W., Moss, B. (Eds.), Climate Change Impacts on Freshwater Ecosystems. Wiley-Blackwell, pp. 84–118.

He, Y., Jouaux, A., Ford, S.E., Lelong, C., Sourdaine, P., Mathieu, M., Guo, X., 2015. Transcriptome analysis reveals strong and complex antiviral response in a mollusc. Fish & Shellfish Immunology, SI: Molluscan Immunity 46, 131–144. https://doi.org/10.1016/j.fsi.2015.05.023

His, E., Beiras, R., Seaman, M.N.L., 1999. The Assessment of Marine Pollution - Bioassays with Bivalve Embryos and Larvae, in: A.J. Southward, P.A.T. and C.M.Y. (Ed.), Advances in Marine Biology. Academic Press, pp. 1–178.

His, E., Robert, R., Dinet, A., 1989. Combined effects of temperature and salinity on fed and starved larvae of the mediterranean mussel Mytilus galloprovincialis and the Japanese oyster Crassostrea gigas. Marine Biology. 100, 455–463. https://doi.org/10.1007/BF00394822

His, E., Seaman, M.N.L., Beiras, R., 1997. A simplification the bivalve embryogenesis and larval development bioassay method for water quality assessment. Water Research 31, 351–355. https://doi.org/10.1016/S0043-1354(96)00244-8

Hoegh-Guldberg, O., Bruno, J.F., 2010. The Impact of Climate Change on the World's Marine Ecosystems. Science 328, 1523–1528. https://doi.org/10.1126/science.1189930

Hrs-Brenko, M., Claus, C., Bubic, S., 1977. Synergistic effects of lead, salinity and temperature on embryonic development of the mussel Mytilus galloprovincialis. Marine Biology. 44, 109–115. https://doi.org/10.1007/BF00386951

Hsiao, S.-T., Chuang, S.-C., Chen, K.-S., Ho, P.-H., Wu, C.-L., Chen, C.A., 2016. DNA barcoding reveals that the common cupped oyster in Taiwan is the Portuguese oyster *Crassostrea angulata* (Ostreoida; Ostreidae), not *C. gigas*. Scientific Reports 6, 34057. https://doi.org/10.1038/srep34057

Hüning, A.K., Melzner, F., Thomsen, J., Gutowska, M.A., Krämer, L., Frickenhaus, S., Rosenstiel, P., Pörtner, H.-O., Philipp, E.E.R., Lucassen, M., 2013. Impacts of seawater acidification on mantle gene expression patterns of the Baltic Sea blue mussel: implications for shell formation and energy metabolism. Marine Biology. 160, 1845–1861. https://doi.org/10.1007/s00227-012-1930-9

Hurd, T.R., Costa, N.J., Dahm, C.C., Beer, S.M., Brown, S.E., Filipovska, A., Murphy, M.P., 2005. Glutathionylation of Mitochondrial Proteins. Antioxidants & Redox Signaling 7, 999–1010. https://doi.org/10.1089/ars.2005.7.999

Huvet, A., Fabioux, C., McCombie, H., Lapegue, S., Boudry, P., 2004. Natural hybridization between genetically differentiated populations of Crassostrea gigas and C. angulata highlighted by sequence variation in flanking regions of a microsatellite locus. Marine Ecology Progress Series 272, 141–152.

IPCC, 2014: Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

ISO 2015. 17244 Water quality -- Determination of the toxicity of water samples on the embryo-larval development of Japanese oyster (Crassostrea gigas) and mussel (Mytilus edulis or Mytilus galloprovincialis)

Ivanina, A.V., Nesmelova, I., Leamy, L., Sokolov, E.P., Sokolova, I.M., 2016. Intermittent hypoxia leads to functional reorganization of mitochondria and affects cellular bioenergetics in marine molluscs. Journal of Experimental Biology. 219, 1659–1674. https://doi.org/10.1242/jeb.134700

Ivanina, A.V., Sokolova, I.M., 2015. Interactive effects of metal pollution and ocean acidification on physiology of marine organisms. Current Zoology. 61, 653–668. https://doi.org/10.1093/czoolo/61.4.653

Ivanina, A.V., Sokolova, I.M., Sukhotin, A.A., 2008. Oxidative stress and expression of chaperones in aging mollusks. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 150, 53–61. https://doi.org/10.1016/j.cbpb.2008.01.005

Iwasaki, Y.W., Siomi, M.C., Siomi, H., 2015. PIWI-interacting RNA: its biogenesis and functions. Annual review of biochemistry 84, 405–433.

Johansson, L.H., Håkan Borg, L.A., 1988. A spectrophotometric method for determination of catalase activity in small tissue samples. Analytical Biochemistry 174, 331–336. https://doi.org/10.1016/0003-2697(88)90554-4

Kala, S.V., Kala, G., Prater, C.I., Sartorelli, A.C., Lieberman, M.W., 2004. Formation and urinary excretion of arsenic triglutathione and methylarsenic diglutathione. Chemical Research in Toxicology. 17, 243–249. https://doi.org/10.1021/tx0342060

Kashina, A., 2014. Protein arginylation, a global biological regulator that targets actin cytoskeleton and the muscle. Anatatomical Record (Hoboken). 297, 1630–1636. https://doi.org/10.1002/ar.22969

Kasyanov, V.L., others, 2001. Reproductive strategy of marine bivalves and echinoderms. Science Publishers, Inc. ISBN 157808136X.

Khan, B., Ringwood, A.H., 2016. Cellular biomarker responses to hypoxia in eastern oysters and Atlantic ribbed marsh mussels. Marine Ecology Progress Series 546, 123–133. https://doi.org/10.3354/meps11622

Ki, J.-S., Raisuddin, S., Lee, K.-W., Hwang, D.-S., Han, J., Rhee, J.-S., Kim, I.-C., Park, H.G., Ryu, J.-C., Lee, J.-S., 2009. Gene expression profiling of copper-induced responses in the intertidal copepod Tigriopus japonicus using a 6K oligochip microarray. Aquatic Toxicology 93, 177–187. https://doi.org/10.1016/j.aquatox.2009.04.004

King, F.D., Packard, T.T., 1975. Respiration and the activity of the respiratory electron transport system in marine zooplankton1. Limnology and Oceanography. 20, 849–854. https://doi.org/10.4319/lo.1975.20.5.0849

Knezovich, J.P., Harrison, F.L., Tucker, J.S., 1981. The influence of organic chelators on the toxicity of copper to embryos of the pacific oyster,Crassostrea gigas. Archives of Environmental Contamination and. Toxicology. 10, 241–249. https://doi.org/10.1007/BF01055625

Knight, K., 2017. High and dry oysters at most risk from climate change. Journal of Experimental Biology 220, 734–734. https://doi.org/10.1242/jeb.157974

Kobayashi, Y., Cui, X., Hirano, S., 2005. Stability of arsenic metabolites, arsenic triglutathione [As(GS)3] and methylarsenic diglutathione [CH3As(GS)2], in rat bile. Toxicology 211, 115–123. https://doi.org/10.1016/j.tox.2005.03.001

Ko, G.W.K., Dineshram, R., Campanati, C., Chan, V.B.S., Havenhand, J., Thiyagarajan, V., 2014. Interactive Effects of Ocean Acidification, Elevated Temperature, and Reduced Salinity on Early-Life Stages of the Pacific Oyster. Environmental Science and Technology. 48, 10079–10088. https://doi.org/10.1021/es501611u

Kohlmeyer, U., Kuballa, J., Jantzen, E., 2002. Simultaneous separation of 17 inorganic and organic arsenic compounds in marine biota by means of high-performance liquid chromatography/inductively coupled plasma mass spectrometry. Rapid Communication in Mass Spectrometry. 16, 965–974. https://doi.org/10.1002/rcm.671

Krassoi, F.R., Brown, K.R., Bishop, M.J., Kelaher, B.P., Summerhayes, S., 2008. Condition-specific competition allows coexistence of competitively superior exotic oysters with native oysters. Journal of Animal Ecology 77, 5–15. https://doi.org/10.1111/j.1365-2656.2007.01316.x

Kumar, A., Birnbaum, M.D., Patel, D.M., Morgan, W.M., Singh, J., Barrientos, A., Zhang, F., 2016. Posttranslational arginylation enzyme Ate1 affects DNA mutagenesis by regulating stress response. Cell Death and Disease. 7, e2378. https://doi.org/10.1038/cddis.2016.284

Kuo, M.T., Wei, Y., Yang, X., Tatebe, S., Liu, J., Troncoso, P., Sahin, A., Ro, J.Y., Hamilton, S.R., Savaraj, N., 2006. Association of fragile site-associated (FSA) gene expression with epithelial differentiation and tumor development. Biochemical and Biophysical Research Communications 340, 887–893. https://doi.org/10.1016/j.bbrc.2005.12.088

Lannig, G., Flores, J.F., Sokolova, I.M., 2006. Temperature-dependent stress response in oysters, Crassostrea virginica: pollution reduces temperature tolerance in oysters. Aquatic Toxicology. 79, 278–287. https://doi.org/10.1016/j.aquatox.2006.06.017

Lapaille, M., Thiry, M., Perez, E., González-Halphen, D., Remacle, C., Cardol, P., 2010. Loss of mitochondrial ATP synthase subunit beta (Atp2) alters mitochondrial and chloroplastic function and morphology in Chlamydomonas. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1797, 1533–1539. https://doi.org/10.1016/j.bbabio.2010.04.013

Lazoski, C., Gusmao, J., Boudry, P., Sole-Cava, A.M., 2011. Phylogeny and phylogeography of Atlantic oyster species: evolutionary history, limited genetic connectivity and isolation by distance. Marine Ecology-progress Series 426, 197–212. https://doi.org/10.3354/meps09035

Le Dantec, J., 1968. Ecologie et reproduction de l'huitre portugaise (Crassostrea Angulata LAMARCK) dans le bassin d'Arcachon et sur la rive gauche de la Gironde. Revue des Travaux de l'Institut des Pêches Maritimes 32, 237–362.

Leermakers, M., Baeyens, W., De Gieter, M., Smedts, B., Meert, C., De Bisschop, H.C., Morabito, R., Quevauviller, P., 2006. Toxic arsenic compounds in environmental samples: Speciation and validation. TrAC Trends in Analytical Chemistry 25, 1–10. https://doi.org/10.1016/j.trac.2005.06.004

Le Moullac, G., Quéau, I., Le Souchu, P., Pouvreau, S., Moal, J., René Le Coz, J., François Samain, J., 2007. Metabolic adjustments in the oyster Crassostrea gigas according to oxygen level and temperature. Marine Biology Research 3, 357–366.

Lenihan, H.S., Peterson, C.H., 1998. How Habitat Degradation Through Fishery Disturbance Enhances Impacts of Hypoxia on Oyster Reefs. Ecological Applications 8, 128–140. https://doi.org/10.1890/1051-0761(1998)008[0128:HHDTFD]2.0.CO;2

Le Roy, N., Jackson, D.J., Marie, B., Ramos-Silva, P., Marin, F., 2014. The evolution of metazoan α-carbonic anhydrases and their roles in calcium carbonate biomineralization. Frontiers in Zoology 11, 75. https://doi.org/10.1186/s12983-014-0075-8

Lesser, M.P., 2006. OXIDATIVE STRESS IN MARINE ENVIRONMENTS: Biochemistry and Physiological Ecology. Annual Review of Physiology 68, 253–278. https://doi.org/10.1146/annurev.physiol.68.040104.110001

Lesser, M.P., Bailey, M.A., Merselis, D.G., Morrison, J.R., 2010. Physiological response of the blue mussel Mytilus edulis to differences in food and temperature in the Gulf of Maine. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 156, 541–551. https://doi.org/10.1016/j.cbpa.2010.04.012

Lesser, M.P., Kruse, V.A., 2004. Seasonal temperature compensation in the horse mussel, Modiolus modiolus: metabolic enzymes, oxidative stress and heat shock proteins. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 137, 495–504. https://doi.org/10.1016/j.cbpb.2003.10.022

Leverett, D., Thain, J., 2013. ICES Techniques in Marine Environmental Sciences: Oyster Embryo-larval Bioassay. No 54.

Levinton, J., Doall, M., Ralston, D., Starke, A., Allam, B., 2011. Climate Change, Precipitation and Impacts on an Estuarine Refuge from Disease. PLOS ONE 6, e18849. https://doi.org/10.1371/journal.pone.0018849

Li, A., Li, L., Song, K., Wang, W., Zhang, G., 2017. Temperature, energy metabolism, and adaptive divergence in two oyster subspecies. Ecology and Evolution 7, 6151–6162. https://doi.org/10.1002/ece3.3085

Libralato, G., Ghirardini Annamaria, V., Francesco, A., 2010. How toxic is toxic? A proposal for wastewater toxicity hazard assessment. Ecotoxicology and Environmental Safety 73, 1602–1611. https://doi.org/10.1016/j.ecoenv.2010.03.007

Libralato, G., Losso, C., Arizzi Novelli, A., Citron, M., Della Sala, S., Zanotto, E., Cepak, F., Volpi Ghirardini, A., 2008. Ecotoxicological evaluation of industrial port of Venice (Italy) sediment samples after a decontamination treatment. Environmental Pollution 156, 644–650. https://doi.org/10.1016/j.envpol.2008.06.025

Libralato, G., Losso, C., Avezzù, F., Volpi Ghirardini, A., 2009. Influence of the salinity adjustment methods, salts and brine, on the toxicity of wastewater samples to mussel embryos. Environmental Technology 30, 85–91. https://doi.org/10.1080/09593330802505094

Lin, M.-C., Galletta, B.J., Sept, D., Cooper, J.A., 2010. Overlapping and distinct functions for cofilin, coronin and Aip1 in actin dynamics in vivo. Journal of Cell Science. 123, 1329–1342. https://doi.org/10.1242/jcs.065698

Lionetto, M.G., Caricato, R., Erroi, E., Giordano, M.E., Schettino, T., 2006. Potential application of carbonic anhydrase activity in bioassay and biomarker studies. Chemistry and Ecology 22, S119–S125. https://doi.org/10.1080/02757540600670661

Lionetto, M.G., Caricato, R., Giordano, M.E., Erroi, E., Schettino, T., 2012. Carbonic Anhydrase as Pollution Biomarker: An Ancient Enzyme with a New Use. International Journal of Environmental Research and Public Health 9, 3965–3977. https://doi.org/10.3390/ijerph9113965

Lionetto, M.G., Giordano, M.E., Vilella, S., Schettino, T., 2000. Inhibition of eel enzymatic activities by cadmium. Aquatic Toxicology 48, 561–571.

Lira, G.M., Pascoal, J.C.M., Torres, E.A.F.S., Soares, R.A.M., Mendonça, S., Sampaio, G.R., Correia, M.S., Cabral, C.C.V.Q., Cabral Júnior, C.R., López, A.M.Q., 2013. Influence of seasonality on the chemical composition of oysters (Crassostrea rhizophorae). Food Chemistry 138, 786–790. https://doi.org/10.1016/j.foodchem.2012.11.088

Livingstone, D.R., 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. Revue de Medecine Veterinaire 154, 427–430.

Lockwood, B.L., Sanders, J.G., Somero, G.N., 2010. Transcriptomic responses to heat stress in invasive and native blue mussels (genus Mytilus): molecular correlates of invasive success. Journal of Experimental Biology. 213, 3548–3558. https://doi.org/10.1242/jeb.046094

Lüchmann, K.H., Clark, M.S., Bainy, A.C.D., Gilbert, J.A., Craft, J.A., Chipman, J.K., Thorne, M.A.S., Mattos, J.J., Siebert, M.N., Schroeder, D.C., 2015. Key metabolic pathways involved in xenobiotic biotransformation and stress responses revealed by transcriptomics of the mangrove oyster Crassostrea brasiliana. Aquatic Toxicology 166, 10–20. https://doi.org/10.1016/j.aquatox.2015.06.012

Luo, L., Ke, C., Guo, X., Shi, B., Huang, M., 2014. Metal accumulation and differentially expressed proteins in gill of oyster (Crassostrea hongkongensis) exposed to long-term heavy metal-contaminated estuary. Fish & Shellfish Immunology 38, 318–329. https://doi.org/10.1016/j.fsi.2014.03.029

Luo, S., Lu, J., 2017. Silencing of Transposable Elements by piRNAs in Drosophila: An Evolutionary Perspective. Genomics, Proteomics & Bioinformatics, RNA Epigenetics (I) 15, 164–176. https://doi.org/10.1016/j.gpb.2017.01.006

Macías-Mayorga, D., Laiz, I., Moreno-Garrido, I., Blasco, J., 2015. Is oxidative stress related to cadmium accumulation in the Mollusc Crassostrea angulata? Aquatic Toxicology 161, 231–241. https://doi.org/10.1016/j.aquatox.2015.02.007

MacInnes, J.R., Calabrese, A., 1979. Combined effects of salinity, temperature, and copper on embryos and early larvae of the American oyster,Crassostrea virginica. Archives of Environmental Contamination and Toxicology 8, 553–562. https://doi.org/10.1007/BF01055036

Mamindy-Pajany, Y., Hurel, C., Géret, F., Galgani, F., Battaglia-Brunet, F., Marmier, N., Roméo, M., 2013. Arsenic in marine sediments from French Mediterranean ports: Geochemical partitioning, bioavailability and ecotoxicology. Chemosphere 90, 2730–2736. https://doi.org/10.1016/j.chemosphere.2012.11.056

Mamindy-Pajany, Y., Libralato, G., Roméo, M., Hurel, C., Losso, C., Ghirardini, A.V., Marmier, N., 2010. Ecotoxicological evaluation of Mediterranean dredged sediment ports based on elutriates with oyster embryotoxicity tests after composting process. Water Research 44, 1986–1994. https://doi.org/10.1016/j.watres.2009.11.056

Mandal, B.K., Suzuki, K.T., 2002. Arsenic round the world: A review. Talanta 58, 201–235. https://doi.org/10.1016/S0039-9140(02)00268-0

Mani, S.R., Juliano, C.E., 2013. Untangling the Web: The Diverse Functions of the PIWI/piRNA Pathway. Mol Reprod Dev 80, 632–664. https://doi.org/10.1002/mrd.22195

Martin, M., Osborn, K.E., Billig, P., Glickstein, N., 1981. Toxicities of ten metals to Crassostrea gigas and Mytilus edulis embryos and Cancer magister larvae. Marine Pollution Bulletin 12, 305–308. https://doi.org/10.1016/0025-326X(81)90081-3

Martino, R.C., Cruz, G.M. da, 2004. Proximate composition and fatty acid content of the mangrove oyster Crassostrea rhizophorae along the year seasons. Brazilian Archives of Biology and Technology 47, 955–960. https://doi.org/10.1590/S1516-89132004000600015

Matoo, O.B., Ivanina, A.V., Ullstad, C., Beniash, E., Sokolova, I.M., 2013. Interactive effects of elevated temperature and CO2 levels on metabolism and oxidative stress in two common marine bivalves (Crassostrea virginica and Mercenaria mercenaria). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 164, 545–553. https://doi.org/10.1016/j.cbpa.2012.12.025

Matozzo, V., Chinellato, A., Munari, M., Bressan, M., Marin, M.G., 2013. Can the combination of decreased pH and increased temperature values induce oxidative stress in the clam Chamelea gallina and the mussel Mytilus galloprovincialis? Marine Pollution Bulletin. 72, 34–40. https://doi.org/10.1016/j.marpolbul.2013.05.004

Mckindsey, C.W., Landry, T., O\uc0\u8217{}beirn, F.X., Davies, I.M., 2007. Bivalve aquaculture and exotic species: a review of ecological considerations and management issues. Journal of Shellfish Research 26, 281\uc0\u8211{}294. https://doi.org/10.2983/0730-8000(2007)26[281:BAAESA]2.0.CO;2\.

McKay, R.M., McKay, J.P., Avery, L., Graff, J.M., 2003. C. elegans: A Model for Exploring the Genetics of Fat Storage. Developmental Cell 4, 131–142. https://doi.org/10.1016/S1534-5807(02)00411-2

Mehrbach, C., Culberson, C.H., Hawley, J.E., Pytkowicx, R.M., 1973. Measurement of the Apparent Dissociation Constants of Carbonic Acid in Seawater at Atmospheric Pressure1. Limnology and Oceanography. 18, 897–907. https://doi.org/10.4319/lo.1973.18.6.0897

Melo, C.M.R., Silva, F.C., Gomes, C.H.A.M., Solé-Cava, A.M., Lazoski, C., 2010. Crassostrea gigas in natural oyster banks in southern Brazil. Biol Invasions 12, 441–449. https://doi.org/10.1007/s10530-009-9475-7

Melo, A.G.C. de, Varela, E.S., Beasley, C.R., Schneider, H., Sampaio, I., Gaffney, P.M., Reece, K.S., Tagliaro, C.H., 2010. Molecular identification, phylogeny and geographic distribution of Brazilian mangrove oysters (Crassostrea). Genetics and Molecular Biology 33, 564–572. https://doi.org/10.1590/S1415-47572010000300030

Melwani, A.R., Thompson, E.L., Raftos, D.A., 2016. Differential proteomic response of Sydney rock oysters (Saccostrea glomerata) to prolonged environmental stress. Aquatic Toxicology 173, 53–62. https://doi.org/10.1016/j.aquatox.2016.01.003

Melzner, F., Thomsen, J., Koeve, W., Oschlies, A., Gutowska, M.A., Bange, H.W., Hansen, H.P., Körtzinger, A., 2013. Future ocean acidification will be amplified by hypoxia in coastal habitats. Marine Biology 160, 1875–1888.

Meng, J., Zhu, Q., Zhang, L., Li, C., Li, L., She, Z., Huang, B., Zhang, G., 2013. Genome and transcriptome analyses provide insight into the euryhaline adaptation mechanism of Crassostrea gigas. PLoS One 8, e58563.

Meng, X., Tian, X., Nie, G., Wang, J., Liu, M., Jiang, K., Wang, B., Guo, Q., Huang, J., Wang, L., 2015. The transcriptomic response to copper exposure in the digestive gland of Japanese scallops (Mizuhopecten yessoensis). Fish & Shellfish Immunology 46, 161–167. https://doi.org/10.1016/j.fsi.2015.05.022

Menzel, W., 1991. Estuarine and Marine Bivalve Mollusk Culture. CRC Press.

Metcalfe, N.B., Alonso-Alvarez, C., n.d. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Functional Ecology 24, 984–996. https://doi.org/10.1111/j.1365-2435.2010.01750.x

Michaelidis, B., Ouzounis, C., Paleras, A., Prtner, H.O., 2005. Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels Mytilus galloprovincialis. Marine Ecology Progress Series. 293, 109–118. https://doi.org/10.3354/meps293109

Miller, A.W., Reynolds, A.C., Sobrino, C., Riedel, G.F., 2009. Shellfish Face Uncertain Future in High CO2 World: Influence of Acidification on Oyster Larvae Calcification and Growth in Estuaries. PLOS ONE 4, e5661. https://doi.org/10.1371/journal.pone.0005661

Miossec, L., Le Deuff, R.-M., Goulletquer, P., 2009. Alien species alert: Crassostrea gigas (Pacific oyster). ICES Cooperative Research Report 299.

Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Amado, L.L., Martins, C.M.G., Pinho, G.L.L., Chaves, I.S., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007. Pollution biomarkers in estuarine animals: critical review and new perspectives. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 146, 221–234.

Moreira, A., Figueira, E., Soares, A.M.V.M., Freitas, R., 2016. Salinity influences the biochemical response of Crassostrea angulata to Arsenic. Environmental Pollution 214, 756–766. https://doi.org/10.1016/j.envpol.2016.04.036

Moreira, A., Freitas, R., Figueira, E., Volpi Ghirardini, A., Soares, A.M.V.M., Radaelli, M., Guida, M., Libralato, G., 2018. Combined effects of arsenic, salinity and temperature on Crassostrea gigas embryotoxicity. Ecotoxicology and Environmental Safety 147, 251–259. https://doi.org/10.1016/j.ecoenv.2017.08.043

Moya, A., Huisman, L., Ball, E.E., Hayward, D.C., Grasso, L.C., Chua, C.M., Woo, H.N., Gattuso, J.-P., Forêt, S., Miller, D.J., 2012. Whole Transcriptome Analysis of the Coral Acropora millepora Reveals Complex Responses to CO2-driven Acidification during the Initiation of Calcification. Molecular Ecology 21, 2440–2454. https://doi.org/10.1111/j.1365-294X.2012.05554.x

Muraeva, O.A., Maltseva, A.L., Mikhailova, N.A., Granovitch, A.I., 2016. Mechanisms of adaption to salinity stress in marine gastropods <Emphasis Type="Italic">Littorina saxatilis</Emphasis>: a proteomic analysis. Cell and Tissue Biology. 10, 160–169. https://doi.org/10.1134/S1990519X16020085

Muralidharan, S., Thompson, E., Raftos, D., Birch, G., Haynes, P.A., 2012. Quantitative proteomics of heavy metal stress responses in Sydney rock oysters. Proteomics 12, 906–921. https://doi.org/10.1002/pmic.201100417

Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. Biochemical Journal 417, 1–13.

Nardi, A., Mincarelli, L.F., Benedetti, M., Fattorini, D., d'Errico, G., Regoli, F., 2017. Indirect effects of climate changes on cadmium bioavailability and biological effects in the Mediterranean mussel Mytilus galloprovincialis. Chemosphere 169, 493–502. https://doi.org/10.1016/j.chemosphere.2016.11.093

Neff, J.M., n.d. Ecotoxicology of arsenic in the marine environment. Environmental Toxicology and Chemistry 16, 917–927. https://doi.org/10.1002/etc.5620160511

Nemova, N.N., Fokina, N.N., Nefedova, Z.A., Ruokolainen, T.R., Bakhmet, I.N., 2013. Modifications of gill lipid composition in littoral and cultured blue mussels Mytilus edulis L. under the influence of ambient salinity. Polar Record 49, 272–277. https://doi.org/10.1017/S0032247412000629

Neto, R.M., Zeni, T.O., Ludwig, S., Horodesky, A., Girotto, M.V.F., Castilho-Westphal, G.G., Ostrensky, A., 2013. Influence of environmental variables on the growth and reproductive cycle of Crassostrea (Mollusca, Bivalvia) in Guaratuba Bay, Brazil. Invertebrate Reproduction & Development 57, 208–218. https://doi.org/10.1080/07924259.2012.747449

Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., Erwin, K.N., Levin, E.D., 2009. The toxicology of climate change: environmental contaminants in a warming world. Environment international 35, 971–986.

O'Connor, W.A., Lawler, N.F., 2004. Salinity and temperature tolerance of embryos and juveniles of the pearl oyster, Pinctada imbricata Röding. Aquaculture 229, 493–506. https://doi.org/10.1016/S0044-8486(03)00400-9

Pace, D.A., Marsh, A.G., Leong, P.K., Green, A.J., Hedgecock, D., Manahan, D.T., 2006. Physiological bases of genetically determined variation in growth of marine invertebrate larvae: A study of growth heterosis in the bivalve Crassostrea gigas. Journal of Experimental Marine Biology and Ecology 335, 188\uc0\u8211{}209. https://doi.org/10.1016/j.jembe.2006.03.005.

Padilla, D.K., 2010. Context-dependent Impacts of a Non-native Ecosystem Engineer, the Pacific Oyster Crassostrea gigas. Integrative and Comparative Biology. 50, 213–225. https://doi.org/10.1093/icb/icq080

Pannunzio, T.M., Storey, K.B., 1998. Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod Littorina littorea. Journal of Experimental Marine Biology and Ecology 221, 277–292.

Parker, L.M., Ross, P.M., O'Connor, W.A., 2010. Comparing the effect of elevated pCO2 and temperature on the fertilization and early development of two species of oysters. Marine Biology. 157, 2435–2452. https://doi.org/10.1007/s00227-010-1508-3

Parker, L.M., Ross, P.M., O'Connor, W.A., Borysko, L., Raftos, D.A., Pörtner, H.-O., 2012. Adult exposure influences offspring response to ocean acidification in oysters. Global Change Biology. 18, 82–92. https://doi.org/10.1111/j.1365-2486.2011.02520.x

Parker, L.M., Ross, P.M., O'Connor, W.A., Pörtner, H.O., Scanes, E., Wright, J.M., 2013. Predicting the Response of Molluscs to the Impact of Ocean Acidification. Biology (Basel) 2, 651–692. https://doi.org/10.3390/biology2020651

Pechenik, J.A., 1999. On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. Marine Ecology Progress Series. 177, 269–297. https://doi.org/10.3354/meps177269

Péden, R., Rocher, B., Chan, P., Vaudry, D., Poret, A., Olivier, S., Le Foll, F., Bultelle, F., 2016. Consequences of acclimation on the resistance to acute thermal stress: Proteomic focus on mussels from pristine site. Marine Environmental Research, 18th International Symposium on Pollutant Responses in Marine Organisms (PRIMO18) 121, 64–73. https://doi.org/10.1016/j.marenvres.2016.02.006

Peña-Llopis, S., Ferrando, M.D., Peña, J.B., 2002. Impaired glutathione redox status is associated with decreased survival in two organophosphate-poisoned marine bivalves. Chemosphere 47, 485–497. https://doi.org/10.1016/S0045-6535(01)00323-X

Philippart, C.J.M., Anadón, R., Danovaro, R., Dippner, J.W., Drinkwater, K.F., Hawkins, S.J., Oguz, T., O'Sullivan, G., Reid, P.C., 2011. Impacts of climate change on European marine ecosystems: Observations, expectations and indicators. Journal of Experimental Marine Biology and Ecology, Global change in marine ecosystems 400, 52–69. https://doi.org/10.1016/j.jembe.2011.02.023

Philipp, E., Brey, T., Pörtner, H.-O., Abele, D., 2005. Chronological and physiological ageing in a polar and a temperate mud clam. Mech. Ageing Dev. 126, 598–609. https://doi.org/10.1016/j.mad.2004.12.003

Philipp, E.E.R., Schmidt, M., Gsottbauer, C., Sänger, A.M., Abele, D., 2008. Size- and age-dependent changes in adductor muscle swimming physiology of the scallop Aequipecten opercularis. Journal of Experimental Biology. 211, 2492–2501. https://doi.org/10.1242/jeb.015966

Piacentini, L., Fanti, L., Specchia, V., Bozzetti, M.P., Berloco, M., Palumbo, G., Pimpinelli, S., 2014. Transposons, environmental changes, and heritable induced phenotypic variability. Chromosoma 123, 345–354. https://doi.org/10.1007/s00412-014-0464-y

Pisias, N.G., Mix, A.C., Heusser, L., 2001. Millennial scale climate variability of the northeast Pacific Ocean and northwest North America based on radiolaria and pollen. Quaternary Science Reviews 20, 1561–1576. https://doi.org/10.1016/S0277-3791(01)00018-X

Pörtner, H.-O., 2010. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. Journal of Experimental Biology. 213, 881–893. https://doi.org/10.1242/jeb.037523

Pörtner, H.O., Bennett, A.F., Bozinovic, F., Clarke, A., Lardies, M.A., Lucassen, M., Pelster, B., Schiemer, F., Stillman, J.H., 2006. Trade-Offs in Thermal Adaptation: The Need for a Molecular to Ecological Integration. Physiological and Biochemical Zoology: Ecological and Evolutionary Approaches 79, 295–313. https://doi.org/10.1086/499986

Pörtner, H.O., Farrell, A.P., 2008. Physiology and Climate Change. Science 322, 690–692. https://doi.org/10.1126/science.1163156

Pörtner, H.O., Langenbuch, M., Michaelidis, B., 2005. Synergistic effects of temperature extremes, hypoxia, and increases in CO2 on marine animals: From Earth history to global change. Journal of Geophysical Research. 110, C09S10. https://doi.org/10.1029/2004JC002561

Pörtner, H.O., Reipschläger, A., Heisler, N., 1998. Acid-base regulation, metabolism and energetics in sipunculus nudus as a function of ambient carbon dioxide level. Journal of Experimental Biology 201, 43–55.

Przeslawski, R., Ahyong, S., Byrne, M., Wörheide, G., Hutchings, P., 2008. Beyond corals and fish: the effects of climate change on noncoral benthic invertebrates of tropical reefs. Global Change Biology 14, 2773–2795. https://doi.org/10.1111/j.1365-2486.2008.01693.x

Ragsdale, S.W., Yi, L., 2011. Thiol/Disulfide Redox Switches in the Regulation of Heme Binding to Proteins. Antioxidants and Redox Signaling. 14, 1039–1047. https://doi.org/10.1089/ars.2010.3436

Rahman, I., Kode, A., Biswas, S.K., 2007. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nature Protocols 1, 3159–3165. https://doi.org/10.1038/nprot.2006.378

Rainbow, P.S., 1997. Trace metal accumulation in marine invertebrates: marine biology or marine chemistry? Journal of the Marine Biological Association of the United Kingdom 77, 195–210.

Raper, S.C.B., Braithwaite, R.J., 2006. Low sea level rise projections from mountain glaciers and icecaps under global warming. Nature 439, 311–313. https://doi.org/10.1038/nature04448

Raven, J., Caldeira, K., Elderfield, H., Hoegh-Guldberg, O., Liss, P., Riebesell, U., Shepherd, J., Turley, C., Watson, A., 2005. Ocean acidification due to increasing atmospheric carbon dioxide. The Royal Society.

Regoli, F., 2011. Chemical Pollutants and the Mechanisms of Reactive Oxygen Species Generation in Aquatic Organisms, in: Oxidative Stress in Aquatic Ecosystems. Wiley-Blackwell, pp. 308–316.

Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Marine environmental research 93, 106–117.

Regoli, F., Hummel, H., Amiard-Triquet, C., Larroux, C., Sukhotin, A., 1998. Trace metals and variations of antioxidant enzymes in Arctic bivalve populations. Archives of Environmental Contamation and Toxicology. 35, 594–601.

Regoli, F., Nigro, M., Bertoli, E., Principato, G., Orlando, E., 1997. Defenses against oxidative stress in the Antarctic scallop Adamussium colbecki and effects of acute exposure to metals, in: Naumov, A.D., Hummel, H., Sukhotin, A.A., Ryland, J.S. (Eds.), Interactions and Adaptation Strategies of Marine Organisms, Developments in Hydrobiology. Springer Netherlands, pp. 139–144.

Reipschläger, A., Pörtner, H.O., 1996. Metabolic depression during environmental stress: the role of extracellular versus intracellular pH in Sipunculus nudus. Journal of Experimental Biology 199, 1801–1807.

Ren, J., Liu, X., Jiang, F., Guo, X., Liu, B., 2010. Unusual conservation of mitochondrial gene order in Crassostreaoysters: evidence for recent speciation in Asia. BMC Evolutionary Biology 10, 394. https://doi.org/10.1186/1471-2148-10-394

Reise, K., Buschbaum, C., Büttger, H., Rick, J., Wegner, K.M., 2017. Invasion trajectory of Pacific oysters in the northern Wadden Sea. Mar Biol 164. https://doi.org/10.1007/s00227-017-3104-2

Riba, I., Del, V., Forja, J.M., Gómez-Parra, A., 2004. The influence of pH and salinity on the toxicity of heavy metals in sediment to the estuarine clam Ruditapes philippinarum. Environmental Toxicology and Chemistry 23, 1100–1107. https://doi.org/10.1897/023-601

Ridge, J.T., Rodriguez, A.B., Fodrie, F.J., 2017. Evidence of exceptional oyster-reef resilience to fluctuations in sea level. Ecology and Evolotion. 7, 10409–10420. https://doi.org/10.1002/ece3.3473

Ries, J.B., Cohen, A.L., McCorkle, D.C., 2009. Marine calcifiers exhibit mixed responses to CO2-induced ocean acidification. Geology 37, 1131–1134. https://doi.org/10.1130/G30210A.1

Ringwood, A.H., Conners, D.E., Keppler, C.J., 1999. Cellular responses of oysters, Crassostrea virginica, to metal-contaminated sediments. Marine Environmental Research. 48, 427–437. https://doi.org/10.1016/S0141-1136(99)00062-8

Ringwood, A.H., Keppler, C.J., 2002. Water quality variation and clam growth: Is pH really a non-issue in estuaries? Estuaries. 25, 901–907. https://doi.org/10.1007/BF02691338

Ristori, C.A., Iaria, S.T., Gelli, D.S., Rivera, I.N.G., 2007. Pathogenic bacteria associated with oysters (Crassostrea brasiliana) and estuarine water along the south coast of Brazil. International Journal of Environmental Health Research. 17, 259–269. https://doi.org/10.1080/09603120701372169

Rivera-Ingraham, G.A., Lignot, J.-H., 2017. Osmoregulation, bioenergetics and oxidative stress in coastal marine invertebrates: raising the questions for future research. Journal of Experimental Biology. 220, 1749–1760. https://doi.org/10.1242/jeb.135624

Rivera-Ingraham, G.A., Rocchetta, I., Meyer, S., Abele, D., 2013. Oxygen radical formation in anoxic transgression and anoxia-reoxygenation: foe or phantom? Experiments with a hypoxia tolerant bivalve. Marine Environmental Research. 92, 110–119. https://doi.org/10.1016/j.marenvres.2013.09.007

Robbins, L.L., Hansen, M.E., Kleypas, J.A., Meylan, S.C., 2010. CO2calc: A User-Friendly Seawater Carbon Calculator for Windows, Mac OS X, and iOS (iPhone) (USGS Numbered Series No. 2010–1280) Open-File Report U.S. Geological Survey, Reston, VA

Robinson, H.W., Hogden, C.G., 1940. The biuret reaction in the determination of serum proteins. 1. A study of the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration. Journal of Biological Chemistry 135, 707–725.

Robins, P.E., Skov, M.W., Lewis, M.J., Giménez, L., Davies, A.G., Malham, S.K., Neill, S.P., McDonald, J.E., Whitton, T.A., Jackson, S.E., Jago, C.F., 2016. Impact of climate change on UK estuaries: A review of past trends and potential projections. Estuarine, Coastal and Shelf Science 169, 119–135. https://doi.org/10.1016/j.ecss.2015.12.016

Ruano, F., 1997. Fisheries and farming of important marine bivalves in Portugal. The history, present condition and future of the molluscan fisheries of North and Central America and Europe 3, 191–200.

Ruesink, J.L., Feist, B.E., Harvey, C.J., Hong, J.S., Trimble, A.C., Wisehart, L.M., 2006. Changes in productivity associated with four introduced species: ecosystem transformation of a “pristine” estuary. Marine Ecology Progress Series. 311, 203–215. https://doi.org/10.3354/meps311203

Ryan, C.P., Brownlie, J.C., Whyard, S., 2016. Hsp90 and Physiological Stress Are Linked to Autonomous Transposon Mobility and Heritable Genetic Change in Nematodes. Genome Biology and Evolution 8, 3794–3805. https://doi.org/10.1093/gbe/evw284

Rybakin, V., Clemen, C.S., 2005. Coronin proteins as multifunctional regulators of the cytoskeleton and membrane trafficking. Bioessays 27, 625–632. https://doi.org/10.1002/bies.20235

Samain, J.-F., McCombie, H., 2008. Summer Mortality of Pacific Oyster Crassostrea Gigas: The Morest Project. Editions Quae.

Samuel, S., Kathirvel, R., Jayavelu, T., Chinnakkannu, P., 2005. Protein oxidative damage in arsenic induced rat brain: influence of dl-α-lipoic acid. Toxicology Letters 155, 27–34. https://doi.org/10.1016/j.toxlet.2004.08.001

Sappal, R., MacDougald, M., Fast, M., Stevens, D., Kibenge, F., Siah, A., Kamunde, C., 2015. Alterations in mitochondrial electron transport system activity in response to warm acclimation, hypoxia-reoxygenation and copper in rainbow trout, Oncorhynchus mykiss. Aquatic Toxicology 165, 51–63. https://doi.org/10.1016/j.aquatox.2015.05.014

Schiedek, D., Sundelin, B., Readman, J.W., Macdonald, R.W., 2007. Interactions between climate change and contaminants. Marine Pollution Bulletin. 54, 1845–1856. https://doi.org/10.1016/j.marpolbul.2007.09.020

Schropp, S.J., Graham Lewis, F., Windom, H.L., Ryan, J.D., Calder, F.D., Burney, L.C., 1990. Interpretation of metal concentrations in estuarine sediments of Florida using aluminum as a reference element. Estuaries and Coasts 13, 227–235.

Sharma, V.K., Sohn, M., 2009. Aquatic arsenic: Toxicity, speciation, transformations, and remediation. Environment International 35, 743–759. https://doi.org/10.1016/j.envint.2009.01.005

Sherwood, C.L., Liguori, A.E., Olsen, C.E., Lantz, R.C., Burgess, J.L., Boitano, S., 2013. Arsenic Compromises Conducting Airway Epithelial Barrier Properties in Primary Mouse and Immortalized Human Cell Cultures. PLOS ONE 8, e82970. https://doi.org/10.1371/journal.pone.0082970

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V., Mann, M., 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nature Protocols. 1, 2856–2860. https://doi.org/10.1038/nprot.2006.468

Shiomi, K., Sugiyama, Y., Shimakura, K., Nagashima, Y., 1996. Retention and biotransformation of arsenic compounds administered intraperitoneally to carp. Fisheries science 62, 261–266.

Silacci, P., Mazzolai, L., Gauci, C., Stergiopulos, N., Yin, H.L., Hayoz, D., 2004. Gelsolin superfamily proteins: key regulators of cellular functions. CMLS, Cell and Molecular Life Sciences. 61, 2614–2623. https://doi.org/10.1007/s00018-004-4225-6

Simčič, T., Pajk, F., Jaklič, M., Brancelj, A., Vrezec, A., 2014. The thermal tolerance of crayfish could be estimated from respiratory electron transport system activity. Journal of Thermal Biology 41, 21–30. https://doi.org/10.1016/j.jtherbio.2013.06.003

Singh, S., Brocker, C., Koppaka, V., Chen, Y., Jackson, B.C., Matsumoto, A., Thompson, D.C., Vasiliou, V., 2013. Aldehyde dehydrogenases in cellular responses to oxidative/electrophilicstress. Free Radical Biology and Medicine 56, 89–101. https://doi.org/10.1016/j.freeradbiomed.2012.11.010

Skaggs, H.S., Henry, R.P., 2002. Inhibition of carbonic anhydrase in the gills of two euryhaline crabs, Callinectes sapidus and Carcinus maenas, by heavy metals. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 133, 605–612. https://doi.org/10.1016/S1532-0456(02)00175-8

Small, J.V., 1988. The actin cytoskeleton. Electron Microscopy Reviews 1, 155–174. https://doi.org/10.1016/S0892-0354(98)90010-7

Smith, J.B., Schneider, S.H., Oppenheimer, M., Yohe, G.W., Hare, W., Mastrandrea, M.D., Patwardhan, A., Burton, I., Corfee-Morlot, J., Magadza, C.H.D., Füssel, H.-M., Pittock, A.B., Rahman, A., Suarez, A., Ypersele, J.-P. van, 2009. Assessing dangerous climate change through an update of the Intergovernmental Panel on Climate Change (IPCC) “reasons for concern.” PNAS 106, 4133–4137. https://doi.org/10.1073/pnas.0812355106

Smith, R., 2009. Two-Dimensional Electrophoresis: An Overview, in: Two-Dimensional Electrophoresis Protocols, Methods in Molecular Biology. Humana Press, pp. 2–17.

Sokolova, I.M., 2013. Energy-Limited Tolerance to Stress as a Conceptual Framework to Integrate the Effects of Multiple Stressors. Integrative and Comparative Biology 53, 597–608. https://doi.org/10.1093/icb/ict028

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Marine Environmental Research 79, 1–15. https://doi.org/10.1016/j.marenvres.2012.04.003

Sokolova, I.M., Lannig, G., 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. Climate Research 37, 181–201.

Sokolov, E.P., Sokolova, I.M., 2018. Compatible osmolytes modulate mitochondrial function in a marine osmoconformer Crassostrea gigas (Thunberg, 1793). Mitochondrion. https://doi.org/10.1016/j.mito.2018.02.002

Solomon, S., 2007. Climate change 2007-the physical science basis: Working group I contribution to the fourth assessment report of the IPCC. Cambridge University Press.

Somero, G.N., 2012. The Physiology of Global Change: Linking Patterns to Mechanisms. Annual Review of Marine Science 4, 39–61. https://doi.org/10.1146/annurev-marine-120710-100935

Srivastava, R., Prasadareddy Kajuluri, L., Pathak, N., Gupta, C.M., Sahasrabuddhe, A.A., 2015. Oligomerization of coronin: Implication on actin filament length in Leishmania. Cytoskeleton (Hoboken) 72, 621–632. https://doi.org/10.1002/cm.21269

Storey, K.B., 1998. Survival under stress: molecular mechanisms of metabolic rate depression in animals. South African Journal of Zoology 33, 55–64.

Strobel, A., Graeve, M., Poertner, H.O., Mark, F.C., 2013. Mitochondrial Acclimation Capacities to Ocean Warming and Acidification Are Limited in the Antarctic Nototheniid Fish, Notothenia rossii and Lepidonotothen squamifrons. PLOS ONE 8, e68865. https://doi.org/10.1371/journal.pone.0068865

Sukhotin, A.A., Abele, D., Pörtner, H.-O., 2002. Growth, metabolism and lipid peroxidation in Mytilus edulis L.: age and size effects. Marine ecology-progress series 226, 223–234.

Sutherland, W.J., Pullin, A.S., Dolman, P.M., Knight, T.M., 2004. The need for evidence-based conservation. Trends in Ecology & Evolution 19, 305–308. https://doi.org/10.1016/j.tree.2004.03.018

Talmage, S.C., Gobler, C.J., 2011. Effects of Elevated Temperature and Carbon Dioxide on the Growth and Survival of Larvae and Juveniles of Three Species of Northwest Atlantic Bivalves. PLoS One 6. https://doi.org/10.1371/journal.pone.0026941

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular and Biological Evolution. 30, 2725–2729. https://doi.org/10.1093/molbev/mst197

Taris, N., Ernande, B., McCombie, H., Boudry, P., 2006. Phenotypic and genetic consequences of size selection at the larval stage in the Pacific oyster (Crassostrea gigas). Journal of Experimental Marine Biology and Ecology 333, 147–158. https://doi.org/10.1016/j.jembe.2005.12.007

Telesh, I.V., Khlebovich, V.V., 2010. Principal processes within the estuarine salinity gradient: A review. Marine Pollution Bulletin, Estuarine Ecosystems: Structure, Function and Management (ECSA-42 Symposium in Russia) 61, 149–155. https://doi.org/10.1016/j.marpolbul.2010.02.008

Tewksbury, J.J., Huey, R.B., Deutsch, C.A., 2008. Putting the Heat on Tropical Animals. Science 320, 1296–1297. https://doi.org/10.1126/science.1159328

Thain, J.E., 1991. Biological effects of contaminants in oyster (Crassostrea gigas) embryo bioassay. ICES, Copenhagen.

Thiyagarajan, V., Ko, G.W.K., 2012. Larval growth response of the Portuguese oyster (Crassostrea angulata) to multiple climate change stressors. Aquaculture 370, 90–95.

Thomas, D.J., Styblo, M., Lin, S., 2001. The Cellular Metabolism and Systemic Toxicity of Arsenic. Toxicology and Applied Pharmacology 176, 127–144. https://doi.org/10.1006/taap.2001.9258

Thompson, E.L., O'Connor, W., Parker, L., Ross, P., Raftos, D.A., 2015. Differential proteomic responses of selectively bred and wild-type Sydney rock oyster populations exposed to elevated CO2. Molecular Ecology 24, 1248–1262. https://doi.org/10.1111/mec.13111

Thompson, E.L., Parker, L., Amaral, V., Bishop, M.J., O'Connor, W.A., Raftos, D.A., 2016. Wild populations of Sydney rock oysters differ in their proteomic responses to elevated carbon dioxide. Marine and Freshwater Research. 67, 1964–1972. https://doi.org/10.1071/MF15320

Thompson, E.L., Taylor, D.A., Nair, S.V., Birch, G., Haynes, P.A., Raftos, D.A., 2012. Proteomic discovery of biomarkers of metal contamination in Sydney Rock oysters (Saccostrea glomerata). Aquatic toxicology 109, 202–212.

Timmins-Schiffman, E., Coffey, W.D., Hua, W., Nunn, B.L., Dickinson, G.H., Roberts, S.B., 2014. Shotgun proteomics reveals physiological response to ocean acidification in Crassostrea gigas. BMC Genomics 15, 951. https://doi.org/10.1186/1471-2164-15-951

Tomanek, L., 2015. Proteomic responses to environmentally induced oxidative stress. Journal of Experimental Biology 218, 1867–1879. https://doi.org/10.1242/jeb.116475

Tomanek, L., 2014. Proteomics to study adaptations in marine organisms to environmental stress. Journal of Proteomics, Special Issue: Proteomics of non-model organisms 105, 92–106. https://doi.org/10.1016/j.jprot.2014.04.009

Tomanek, L., 2012. Environmental Proteomics of the Mussel Mytilus: Implications for Tolerance to Stress and Change in Limits of Biogeographic Ranges in Response to Climate Change. Integrative and Comparative Biology. 52, 648–664. https://doi.org/10.1093/icb/ics114

Tomanek, L., Zuzow, M.J., 2010. The proteomic response of the mussel congeners Mytilus galloprovincialis and M. trossulus to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. Journal of Experimental Biology 213, 3559–3574. https://doi.org/10.1242/jeb.041228

Tomanek, L., Zuzow, M.J., Ivanina, A.V., Beniash, E., Sokolova, I.M., 2011. Proteomic response to elevated P_{CO2} level in eastern oysters, *Crassostrea virginica*: evidence for oxidative stress. Journal of Experimental Biology 214, 1836–1844. https://doi.org/10.1242/jeb.055475

Townsend, D.M., Tew, K.D., 2003. The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene 22, 7369–7375. https://doi.org/10.1038/sj.onc.1206940

Tresguerres, M., 2016. Novel and potential physiological roles of vacuolar-type H+-ATPase in marine organisms. Journal of Experimental Biology 219, 2088–2097. https://doi.org/10.1242/jeb.128389

Trevisan, R., Mello, D.F., Uliano-Silva, M., Delapedra, G., Arl, M., Dafre, A.L., 2014. The biological importance of glutathione peroxidase and peroxiredoxin backup systems in bivalves during peroxide exposure. Marine Environmental Research 101, 81–90. https://doi.org/10.1016/j.marenvres.2014.09.004

Turner, A.J., Isaac, R.E., Coates, D., 2001. The neprilysin (NEP) family of zinc metalloendopeptidases: Genomics and function. Bioessays 23, 261–269. https://doi.org/10.1002/1521-1878(200103).

USEPA, 1991. Methods for Aquatic Toxicity Identification Evaluations—Phase I Toxicity Characterization Procedures. EPA-600-6-91-003, US Environmental Protection Agency, Washington, DC.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicology and Environmental Safety 64, 178–189. https://doi.org/10.1016/j.ecoenv.2005.03.013

Velez, C., Figueira, E., Soares, A.M.V.M., Freitas, R., 2016a. Native and introduced clams biochemical responses to salinity and pH changes. Science of The Total Environment 566–567, 260–268. https://doi.org/10.1016/j.scitotenv.2016.05.019

Velez, C., Teixeira, M., Wrona, F.J., Soares, A.M.V.M., Figueira, E., Freitas, R., 2016b. Clam Ruditapes philippinarum recovery from short-term exposure to the combined effect of salinity shifts and Arsenic contamination. Aquatic Toxicology. 173, 154–164. https://doi.org/10.1016/j.aquatox.2016.01.007

Ventura-Lima, J., Ramos, P.B., Fattorini, D., Regoli, F., Ferraz, L., de Carvalho, L.M., Monserrat, J.M., 2011. Accumulation, biotransformation, and biochemical responses after exposure to arsenite and arsenate in the estuarine polychaete Laeonereis acuta (Nereididae). Environmental Science and Pollution Research 18, 1270–1278.

Verlecar, X.N., Jena, K.B., Chainy, G.B.N., 2007. Biochemical markers of oxidative stress in Perna viridis exposed to mercury and temperature. Chemico-Biological Interactions 167, 219–226. https://doi.org/10.1016/j.cbi.2007.01.018

Verween, A., Vincx, M., Degraer, S., 2007. The effect of temperature and salinity on the survival of Mytilopsis leucophaeata larvae (Mollusca, Bivalvia): The search for environmental limits. Journal of Experimental Marine Biology and Ecology 348, 111–120. https://doi.org/10.1016/j.jembe.2007.04.011

Viarengo, A., Canesi, L., Pertica, M., Livingstone, D.R., Orunesu, M., 1991. Age-related lipid peroxidation in the digestive gland of mussels: The role of the antioxidant defence systems. Experientia 47, 454–457. https://doi.org/10.1007/BF01959942

Virvilis, C., Angelidis, P., 2006. Presence of the parasite Marteilia sp. in the flat oyster (Ostrea edulis L) in Greece. Aquaculture 259, 1–5. https://doi.org/10.1016/j.aquaculture.2006.05.008

Wang, Q., Cao, R., Ning, X., You, L., Mu, C., Wang, C., Wei, L., Cong, M., Wu, H., Zhao, J., 2016. Effects of ocean acidification on immune responses of the Pacific oyster Crassostrea gigas. Fish & Shellfish Immunology 49, 24–33. https://doi.org/10.1016/j.fsi.2015.12.025

Wang, X., Mu, X., Zhang, J., Huang, Q., Alamdar, A., Tian, M., Liu, L., Shen, H., 2015. Serum metabolomics reveals that arsenic exposure disrupted lipid and amino acid metabolism in rats: a step forward in understanding chronic arsenic toxicity. Metallomics 7, 544–552. https://doi.org/10.1039/c5mt00002e

Warrier, R.R., Lalitha, S., Savitha, C., 2014. A Modified Assay of Carbonic Anhydrase Activity in Tree Species. BBR - Biochemistry and Biotechnology Reports 3, 48–55. https://doi.org/10.5433/2316-5200.2014v3n1p48

Weis, V.M., 1991. The Induction of Carbonic Anhydrase in the Symbiotic Sea Anemone Aiptasia pulchella. Biology Bulletin. 180, 496–504. https://doi.org/10.2307/1542351

Weis, V.M., Reynolds, W.S., 1999. Carbonic Anhydrase Expression and Synthesis in the Sea Anemone Anthopleura elegantissima Are Enhanced by the Presence of Dinoflagellate Symbionts. Physiological and Biochemical Zoology 72, 307–316. https://doi.org/10.1086/316674

Weng, N., Wang, W.-X., 2017. Dynamics of maternally transferred trace elements in oyster larvae and latent growth effects. Scientific Reports 7, 3580. https://doi.org/10.1038/s41598-017-03753-2

Willson, L.L., Burnett, L.E., 2000. Whole animal and gill tissue oxygen uptake in the Eastern oyster, Crassostrea virginica: Effects of hypoxia, hypercapnia, air exposure, and infection with the protozoan parasite Perkinsus marinus1. Journal of Experimental Marine Biology and Ecology 246, 223–240. https://doi.org/10.1016/S0022-0981(99)00183-5

Wu, H., Zhang, X., Wang, Q., Li, L., Ji, C., Liu, X., Zhao, J., Yin, X., 2013. A metabolomic investigation on arsenic-induced toxicological effects in the clam Ruditapes philippinarum under different salinities. Ecotoxicology and Environmental Safety 90, 1–6. https://doi.org/10.1016/j.ecoenv.2012.02.022

Xu, T., Pagadala, V., Mueller, D.M., 2015. Understanding structure, function, and mutations in the mitochondrial ATP synthase. Microbial Cell. 2, 105–125. https://doi.org/10.15698/mic2015.04.197

Yamamoto, T., Takano, N., Ishiwata, K., Ohmura, M., Nagahata, Y., Matsuura, T., Kamata, A., Sakamoto, K., Nakanishi, T., Kubo, A., Hishiki, T., Suematsu, M., 2014. Reduced methylation of PFKFB3 in cancer cells shunts glucose towards the pentose phosphate pathway. Nature Communications. 5, 3480. https://doi.org/10.1038/ncomms4480

Yoshikawa, H., 1959. Glycogen. Rinsho Ikagaku Kyodoisho, Tokyo 150–152.

Yu, L.-R., Stewart, N.A., Veenstra, T.D., 2010. Chapter 8 - Proteomics: The Deciphering of the Functional Genome, in: Ginsburg, G.S., Willard, H.F. (Eds.), Essentials of Genomic and Personalized Medicine. Academic Press, San Diego, pp. 89–96.

Yurista, P.M., 1999. A model for temperature correction of size-specific respiration in Bythotrephes cederstroemi and Daphnia middendorffiana. Journal of Plankton Research 21, 721–734.

Zacchi, F.L., de Lima, D., Flores-Nunes, F., Mattos, J.J., Lüchmann, K.H., de Miranda Gomes, C.H.A., Bícego, M.C., Taniguchi, S., Sasaki, S.T., Dias Bainy, A.C., 2017. Transcriptional changes in oysters Crassostrea brasiliana exposed to phenanthrene at different salinities. Aquatic Toxicology 183, 94–103. https://doi.org/10.1016/j.aquatox.2016.12.016

Zanette, J., de Almeida, E.A., da Silva, A.Z., Guzenski, J., Ferreira, J.F., Di Mascio, P., Marques, M.R.F., Bainy, A.C.D., 2011. Salinity influences glutathione S-transferase activity and lipid peroxidation responses in the Crassostrea gigas oyster exposed to diesel oil. Science of The Total Environment 409, 1976–1983. https://doi.org/10.1016/j.scitotenv.2011.01.048

Zanette, J., Monserrat, J.M., Bianchini, A., 2006. Biochemical biomarkers in gills of mangrove oyster Crassostrea rhizophorae from three Brazilian estuaries. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology. 143, 187–195. https://doi.org/10.1016/j.cbpc.2006.02.001

Zhang, G., Fang, X., Guo, X., Li, L., Luo, R., Xu, F., Yang, P., Zhang, L., Wang, X., Qi, H., Xiong, Z., Que, H., Xie, Y., Holland, P.W.H., Paps, J., Zhu, Y., Wu, F., Chen, Y., Wang, J., Peng, C., Meng, J., Yang, L., Liu, J., Wen, B., Zhang, N., Huang, Z., Zhu, Q., Feng, Y., Mount, A., Hedgecock, D., Xu, Z., Liu, Y., Domazet-Lošo, T., Du, Y., Sun, X., Zhang, S., Liu, B., Cheng, P., Jiang, X., Li, J., Fan, D., Wang, W., Fu, W., Wang, T., Wang, B., Zhang, J., Peng, Z., Li, Y., Li, N., Wang, J., Chen, M., He, Y., Tan, F., Song, X., Zheng, Q., Huang, R., Yang, H., Du, X., Chen, L., Yang, M., Gaffney, P.M., Wang, S., Luo, L., She, Z., Ming, Y., Huang, W., Zhang, S., Huang, B., Zhang, Y., Qu, T., Ni, P., Miao, G., Wang, J., Wang, Q., Steinberg, C.E.W., Wang, H., Li, N., Qian, L., Zhang, G., Li, Y., Yang, H., Liu, X., Wang, J., Yin, Y., Wang, J., 2012. The oyster genome reveals stress adaptation and complexity of shell formation. Nature 490, 49–54. https://doi.org/10.1038/nature11413

Zhang, G., Li, L., Meng, J., Qi, H., Qu, T., Xu, F., Zhang, L., 2016. Molecular Basis for Adaptation of Oysters to Stressful Marine Intertidal Environments. Annual Review of Animal Biosciences 4, 357–381. https://doi.org/10.1146/annurev-animal-022114-110903

Zhang, Q.-H., Huang, L., Zhang, Y., Ke, C.-H., Huang, H.-Q., 2013. Proteomic approach for identifying gonad differential proteins in the oyster (Crassostrea angulata) following food-chain contamination with HgCl2. Journal of Proteomics 94, 37–53. https://doi.org/10.1016/j.jprot.2013.08.018

Zhang, W., Guo, Z., Zhou, Y., Liu, H., Zhang, L., 2015. Biotransformation and detoxification of inorganic arsenic in Bombay oyster Saccostrea cucullata. Aquatic Toxicology 158, 33–40. https://doi.org/10.1016/j.aquatox.2014.10.021

Zhang, Y., Sun, J., Mu, H., Li, J., Zhang, Y., Xu, F., Xiang, Z., Qian, P.-Y., Qiu, J.-W., Yu, Z., 2015. Proteomic Basis of Stress Responses in the Gills of the Pacific Oyster Crassostrea gigas. Journal of Proteome Research. 14, 304–317. https://doi.org/10.1021/pr500940s

Zhao, X., Yu, H., Kong, L., Li, Q., 2012. Transcriptomic responses to salinity stress in the Pacific oyster Crassostrea gigas. PloS one 7, e46244.