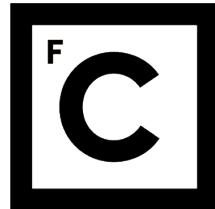


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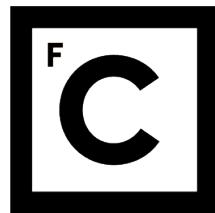
**Effects of ocean warming and acidification on the early stages
of marine fishes**

Doutoramento em Ciências do Mar

Marta Cristina Silva Pimentel da Silva

Tese orientada por:
Professor Doutor Rui Rosa
Professor Doutor Jorge Machado

Documento especialmente elaborado para a obtenção do grau de doutor



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Dedico

À Rita

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LIST OF ABBREVIATIONS AND UNITS

ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
BSA	Bovine Serum Albumin
Ca	Calcium
CAT	Catalase
CDNB	l-chloro-2,4-dinitrobenzene
CO ₂	Carbon Dioxide
CS	Citrate Synthase
CTMax	Critical Thermal Maximum
e.g.	For Example
EDTA	Ethylenediaminetetraacetic acid
etc.	<i>Et cetera</i>
GR	Glutathione reductase
GSH	Reduced glutathione
GST	Glutathione S-Transferase
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HSP	Heat Shock Proteins
HSR	Heat Shock Response
HOAD	3-hydroxyacyl CoA dehydrogenase
IPCC	Intergovernmental Panel on Climate Change
LDH	Lactate Dehydrogenase
LT50	Temperature required for 50% of mortality
LT100	Temperature required for 100% of mortality
MDA	Malondialdehyde
NOAA	National Oceanic and Atmospheric Administration
O ₂	Oxygen
OA	Ocean Acidification
OCR	Oxygen Consumption Rates
p-value	Probability of the test statistic
Q ₁₀	Thermal sensitivity
RMRs	Routine metabolic rates
ROS	Reactive oxygen species
SST	Sea surface temperatures
TBARS	Thiobarbituric acid reactive substances
%	Percentage

List of Abbreviations and Units

±	Approximately
°C	Degree Celsius
atm	Atmosphere
ww	Wet weight
g	Gram(s)
G	Relative centrifugal force or G-force
H	Hour(s)
Kg	Kilogram(s)
L	litre(s)
M	Molar concentration
Mg	Miligram(s)
Min	Minute(s)
mL	Milliliter(s)
µmol	Micromole(s)
mM	Milimolar
mm	Millimetre(s)
rpm	Revolutions per minute
sec	Second(s)
U	Units
λ	Wavelength
µg	Micrograma(s)
µL	Microlitre(s)
µmol	Micromole

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ABSTRACT

The potential susceptibility of fish species to climate-driven changes has been highlighted by an increasing number of studies, yet little is known about fish early-life stages capacity to tolerate future ocean conditions. In this context, the main objectives of this dissertation were to investigate a comprehensive set of biological responses of early-life stages of commercially important fish species with different life strategies, seabream (*Sparus aurata*), meagre (*Argyrosomus regius*), Senegalese sole (*Solea senegalensis*) and dolphinfish (*Coryphaena hippurus*) to future ocean warming (+4°C) and acidification ($\Delta\text{pH}=0.5$) expected for 2100. The current dissertation constitutes the first attempt to address the interacting effects of climate-related conditions in fish early ontogeny. The combination of ocean warming and acidification intensified the effects on many morphological, behavioural, biochemical and physiological features, namely hatching success, skeletal deformities, growth, metabolic and enzymatic profiles, heat shock and antioxidant responses. However, species tolerance to future conditions was shown to be species-specific. Changes on the different features here investigated had severe repercussions on larval survival rates of each fish species. Impacts revealed to be more deleterious for seabream and meagre, the most active species with an associated planktonic life strategy. The higher decrease in survival rates of these species, 51.92% and 50.00% respectively, suggests a lower tolerance than the benthic flatfish (28.44% decrease) to future climate change. Such impairments are expected to affect larval performance, recruitment success, and further influence the abundance of fish stocks and population structure of these species. The main outputs of this dissertation allow stakeholders and policy-makers to take proactive measures to protect endangered and commercially-important species. However, it is worth noting that these species may have the opportunity to adapt to future ocean conditions.

Keywords: Fish early life stages; ocean climate change; skeletal malformations and otoliths; behaviour; ecophysiology; oxidative stress; digestive capabilities.

RESUMO

Numerosos estudos têm vindo a demonstrar a suscetibilidade de diversas espécies de peixes às alterações climáticas, porém pouco se conhece sobre a capacidade de tolerância dos seus estados ontogenéticos iniciais às condições futuras dos oceanos. Neste contexto, a presente dissertação teve como objetivo investigar um conjunto abrangente de respostas biológicas dos estados iniciais de desenvolvimento de várias espécies de peixes, nomeadamente a dourada (*Sparus aurata*), corvina (*Argyrosomus regius*), linguado (*Solea senegalensis*) e doirado (*Coryphaena hippurus*) às condições de aquecimento (+4°C) e acidificação ($\Delta\text{pH}=0,5$) dos oceanos expectáveis para 2100. A presente dissertação constitui a primeira abordagem à investigação do impacto dos efeitos sinergísticos da temperatura e acidificação nestes estados de desenvolvimento. A exposição à combinação destes fatores demonstrou expor os embriões e larvas ao limite da sua capacidade de aclimatação. A interação entre fatores intensificou os efeitos deletérios em muitos dos processos morfológicos, comportamentais, bioquímicos e fisiológicos, nomeadamente na eclosão, desenvolvimento larvar, perfil metabólico e enzimático, resposta antioxidante e ao choque térmico. As alterações observadas tiveram graves repercussões na sobrevivência das larvas. No entanto, a capacidade de tolerância revelou ser específica de cada espécie. Os impactos mais prejudiciais observaram-se nas espécies mais ativas com uma estratégia de vida planctónica. O maior decréscimo ao nível da sobrevivência registado na dourada e na corvina (51,92% e 50,00%, respectivamente) sugere uma menor tolerância por parte destas espécies às alterações climáticas relativamente ao linguado com uma redução de apenas 28,44%. Espera-se que estas alterações induzam impactos profundos no desempenho larvar e sucesso de recrutamento, afetando consequentemente a distribuição e a abundância destas espécies. Esta dissertação constitui um contributo importante para a tomada de decisões proactivas com vista a proteção das espécies mais ameaçadas e/ou comercialmente importantes. Contudo, atendendo ao ritmo das alterações climáticas nos oceanos é crucial avaliar a capacidade de adaptação destas espécies.

Palavras-chave: Estados ontogenéticos iniciais de peixes marinhos; alterações climáticas dos oceanos; malformações esqueléticas e otólitos; comportamento; ecofisiologia; stress oxidativo; capacidades digestivas.

RESUMO ALARGADO

Desde a revolução industrial, a combustão de combustíveis fósseis e processos industriais têm libertado toneladas de dióxido de carbono para a atmosfera. Como consequência, as concentrações atmosféricas de CO₂ têm vindo aumentar a um ritmo invulgarmente rápido, desde níveis pré-industriais de 280µatm até aos atuais níveis de 394µatm. Especialistas preveem que até ao final do século estes níveis possam chegar até 1000µatm, caso as emissões antropogénicas mantenham as mesmas taxas de aumento. O aumento destes gases tem vindo a intensificar a tendência para o aquecimento global e consequentemente para o aquecimento da superfície dos oceanos. Até ao final do século está previsto um aquecimento adicional até 4°C. Os oceanos são um dos maiores reservatórios de CO₂ e absorvem aproximadamente um terço das emissões procedentes das atividades humanas. O CO₂ em combinação com a água dos oceanos provoca a formação de ácido carbónico, alterando assim o delicado equilíbrio químico dos mares. A absorção continua do CO₂ atmosférico para além de causar um declínio no pH dos oceanos, um processo conhecido como acidificação dos oceanos, diminui a concentração do ião carbonato e do estado de saturação do carbonato de cálcio. Estudos recentes indicam que estas alterações ambientais afectam uma série de processos biológicos necessários para o normal desenvolvimento e a sobrevivência de várias espécies. Embora se saiba que o aumento do CO₂ poderá afectar negativamente muitas espécies de invertebrados marinhos que sintetizam exoesqueleto calcário, os seus efeitos em espécies de peixes foram até então erradamente assumidos como negligenciáveis. Os primeiros estados ontogenéticos foram considerados dos mais vulneráveis às alterações climáticas, no entanto, até ao inicio da presente investigação pouco se conhecia sobre a susceptibilidade destes estados de desenvolvimento de espécies de peixes às condições futuras dos oceanos.

Dada a crescente consciênciia internacional sobre o acelerado ritmo das alterações climáticas e da necessidade urgente de uma avaliação das consequências destas alterações sobre os oceanos do mundo, os objectivos principais desta dissertação foram investigar os efeitos das condições de aquecimento (+4°C) e acidificação ($\Delta\text{pH}=0,5$) dos oceanos expectáveis para o final deste século sobre as fases iniciais de vida de diversas espécies de peixes marinhos, nomeadamente a dourada (*Sparus aurata*), corvina (*Argyrosomus regius*), linguado (*Solea senegalensis*) e doirado (*Coryphaena hippurus*). De forma a compreender os mecanismos que

Resumo Alargado

permitirão estas espécies suportar as futuras condições climáticas, foi avaliado um conjunto de respostas biológicas de uma forma integrada, nomeadamente estudo de alterações morfológicas, comportamentais, fisiológicas e bioquímicas, nos estados de desenvolvimento inicial das espécies acima referidas.

As experiências realizadas demonstraram que os cenários futuros de aquecimento e acidificação afectam a capacidade de resistência dos primeiros estados ontogenéticos das espécies de peixes aqui analisadas. Em geral os resultados indicam que as espécies quando expostas às alterações climáticas foram induzidas para fora dos seus limites fisiológicos e de tolerância térmica. A exposição à acidificação demonstrou intensificar os efeitos em muitos dos processos biológicos analisados, diminuindo assim o limites de tolerância das espécies. Contudo, a susceptibilidade às referidas condições revelou que o tipo e intensidade das respostas diferem entre as diferentes espécies estudadas.

O efeito do aumento de temperatura provocou um impacto negativo sobre a sobrevivência embrionária (sucesso de eclosão) e larval da dourada, corvina e linguado, no entanto, os efeitos negativos da acidificação dos oceanos foram intensificados quando combinados com os do aumento da temperatura. A aclimatação, das espécies aqui analisadas, às condições de aquecimento e acidificação revelou que os embriões foram mais tolerantes à exposição a tais condições do que as larvas. A falta de proteção conferida aos embriões pelo ovo juntamente com a existência de vida planctônicas e maiores taxas metabólicas podem ter consequentemente contribuído para a menor tolerância das larvas. Embora não tenham sido detectadas diferenças significativas na sobrevivência embrionária entre espécies, a exposição às condições futuras dos oceanos induziu um decréscimo superior na sobrevivência da dourada e corvina, cerca de 51,92 e de 50,00%, respectivamente, do que no linguado (decréscimo de 28,44%). Este elevado decréscimo da sobrevivência das larvas foi certamente reflexo e consequência das alterações nas diferentes funções biológicas aqui investigadas.

Como expectável, o aumento de temperatura induziu um aumento do consumo de oxigénio e frequência cardíaca, porém a tendência oposta foi observada com a acidificação. A magnitude da depressão metabólica foi bastante similar entre as espécies, tendo o consumo de oxigénio do linguado, doirado e dourada diminuído cerca de 27,20, 21,40 e 21,30%, respectivamente. Juntamente com a depressão metabólica, a diminuição dos batimentos cardíacos (bradicárdia) pode causar perturbações no transporte de oxigênio, limitando o fornecimento de oxigénio

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para os tecidos diminuindo assim capacidade aeróbica celular. A redução dos batimentos cardíacos foi especialmente significativa para as larvas de dourada que apresentaram o maior decréscimo (24,4%). O aquecimento e acidificação dos oceanos induziram também ajustes específicos nas vias metabólicas e na regulação enzimática de cada uma das vias. Especificamente, o aumento de temperatura provocou um aumento na atividade das enzimas mitocondriais (citrate sintase, CS e β -hidroxiacil CoA desidrogenase, HOAD) e glicolíticas (lactato desidrogenase, LDH), enquanto a hipercapnia induziu a inibição da atividade da CS e HOAD e um aumento da atividade da LDH. Estas modificações indicam que ocorreu uma transição de metabolismo aeróbio para o anaeróbico de forma a sustentar as necessidades energéticas das larvas em condições de acidificação. Embora a depressão metabólica como a transição para metabolismo anaeróbico sejam consideradas estratégias adaptativas cruciais para proteger os organismos sob condições de hipercapnia, a longo prazo podem não ser suportáveis nem benéficas para determinados processos, e.g. crescimento. Em condições de acidificação estes mecanismos foram simultaneamente acompanhados por um decréscimo no crescimento das larvas, sendo este mais acentuado na dourada (decréscimo 61,54% superior do que no linguado). A maior vulnerabilidade das larvas às alterações climáticas foi também indicado pelo aumento das deformações esqueléticas assim como pela alteração do tamanho das estruturas sensórias formadas por aragonite (otólitos). Embora a temperatura tenha aumentado a frequência de malformações (aumento de 22,89 e 53,49% no linguado e dourada, respectivamente), a combinação entre temperatura e acidificação demonstrou induzir um aumento adicional nas malformações esqueléticas (aumento de 31,38 e 77,27% no linguado e dourada, respectivamente). A mesma tendência foi observada no tamanho dos otólitos, tendo a temperatura induzido um aumento de 104,02% e a interação da temperatura e acidificação um aumento de 127,73%.

De acordo com os resultados acima descritos, os padrões comportamentais observados foram também afetados pelas condições de aquecimento e acidificação. O comportamento predatório, conforme o esperado, não acompanhou o aumento das taxas metabólicas com o aquecimento, o que pode ter consequências negativas na energia necessária para satisfazer as maiores necessidades energéticas em condições futuras de aquecimento. Contudo, a acidificação dos oceanos teve efeitos ainda mais prejudiciais sobre o comportamento dos peixes do que o aquecimento. Os comportamentos natatórios e predatórios diminuíram drasticamente,

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indicando que as larvas entraram num estado letárgico. Das espécies analisadas, o comportamento natatório do dourado mostrou ser o mais afetado pela acidificação, tendo-se observado um aumento da exaustão desta espécie quando comparado com o comportamento natatório da dourada e corvina (aumento de exaustão na ordem dos 36,60 e 52,90%, respectivamente). Os comportamentos predatórios (ataque e captura de presas) da dourada foram significativamente mais afectados do que os da corvina.

Quando expostos a flutuações ambientais, muitos organismos dispõem de uma primeira linha de proteção contra o stress oxidativo gerado, que é composta pela ativação de mecanismos de proteção fisiológicos, tais como o aumento da resposta ao choque térmico e ativação de enzimas antioxidantes (catalase, CAT e glutathione S- transferase, GST). A capacidade de resposta ao choque térmico é caracterizada pela síntese de proteínas denominadas proteínas de choque térmico (HSP70) que têm a função de reparar e eliminar proteínas danificadas ou desnaturadas. Apesar da ativação destas respostas em condições de aquecimento e acidificação, nomeadamente aumento de cerca de 147,86% da HSP70/HSC70 e ativação de enzimas antioxidantes CAT e GST (aumento de 88,29% e 71,97%, respectivamente), os mecanismos de defesa parecem ter sido insuficientes para lidar com as novas condições tendo sido evidente um elevado aumento do dano celular (indicado pelo aumento da peroxidação lipídica). Para além das alterações acima mencionadas, as condições futuras para os oceanos também afetaram a atividade das enzimas digestivas. A temperatura bem como o pH são factores igualmente importantes que moldam o processo de digestão de diversos organismos. Enquanto o aquecimento produziu um aumento na atividade das enzimas pancreáticas (tripsina) e intestinais (fosfatase alcalina e amilase), a acidificação induziu uma diminuição na atividade das enzimas digestivas.

Posto isto, os resultados obtidos na presente tese fornecem uma valiosa visão de como as futuras condições ambientais podem vir a prejudicar o desenvolvimento embrionário e larval de várias espécies de peixes. Os impactos sobre a sobrevivência larvar revelaram ser mais prejudiciais para as espécies mais ativas com uma estratégia de vida planctônica que apresenta custos metabólicos associados mais elevados. Os profundos impactos negativos causados nestes estados de desenvolvimento colocam em perigo o sucesso ecológico e persistência das espécies, e a longo prazo podem somar-se ainda graves consequências ecológicas e económicas.

LIST OF PAPERS

It is hereby declared that the author of this thesis participate and was responsible for the conception and design of the work, fish larvae rearing, sample collection, laboratory analytical procedures, data analysis and writing of the respective manuscripts. Remaining authors collaborated in some or several of these procedures. All papers published were included with the publishers' agreement. As listed below this thesis comprise a total of five scientific papers, three published in peer-reviewed international journals and two submitted articles in international journals, which can be found from Chapter 2 to 6.

CHAPTER 2

Defective skeletogenesis and oversized otoliths in fish early stages in a changing ocean

Marta S. Pimentel, Filipa Faleiro, Gisela Dionísio, Tiago Repolho, Pedro Pousão-Ferreira, Jorge Machado and Rui Rosa. Published in *The journal of Experimental Biology* (2014) 217: 2062-2070, doi:10.1242/jeb.092635

CHAPTER 3

Foraging behaviour, swimming performance and malformations of early stages of commercially important fishes under ocean acidification and warming

Marta S. Pimentel, Filipa Faleiro, Tiago Marques, Regina Bispo, Gisela Dionísio, Ana Margarida Faria, Jorge Machado, Myron A. Peck, Hans Pörtner, Pedro Pousão-Ferreira, Emanuel J. Gonçalves, Rui Rosa. In review in *Climatic Change*

CHAPTER 4

Impact of ocean acidification in the metabolism and swimming behavior of the dolphinfish (*Coryphaena hippurus*) early larvae

Marta Pimentel, Maria Pegado, Tiago Repolho, Rui Rosa. Published in *Marine Biology* (2014) 161:725-729

CHAPTER 5

Metabolic potential of fish early stages with different life strategies and locomotory abilities under ocean warming and acidification

Marta S. Pimentel, Filipa Faleiro, Jorge Machado, Myron A. Peck, Hans-O. Pörtner, Rui Rosa. In review in *Journal of Comparative Physiology B*

CHAPTER 6

Oxidative stress and digestive enzyme activity of flatfish larvae in a changing ocean

Marta S. Pimentel, Filipa Faleiro, Mário Diniz, Jorge Machado, Pedro Pousão-

Ferreira, Myron A. Peck, Hans O. Pörtner, Rui Rosa. Published in PLoS ONE (2015)

e10(7), 0134082. doi:10.1371/journal.pone.0134082

CHAPTER 1

1. General Introduction

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GENERAL INTRODUCTION

1.1 Global changes

Global emissions and the accumulation in the atmosphere of so-called greenhouse gases (e.g. carbon dioxide, CO₂) rose dramatically during the 20th century, contrasting with the previous 800 thousands of years with relatively stable levels (Fig. 1a). Since the industrial revolution, fossil fuel combustion and industrial processes have released tons of carbon into the atmosphere, actually this value surpasses over six billion metric tons per year. As a consequence, atmospheric CO₂ concentrations have greatly increased from pre-industrial levels of 280 μatm to present-day levels of 398 μatm (IPCC 2013), at a rate of ~1% to 3.4% per year (Le Quéré et al. 2009). Climate experts predict that future levels may reach 1000 μatm by the end of the century (Caldeira and Wickett 2003; IPCC 2013) if anthropogenic emissions remain within the same rates. The impact of climate change is one of the most significant environmental challenges facing the world today, and is primarily driven by the increase CO₂ concentration (IPCC 2013). Carbon accumulation overloads the atmosphere, and the consequently trapped heat cause Earth to warm.

There is an overwhelming scientific consensus that there will be a global warming during the current century and further on. Assessed projections have estimated global average surface temperature to increase by an additional 1.1-6.4°C, and that frequency of heat waves will also increase and become more extreme by the end of the century (Fig. 1b) (IPCC 2013). These increasing CO₂ concentrations in the atmosphere can have three fates, remain in the atmosphere as mentioned above or can be absorbed by the terrestrial biosphere or by the oceans (Le Quéré et al. 2009). By absorbing atmospheric carbon, the terrestrial and ocean sinks mitigate climatic changes, however their efficiency may decrease generating greater climate perturbations.

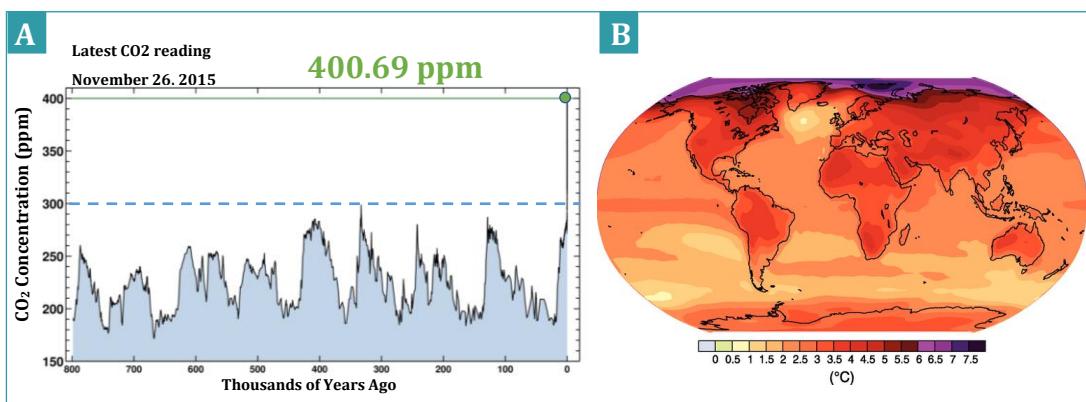


Figure 1. (A) Carbon dioxide concentration levels from previous 800 thousands of years until November 26th 2015 (Graph from NOAA and data from Mauna Loa Observatory); and (B) projected surface temperature changes for the late 21st century (figure SPM.6. from IPCC 2007 report); temperatures are relative to the period 1980-1999.

1.1.1 Oceans

The oceans play a key role in mitigating climatic changes, sequestering heat and carbon from the atmosphere. As a consequence of heat absorption from the atmosphere, oceans are becoming warmer at a rate of approximately 0.1°C over the last decades (IPCC 2013). Climate experts predict oceans temperature to increase by a further 4°C (IPCC 2013). In the last decades, oceans stored more than 90% of the atmosphere heat content (IPCC 2013), however the heat-uptake by the oceans may decrease over time, allowing the atmosphere to warm. Unfortunately, the consequences of increasing CO₂ emissions are not only restricted to global and ocean warming. The oceans represent the major carbon sink, storing the CO₂ from the atmosphere. Over the past centuries, evidences indicate that oceans have absorbed approximately 25% of the anthropogenic CO₂ emissions (Sabine et al. 2004). The continuous CO₂ uptake will change seawater chemistry, when combined with water it forms carbonic acid (H₂CO₃), which dissociated into bicarbonate (HCO₃⁻) and carbonate (CO₃²⁻) ions and releases hydrogen ions (H⁺). The increase of H⁺ concentrations will increase partial pressure of CO₂ (hypercarnia) and will thereby reduce ocean's pH, a process known as ocean acidification (OA). Subsequently, CO₃²⁻ concentrations and saturation states of calcium carbonate (CaCO₃) minerals aragonite (Ω_{ar}) and calcite (Ω_{ca}) on the ocean surface waters will decrease during this process (Feely et al. 2008). Ocean

chemistry can vary between waters and some coastal areas like estuaries and upwelling systems are already experience levels beyond those projected in the offshore surface ocean (Frankignoulle et al. 1998). Since pre-industrial times, ocean's pH has already dropped an average of 0.1 units (Meehl et al. 2007), representing a 30% increase in H⁺ ions, and it is predicted that this process will lead to a further decrease of 0.4–0.5 units (Caldeira and Wickett 2005) (Fig. 2). However, if the international Kyoto Protocol fails to slow global carbon emissions the rate of change is expected to be faster than the experienced over the last 300 million years (Honisch et al. 2012), driving ocean's pH to decrease of approximately 0.77 units (IPCC 2013). Although global warming is recognized to affect the ecological structure and functioning of marine ecosystems, the consequences of ocean acidification for marine ecosystems are only beginning to be revealed.

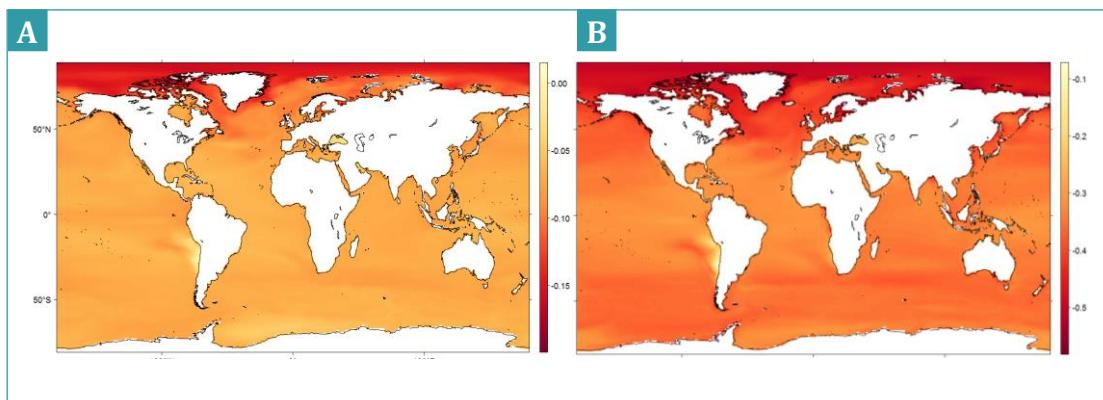


Figure 2. (A) Changes in ocean surface pH (1976-2005 to 2071-2100) for the IPCC AR5, RCP 2.6 scenario; (B) RCP 8.5 scenario, graphs are courtesy from Joana Boavida-Portugal.

1.2 Impacts of climate change on marine biota and ecosystems

Future ocean climate-related changes are expected to challenge many marine organisms across multiple levels of biological organization, from molecular to organismic level, and are predicted to elicit cascading effects on population, community and ecosystems dynamics (Pörtner et al. 2004; Pörtner et al. 2005; Fabry et al. 2008; Pörtner 2008; Brierley and Kingsford 2009; Harvey et al. 2014; Beaugrand et al. 2015). When species persistence is affected by climate change-related conditions, organisms can respond by acclimatizing and adapting to new conditions, or by shifting their geographical distribution. Changes in biodiversity may alter the community structure and possibly disrupt ecological interactions,

enhancing the risk of species and ecosystems extinction. However, species responsiveness to climate change is intrinsically linked to their life stage, mobility, tolerance window and sensitiveness to the different physical factors (Melzner et al. 2009). The research field on the impacts of ocean warming and acidification on marine organisms it is still an emerging issue, given the fast pace of climate change there is an urgent need to fulfill the current knowledge gaps (Wernberg et al. 2012; Todgham and Stillman 2013). Particularly, there is still a limited understanding on the consequences of synergistic effects of multiple stressors, on vulnerability of sensitive life-stages especially early ontogenetic stages, and on the effect of transgenerational adaptation to climate-change related conditions.

1.2.1 Ocean warming

Ocean temperature is changing at unprecedented rates, and the impacts on marine organisms and ecosystems are also likely unprecedented. Temperature is one of the key environmental factors impacting many biological functions and ecological processes in a wide range of marine ectothermic organisms (Pörtner et al. 2006; Brierley and Kingsford 2009). The extent of its impact is extremely variable and it depends on species thermal range and development stage. Additional thermal stress imposed by future ocean warming is expected to especially favor those organisms that do not live close to their thermal limits and are consequently more capable to tolerate temperature changes (Stillman and Somero 2000; Helmuth et al. 2006; Hoegh-Guldberg et al. 2007; Calosi et al. 2008; Tewksbury et al. 2008; Hofmann and Todgham 2010). For example, intertidal organisms and temperate species are likely to have a greater resilience and scope for temperature acclimation, because already experience greater range of temperature fluctuations (Stillman 2003). Acclimation to warming might include phenotypic modifications, namely physiological, behavioural or morphological, to enhance species survival chance in the new environment conditions (Fry 1967; Hazel and Prosser 1974; Randall et al. 2000; Woods and Harrison 2002). Many of these processes are optimal within a narrow thermal tolerance window, which contributes to set performance levels. The limits where performance begins to decrease are denoted as pejus temperature, and within these limits organisms are still capable to induce compensatory mechanisms (Pörtner et al. 2006). However, when temperatures drive organisms' outside thermal optimum, their aerobic scope and performance

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might be reduced and constrained by limited capacity of oxygen supply mechanisms (circulatory and ventilator systems) (Pörtner et al. 2006). The reduction of aerobic scopes will progressively lead to an anaerobic mode of energy production, a compensatory mechanism that only supports survival during short periods (Fig. 3).

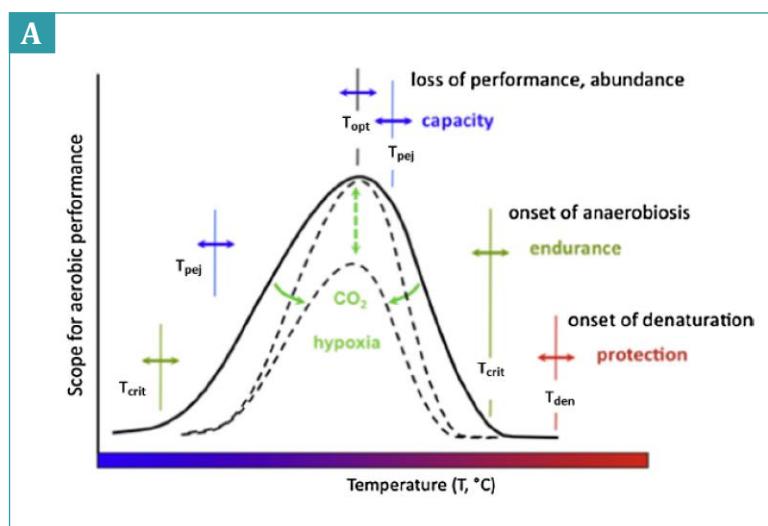


Figure 3. Thermal window of performance and oxygen- and capacity-limited thermal tolerance concept (OCLTT) (Pörtner et al. 2006; Pörtner 2010).

Beyond this point, changing temperatures may lead to a loss in integrity of molecular structures, which progressively activates antioxidant defense and heat-shock response to contribute to extend the period of passive tolerance (Pörtner et al. 2006; Pörtner 2010). As this critical threshold is approached or exceeded, many biological processes, such as metabolism, growth, behaviour, feeding and reproduction, and biochemical mechanisms, are negatively affected and imminent death might be the consequent outcome. These constraints might thereby compromise the overall fitness, survival, distribution and abundance of the species (e.g. Roessig et al. 2004; Hoegh-Guldberg et al. 2007; Pörtner 2010; Byrne 2011; Rosa et al. 2012; Rosa et al. 2013; Rosa et al. 2014a; Vasseur et al. 2014). Responses to climate change are already taking place, in fact, some studies have reported modification in the distribution ranges and phenology of many organisms (Perry et al. 2005; Harley et al. 2006).

1.2.2 Ocean acidification

Exposure to ocean acidification has been shown to narrow the thermal tolerance window of many marine ectothermic animals (Pörtner and Farrell 2008), and consequently intensify the effect of increasing sea surface temperature on physiological processes of marine organisms (Fig. 3), e.g. growth, calcification, reproduction, behaviour and metabolism (Fabry et al. 2008; Pörtner 2008; Melzner et al. 2009; Hofmann et al. 2010; Hofmann and Todgham 2010). Thus marine organisms may be likely more vulnerable to changes in ocean CO₂ concentrations (Metzger et al. 2007; Rosa and Seibel 2008; Rosa et al. 2014a), especially those already near their thermal tolerance limits. The synergism between both environmental stressors may further drive organisms outside their tolerance boundaries, compromising the overall fitness and survival of species. However, until nowadays, few research have focused on the effect of these synergies (Byrne 2011; Flynn et al. 2015).

Estimated levels of ocean acidification have already revealed to negatively influence a diversity of marine organisms and ecosystems. Calcium carbonate (CaCO₃) ions are the key component of the seawater carbonate system that controls calcification rates of shell-building marine animals. Thus, changes in the carbonate chemistry, specifically the reduced availability of CaCO₃ and saturation state of CaCO₃ poses a greater threat to those calcifying organisms who build and rely on carbonate exoskeletons for their existence, e.g. calcareous plankton, oysters, clams, sea urchins and corals. These organism combine calcium ion with carbonate ions from surrounding seawater to produce their shells and skeletons, and a decline in carbonate ions will have a direct effect on the ability of these organisms to produce biogenic carbonate. However consequences do not stop here, major repercussions on the metabolism, growth and reproduction of calcifying organisms have already been reported (e.g. Byrne 2011). Because of the direct implication for calcification processes on calcifying organisms, only recently research has started to focus on the effect of ocean climate change on non-calcifying organisms. Increased CO₂ levels may also be responsible for deleterious effects on the survival, growth, development, behaviour and physiology of non-calcifying marine species, including some molluscs and fishes (Fabry et al. 2008; Rosa and Seibel 2008; Munday et al. 2009; Munday et al. 2011a; Rosa et al. 2012; Stumpp et al. 2012; Jutfelt et al. 2013; Rosa et al. 2013; Rosa et al. 2014a)

1.3 Fishes in a changing ocean

Fish were generally assumed to be quite resilient to future ocean conditions than other marine organisms because they possess an effective acid–base regulatory mechanism. This efficient mechanism is energetically costly but allows fish to maintain their homeostasis and compensate for extra (pH_e) and intracellular (pH_i) disturbances (Toews et al. 1983; Larsen et al. 1997; Hayashi et al. 2004; Michaelidis et al. 2007), by accumulating bicarbonate in body fluids and by exchanging ions across gills (Pörtner et al. 2005; Ishimatsu et al. 2008; Melzner et al. 2009). However, acute exposition to ocean warming and acidification is likely to overwhelm acid-base regulation, with major repercussions for tissues and body fluids pH and oxygen transport. The consequent reduced oxygen carrying oxygen capacity and the extra energy expenditure associated with acid-base regulation might reduce fish aerobic performance (Pörtner and Farrell 2008). In fact, several studies have already reported fish vulnerability to such climate related changes (e.g. Ross et al. 2001; Fifelstad et al. 2003; Ishimatsu et al. 2008; Munday et al. 2010; Esbaugh et al. 2012; Enzor et al. 2013). Most research on biological climate-related impacts has been conducted on non-commercially fish species, and until now only a few related studies have been undertaken on commercially important species, e.g. codfish (Frommel et al. 2014), Atlantic herring (Franke and Clemmesen 2011), mahi-mahi (Bignami et al. 2014) and yellowfin tuna (Bromhead et al. 2015). In comparison to most research performed in juvenile and adult fishes, data on the effects of climate change on early ontogenetic stages is critically lacking, although they are expected to be more prone to environmental stressors than adult fish (reviewed by Pörtner et al. 2005). The less efficient and specialized ion-regulatory mechanisms to maintain internal ionic environment (Morris et al. 1989; Sayer et al. 1993) and also the large surface to volume ratio, increases fish early stages vulnerability to future ocean conditions. As slightly changes on larval performance (e.g. growth and survival) can have cumulative effects on recruitment (Houde 1997), it is thus critical to completely understand the impacts of ocean climate changes on these ontogenetic stages. Their eventual inability to cope and adapt may constitute a bottleneck for species persistence in a changing ocean. Given the importance of larval performance to the year-class success in marine fish populations (Houde 2008; Peck et al. 2012), it is crucial to understand the vulnerability of early stages of fish species to climate-driven changes and the

consequent effects on the distribution and abundance of marine fish stocks. Some studies have already scrutinized fish early life stages vulnerability to ocean's future conditions. Among these studies there is no consistency, while some have reported direct effects on survival, growth, metabolism, otoliths and behaviour (Munday et al. 2009; Franke and Clemmesen 2011; Baumann et al. 2012; Bignami et al. 2013; Frommel et al. 2014), others have found no significant effects of climate change on fish larvae (Munday et al. 2011b; Harvey et al. 2013; Hurst et al. 2013; Maneja et al. 2013), suggesting the specificity of the responses to climate change. Particularly, for some species it has been reported a negative effect on size and growth under ocean acidification (Baumann et al. 2012; Frommel et al. 2014), however, for others faster or null growth has been described under the same conditions (Hurst et al. 2012; Hurst et al. 2013). This inconsistency reveals that besides being stage-specific fish vulnerabilities to climate-related factors might also be species-specific, thereby the impacts of ocean warming and acidification may have more complex impacts on marine food web dynamics, influencing species interactions within ecosystems.

1.4 Fish physiological and biochemical challenges

Species' tolerance to ocean climate change is primarily molded through organism's physiological performance. Thus, to have a more comprehensive and integrated view of the ability of fish to undergo ocean global changes, fish performance should be analyzed by measuring several features such as physiological, biochemical and cellular stress biomarkers. Responses will differ depending on whether the environmental stressors effects are towards or away from the organism's optimum. The capabilities of these features to adapt to ocean climate change may determine species survival under future oceans environment. Recent work indicates that ocean warming and acidification may have adverse consequences on fish physiological and biochemical processes, which may result in changes on biodiversity, trophic interactions, and other ecosystem processes (Society 2005; Kleypas et al. 2006). These approaches help to identify fish thresholds of stress, namely, at which level organisms experience serious limitations.

1.4.1 Acid-base regulation

Teleost fishes are equipped with efficient ion-regulatory machinery for CO₂ excretion and acid-base regulation. This ion-transport system is located on gills, the primary contact point for environmental stressors, which accounts for about 90% or more of the organism's total acid-base and related ion compensation (Claiborne et al. 2002). Specific ATP-consuming ion transporters, such as Na⁺/H⁺, Na⁺/HCO₃⁻, V-type H⁺-ATPase, H⁺/K⁺-ATPase, Na⁺/K⁺-ATPase and Cl⁻/HCO₃⁻, are involved in the acid and base-secretion of fishes (Sullivan et al. 1996; Claiborne et al. 1999; Edwards et al. 2001; Perry et al. 2003; Choe et al. 2004; Tresguerres et al. 2005). Generally the compensatory mechanisms that occur during acid-base regulation are being assumed as responsible for a large number of downstream effects, which may result into physiological and behavioral adjustments. During exposure to increased CO₂ partial pressure (hypercapnia) the environmental CO₂ enters by diffusion across gill epithelia into fish tissues and fluids, causing CO₂ accumulation in their blood. If not actively compensated by HCO₃⁻ accumulation and/or H⁺ secretion blood pH may decrease, resulting into an extracellular acidosis (Melzner et al. 2009). Moreover, plasma pH changes may constrain the capacity of oxygen supply and delivery (Pörtner et al. 2004) and negatively affect marine fishes performance. This regulatory mechanism has been considered as "the first line of defense against hypercapnia disturbances of metabolic and tissue functioning" (Pörtner 2008) and is vital for the preservation of cellular functions and maintenance of homeostasis, especially under acute exposition to high CO₂ environment. Maintenance of intracellular and extracellular pH is essential to avoid metabolic depression (Pörtner 2008) and growth cessation. These hypercapnia-induced disorders can progressively lead to a shift on the energy production mode. Moreover, the concurrently accumulation of HCO₃⁻ and Cl⁻ reduction resultant from acid-base balance regulation in high CO₂ environment (Brauner and Baker 2009) may interfere with the function of brain neurotransmitters, GABA-A receptors.

Although the knowledge about fish adults and juveniles acid-base regulation in response to hypercapnia is wider, little is known about the mechanisms and pathways of acid-base regulation in larval fishes (Brauner 2008).

1.4.2 Metabolic adjustments

Metabolic adjustments are likely the consequence of acid-base regulatory imbalance and hypercapnia-induced extracellular acidosis (Heuer and Grosell 2014), therefore metabolic responses measured as oxygen consumption rate may give a clear indication of fish physiological performance and potential resilience of organisms exposed to climate-related conditions (Sokolova et al. 2012). Fish metabolic rates are intrinsically reliant on environmental temperature. Within organisms thermal window, a slightly increase in temperature may be beneficial as there is more energy available for biological processes and biochemical reactions. However, increased temperature accelerates organism's metabolic demand for oxygen ($Q_{10} = 2\text{-}3$, at normal operating temperatures) (Moran and Woods 2007) up to a point. Beyond this temperature threshold (pejus temperature, T_p), the maximum capacity for oxygen uptake, namely cardiac and ventilatory capacities, can no longer keep pace with the increase metabolic demands (Pörtner and Knust 2007). It is important to note that the increase in metabolic demands differs within different life history stages. Early ontogenetic stages are recognized to be more challenged due to their narrow metabolic scope than juveniles or adults (Cunha et al. 2007), which may result for example in the reallocation of metabolic resources away from somatic growth. The continued increase in temperature above organism's critical thresholds enhances thermal limitation through the loss of aerobic scope, leading to anaerobic respiration alongside with protein denaturation, permanent inactivation of enzymes, growth cessation, and eventual death (Katersky and Carter 2007; Wang and Overgaard 2007).

As previously mentioned, exposure to ocean acidification narrows the thermal tolerance window of many organisms (Pörtner and Farrell 2008), including fishes. When organisms are outside their thermal tolerance window are likely to be more vulnerable to changes in ocean CO_2 concentrations (e.g. Metzger et al. 2007; Rosa and Seibel 2008), compromising some physiological processes (e.g. metabolism). Earlier studies demonstrated that exposure to ocean acidification can induce acidosis (e.g. Reipschläger and Pörtner 1996) which is believed to suppress the metabolic rates of many species (Langenbuch and Pörtner 2003; Michaelidis et al. 2007; Fabry et al. 2008; Pörtner 2008). Metabolic suppression is characterized by shutting down expensive processes, e.g. protein synthesis, growth and specific ATP-consuming ion transporters, Na^+/K^+ ATPase, a strategy known to limit aerobic

scope and reduce animal fitness (Brett 1958). Hypercapnia-induced acidosis also inhibits activities of metabolic enzymes (Somero 1985), and therefore variations on aerobic performance of organisms can be also a reflect of adjustments on a specific metabolic pathway (Pörtner and Farrell 2008; Pörtner 2010; Pörtner 2012; Strobel et al. 2013), namely the inhibition of enzymes of the Krebs cycle (e.g. citrate synthase) and the contra-balanced over-expression of anaerobic enzymes (e.g. lactate dehydrogenase). The increase in glycolytic potential and metabolic suppression are common tactics used by organisms to enhance tolerance and survival under future environmental ocean conditions (Hochachka and Somero 2002), however are likely to be a tradeoffs not bearable on longer time-scales.

1.4.3 Behaviour

Most research on the effects of ocean acidification on fish has focused on behaviour and sensory systems. These impairments have been widely studied in numerous species with different life stages and in geographic distributions (Claiborne et al. 2002; Ferrari et al. 2011; Domenici et al. 2012; Ferrari et al. 2012). Exposure to hypercapnia has been described to impair olfactory and chemosensory abilities of a wide range marine fish species, through disruption of the acid-base homeostasis. The adjustment of new acid-base homeostasis under hypercapnia can cause alterations on GABA-A receptors functions in some brain neurotransmitter, reversing GABAA receptor function (Nilsson et al. 2012). This inhibitory receptor is an ion channel with conductivity for HCO_3^- and Cl^- (Bormann et al. 1987) that under high ambient CO_2 might become excitatory (Nilsson et al. 2012), and affects several behaviours of marine organisms (e.g. Simpson et al. 2011; Ferrari et al. 2012; Leduc et al. 2013; Munday 2014). Overall, under future ocean scenario fish exhibit riskier behaviour, mostly by decreasing anxiety and increasing boldness (Munday et al. 2014; Ou et al. 2015). Behaviour impairments may have directly consequences for the timing of settlement, dispersal, habitat selection, social interactions predator avoidance and individual fitness (Cripps et al. 2011; Devine et al. 2012a; Devine et al. 2012b; Munday et al. 2012).

1.4.4 Digestion

Temperature and pH are among the major factors influencing the biochemical reactions involved in the digestion process. The enzymatic regulation plays a vital role in digestion, absorption and nutrients transition (e.g. Swarup 1981), thus a correct development of the digestive system is essential to transform macronutrients from food into a form that can be easily digested, absorbed and assimilated, in order to supply dietary nutrients required for normal growth and development (Zambonino-Infante and Cahu 2001). The digestive enzymes (pancreatic and brush border intestinal enzymes) are part of the metabolic regulatory mechanisms (Hochachka and Somero 2002) and are thus widely used in studies as markers of fish larval development and as indicators of fish condition and physiological state (Zambonino-Infante and Cahu 2001; Fernández et al. 2008; Zambonino-Infante et al. 2008; Fernández et al. 2009). Any modifications on stability of digestive enzymes can therefore influence fish metabolism and adaptive capacity (Wang et al. 2001). Until now, the impact of future ocean climate-related changes on the digestive efficiency and enzymatic activity of marine organisms had only been investigated on marine invertebrates (Bechmann et al. 2011; Stumpp et al. 2013; Rosa et al. 2014b). However, very few information exists on how predicted ocean warming and acidification affects the digestive processes of marine fish early stages. Morphological abnormalities in the digestive system (namely gut and pancreas) of teleost early stages under ocean acidification have already been reported (Frommel et al. 2012; Frommel et al. 2014), yet the association between altered functional development and digestive enzymatic impairments is still to be proven.

1.4.5 Antioxidant defense

All living organisms respond to environmental disturbances by adjusting physiological protective mechanisms, such as antioxidant defense and heat-shock response (Tomanek 2010). Environmental stress, including ocean warming and acidification, can enhance the formation of reactive oxygen species (ROS) and the decrease efficiency of ROS elimination systems raises oxidative stress. ROS are molecules and free radicals, such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO\cdot$), and are naturally produced as byproducts of

aerobic respiration (Cadenas 1989; Pannunzio and Storey 1998; Lesser 2006). Mitochondria are the major source of ROS within most cells, mostly formed at the electron transport. These active species play a major role in homeostasis and redox signaling from the organelle to the rest of the cell, however within stressful conditions they can also affect cellular integrity (Lesser 2011), namely by oxidative damage to proteins, DNA and lipids (Halliwell 2006; Halliwell and Gutteridge 2006). Lipid peroxidation is the most frequent injury processes where ROS react with unsaturated fatty acids present in cellular membranes (Lesser 2011), a common target for free radicals. This typically occurs as a chain reaction where a free radical will capture hydrogen from the lipid molecules to initiate and to further propagate the chain reaction. Consequently, this will lead to an increase of malondialdehyde (MDA) levels, a specific end product of the oxidative degradation process of lipids. Organisms often rely on an efficient antioxidant mechanism, characterized by a powerful repertoire of antioxidant enzymes that can together detoxify ROS production (Abele and Puntarulo 2004), a paramount mechanism to the survival of all aerobic life forms. This set of antioxidant enzymes includes, superoxide dismutase (SOD), which converts O₂ in H₂O₂; catalase (CAT), which converts H₂O₂ to H₂O and O₂ to prevent H₂O₂ accumulation in cells and tissues; glutathione reductase (GR) is responsible to supply reduced glutathione (GSH) to cells; GSH reacts with O₂, O₂^{•-} and OH[•] and can also break the free-radical chain reactions; glutathione peroxidise (GPX) catalyzes the oxidation of GSH with H₂O₂, and glutathione-S-transferase (GST), which, in association with GSH, converts xenobiotics into other conjugates as part of a detoxification route (Lesser 2006). Additionally when exposed to environmental stressors, organisms display a heat shock response (HSR), which involves the induction of heat shock proteins (HSPs) to protect cells from ROS. HSP are a highly conversed group of molecular chaperones essential to repair, refold, and eliminate damaged or denatured proteins (Tomanek 2010). These molecular chaperones have been classified into different families, according to their molecular size (e.g. HSP70), and cooperate in proteins protection from environmental stressors. Overall, the antioxidant response and HSR are critical biological tools to improve organism's survival, however it is achieved at the cost of significantly energetic investments into cellular protection and maintenance (Somero 2002; Hofmann 2005; Dong et al. 2008).

1.5 Objectives and thesis outline

Given the growing international awareness about the fast pace of climate change and the urgent need for an assessment of the consequences of climate change on the world's oceans, the main goal of this thesis was to gain predictive power to characterize and quantify the effects of climate change on fish early ontogenetic stages. Overall, I aimed to investigate the effects of projected ocean warming (+ 4°C) and acidification ($\Delta\text{pH}=0.5$) and their potential synergistic effects on the early life stages of several marine fish species with different life strategies, *Sparus aurata*, *Argyrosomus regius* and *Coryphaena hippurus* (pelagic continuous-swimmers) and *Solea senegalensis* (benthonic poor-swimmers), and to understand the mechanisms used by them to withstand (nor not) the future changes. This thesis attempted to integrate information in a comprehensive and integrative view of several biological responses of fish early stages to climate change, namely: phenotypic altering of developmental, skeletal, behavioral and ecophysiological features. The thesis is composed by seven chapters and includes five scientific papers published or *in press* in peer-reviewed international journals, which can be found from chapter 2 to 6. Specifically, the main objectives of the chapters are presented below:

- i. Investigate how the future predictions of ocean warming and acidification affects the development of early life stages of marine fish species, namely hatching success, larval survival and growth rates (Chapter 2 and 3);
- ii. Evaluate the effects of ocean warming and acidification on the otoliths, incidences of skeletal and body malformations (Chapter 2 and 3);
- iii. Analyze the impact of ocean warming and acidification on behaviour patterns of larvae of several of marine fish species (Chapter 3 and 4);
- iv. Evaluate the effects of ocean warming and acidification on the metabolic rates and thermal tolerance limits response of early life stages of marine fish species (Chapter 2, 3 and 4);
- v. Understanding possible shifts in metabolic pathways within species with different life strategies and locomotory abilities (Chapter 5);
- vi. Evaluate the heat shock response and antioxidant defense mechanism to prevent cellular damage during larvae exposure to ocean warming and acidification (Chapter 6);
- vii. Analyze how ocean warming and acidification affects the digestive enzymes activity of fish larvae (Chapter 6).

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CHAPTER 2

Defective skeletogenesis and oversized otoliths in fish early stages in a changing ocean

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RESEARCH ARTICLE

Defective skeletogenesis and oversized otoliths in fish early stages in a changing ocean

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ABSTRACT

Early life stages of many marine organisms are being challenged by rising seawater temperature and CO₂ concentrations, but their physiological responses to these environmental changes still remain unclear. In the present study, we show that future predictions of ocean warming (+4°C) and acidification ($\Delta\text{pH}=0.5$ units) may compromise the development of early life stages of a highly commercial teleost fish, *Solea senegalensis*. Exposure to future conditions caused a decline in hatching success and larval survival. Growth, metabolic rates and thermal tolerance increased with temperature but decreased under acidified conditions. Hypercapnia and warming amplified the incidence of deformities by 31.5% (including severe deformities such as lordosis, scoliosis and kyphosis), while promoting the occurrence of oversized otoliths (109.3% increase). Smaller larvae with greater skeletal deformities and larger otoliths may face major ecophysiological challenges, which might potentiate substantial declines in adult fish populations, putting in jeopardy the species' fitness under a changing ocean.

KEY WORDS: Ocean warming, Acidification, Fish larvae, Ecophysiology, Skeletal deformities

INTRODUCTION

Atmospheric carbon dioxide (CO₂) concentration has increased from pre-industrial levels of 280 μatm to present-day levels of 394 μatm, and it is expected to rise to 730–1000 μatm by the end of the century (Caldeira and Wickett, 2003; Meehl et al., 2007). Continuous CO₂ uptake by the world's oceans is changing the seawater chemistry and is estimated to lead to a drop of 0.4–0.5 units in seawater pH (Caldeira and Wickett, 2005). Concomitantly, the temperature of the oceans is rising, and global sea surface temperature is expected to increase ~4°C by 2100 (Meehl et al., 2007), leading to profound impacts on marine ecosystems. In fact, the predictable rapid rate of climate change will induce thermal stress to coastal marine biota as their thermal tolerance limits are reached or even exceeded. Beyond a certain thermal limit, biological processes such as metabolism, growth, feeding, reproduction and behavior may be affected (Carmona-Osalde et al., 2004; Pörtner and Knust, 2007; Nilsson et al., 2009; Byrne, 2011; Pimentel et al., 2012; Rosa et al., 2012), thus compromising the overall fitness and survival of species.

Additionally, under higher temperatures, marine organisms are likely to be more vulnerable to other environmental stressors such as ocean acidification (Pörtner, 2008; Byrne et al., 2010; Findlay et al., 2010; Parker et al., 2010; Sheppard-Brennan et al., 2010; Byrne, 2011; Rosa et al., 2013; Rosa et al., 2014).

Ocean acidification is considered a major threat to marine organisms as it may lead to acid–base balance disturbances, protein biosynthesis decrease, metabolic depression and growth reduction (Seibel and Walsh, 2001; Pörtner et al., 2004; Langenbuch et al., 2006; Rosa and Seibel, 2008; Baumann et al., 2012). Exposure to elevated CO₂ particularly affects calcifying organisms (Orr et al., 2005; Dupont et al., 2008; Fabry et al., 2008; Talmage and Gobler, 2010), although detrimental effects on survival, growth and respiratory physiology of non-calcifying marine animals have also been observed (Seibel and Walsh, 2001; Rosa and Seibel, 2008; Munday et al., 2009b).

Fish have developed an effective acid–base regulatory mechanism, which allows them to accumulate bicarbonate and exchange ions across gills under hypercapnic conditions (Pörtner et al., 2005; Ishimatsu et al., 2008; Melzner et al., 2009). While this is true for adult organisms, early life stages may not benefit from it, as they lack well-developed and specialized ion-regulatory mechanisms to regulate and maintain their internal ionic environment (Morris, 1989; Sayer et al., 1993). Therefore, early life stages are expected to be the most vulnerable to ocean climate-change-related conditions and their eventual inability to cope and adapt may constitute a bottleneck for species persistence in a changing ocean (Bauman et al., 2012; Frommel et al., 2012). Until now, only a few studies have scrutinized the impact of ocean climate change on fish larvae performance. While some report negligible effects of ocean acidification on fish larvae (Munday et al., 2011b; Hurst et al., 2012; Harvey et al., 2013; Hurst et al., 2013; Maneja et al., 2013), others demonstrate that ocean warming and acidification may have a direct impact on embryonic development, larval growth, metabolism, behavior and survival (Bauman et al., 2012; Franke and Clemmesen, 2011; Frommel et al., 2012; Bignami et al., 2013; Pimentel et al., 2014). More recently, it has also been shown that larval otoliths can be affected by changes in seawater carbonate chemistry (Checkley et al., 2009; Munday et al., 2011a; Bignami et al., 2013), but the impact of hypercapnia on larval fish skeletogenesis still remains unclear.

In the present study, we investigated how the combined effect of warming (+4°C) and high partial pressure of CO₂ ($p\text{CO}_2$; 0.16% CO₂, ~1600 μatm, $\Delta\text{pH}=0.5$) affects the hatching success, larval survival, growth, metabolic rates, thermal tolerance limits and skeletogenesis of early life stages of a flatfish, *Solea senegalensis* Kaup 1858, with major commercial importance. This teleost fish is an environmentally resilient species that inhabits the Western Iberian Upwelling Ecosystem, the northern limit of the Canary Current Upwelling System, one of the four major eastern boundary currents

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of the world, where $p\text{CO}_2$ levels may reach up to $\sim 500 \mu\text{atm}$ (Álvarez-Salgado et al., 1997; Pérez et al., 1999; Borges and Frankignoulle, 2002). Thus, organisms inhabiting such upwelling ecosystem are commonly exposed to seasonal high $p\text{CO}_2$ events, because of the emergence of deep hypercapnic water masses. In these regions, the future $p\text{CO}_2$ levels are thus expected to exceed the forecasted 1000 μatm for 2100 (Meehl et al., 2007).

RESULTS

Hatching success, larval growth and survival

The impact of high $p\text{CO}_2$ and environmental warming on the hatching success, survival, length and growth of *S. senegalensis* larvae is shown in Fig. 1 (see also supplementary material Table S1). Warming had a significant negative impact on the hatching success of sole larvae ($P<0.05$), but neither hypercapnia ($P>0.05$) nor the interaction between hypercapnia and warming had a significant effect ($P>0.05$). The hatching rates decreased from $86.7\pm 5.8\%$ at the present-day scenario to $70.0\pm 10.0\%$ under the future hypercapnic and warming conditions (Fig. 1A).

Survival rates of 30 days post hatching (dph) larvae were also significantly affected (Fig. 1B). Both temperature and $p\text{CO}_2$ had a significant effect ($P<0.001$) on survivorship, which decreased from $45.7\pm 1.9\%$ under control conditions to $32.7\pm 2.6\%$ in the future scenario. However, the interaction between the two variables was not significant ($P>0.05$). The mean length of 30 dph larvae under control conditions was 13.2 ± 1.5 mm (Fig. 1C). Larval growth increased significantly with warming ($P<0.05$), but decreased significantly under acidified conditions ($P<0.05$), with an observed significant interaction effect between these two variables ($P<0.05$). Warming was responsible for increasing length by 48.6 and 46.5% under normocapnic and hypercapnic conditions, respectively. Regardless of temperature, *S. senegalensis* larvae became nearly 22% smaller with increasing CO_2 . As a result, the highest length value (19.4 ± 1.1 mm) was observed under the warming and normocapnic scenario, while the lowest length (10.3 ± 0.9 mm) was found at lower temperature and hypercapnic conditions. An almost identical trend was observed for specific growth rate, which

presented a 23.7–28.4% increase with warming and an 11.9–15.1% decrease with acidification (Fig. 1D). No significant effect of the interaction between these two factors was observed ($P>0.05$).

Oxygen consumption rates, thermal sensitivity and thermal tolerance limits

The effect of warming and high $p\text{CO}_2$ on the metabolic rates and thermal tolerance limits of *S. senegalensis* larvae is presented in Fig. 2 (see also supplementary material Table S2). Temperature had a positive effect ($P<0.05$) on oxygen consumption rates (OCR), upper thermal tolerance limits (LT_{50}) and critical thermal maximum (CT_{\max}), while hypercapnic conditions promoted a significant reduction ($P<0.05$) of these physiological parameters. Even so, no significant effect of the interaction between these two factors was observed ($P>0.05$). The OCR of 30 dph larvae increased with temperature from 23.1 ± 3.2 to $34.8\pm 3.5 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$ and from 16.8 ± 3.8 to $25.3\pm 1.5 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$ under normocapnic and hypercapnic conditions, respectively (Fig. 2A). These findings represent a decrease of 27.3% under acidified conditions. The LT_{50} of 30 dph larvae increased with temperature from 37.5 ± 0.1 to $37.7\pm 0.0^\circ\text{C}$ under normocapnia, and from 36.1 ± 0.1 to $38.8\pm 0.3^\circ\text{C}$ under hypercapnia conditions (Fig. 2B). The CT_{\max} of 30 dph larvae followed a similar pattern as for OCR and LT_{50} , increasing with temperature from 37.0 ± 0.9 to $38.3\pm 0.5^\circ\text{C}$ under normocapnia, and from 35.5 ± 0.6 to $37.3\pm 0.7^\circ\text{C}$ under hypercapnia (Fig. 2C). Additionally, the development stage had a significant effect ($P<0.05$) on metabolic rates and thermal tolerance limits. *Solea senegalensis* hatchlings presented higher OCR and lower LT_{50} and CT_{\max} values in comparison to 30 dph larvae (Fig. 2).

Thermal sensitivity (Q_{10}) of *S. senegalensis* larvae between 18 and 22°C ranged between 1.89 and 2.79 (Table 1). Q_{10} values decreased under acidified conditions and increased with fish age.

Skeletal deformities and otolith morphometrics

Several types of skeletal anomalies were found in 30 dph *S. senegalensis* larvae (Table 2, Fig. 3). Skeletal deformities consisted mainly of vertebral abnormalities, such as fusions (Fig. 3C–G), body

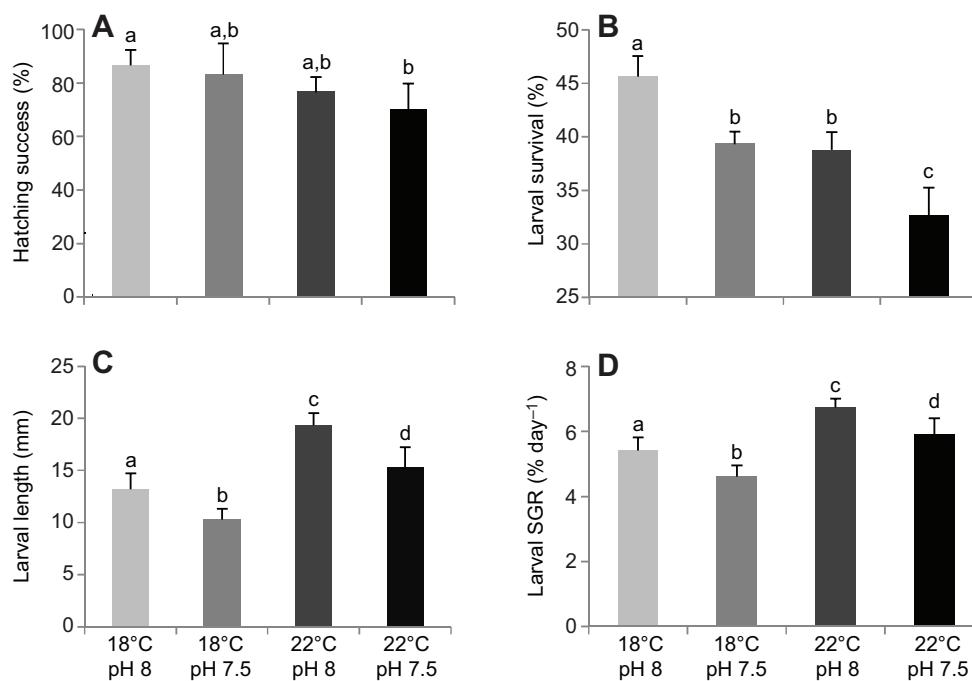


Fig. 1. Effect of ocean warming and acidification on the early life stages of *Solea senegalensis*. (A) Hatching success ($n=30$), (B) survival rate ($n=3$), (C) standard length ($n=60$) and (D) specific growth rate (SGR) ($n=60$) of 30 days post hatching (dph) larvae at different temperature and pH scenarios. Values are given as means \pm s.d. Different letters represent significant differences between the different climate scenarios ($P<0.05$) (more statistical details are available in supplementary material Table S1).

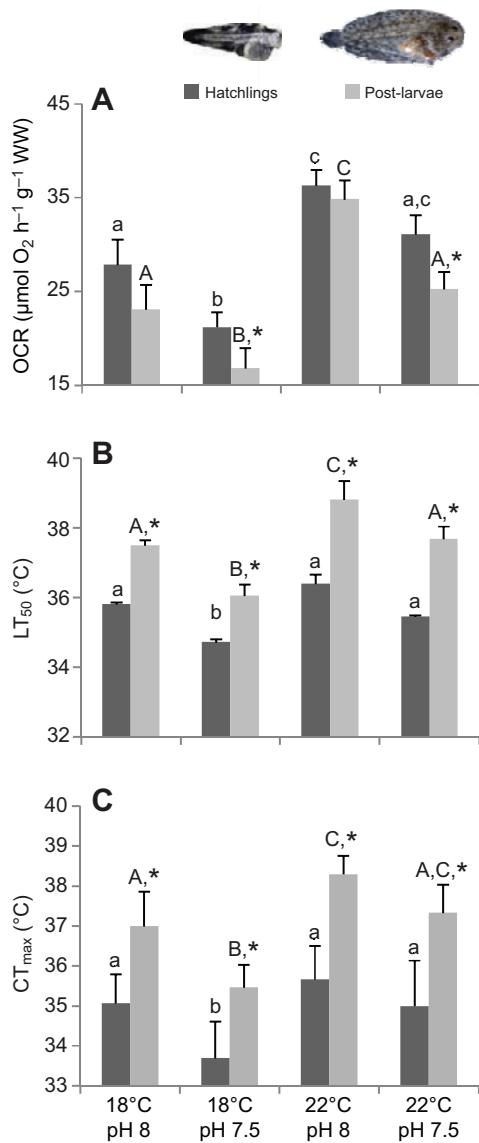


Fig. 2. Impact of ocean warming and acidification on the metabolism and thermal tolerance of *Solea senegalensis* larvae. (A) Oxygen consumption rates (OCR) ($n=9$), (B) upper thermal tolerance limits (LT_{50}) ($n=30$) and (C) critical thermal maximum (CT_{48}) ($n=30$) of 0 and 30 ph larvae (dark and light gray, respectively) at different temperature and pH scenarios. Values are given as means \pm s.d. Different letters (lowercase for hatchlings; uppercase for post-larvae) represent significant differences between the different climate scenarios ($P<0.05$). Asterisks represent significant differences between the two developmental stages ($P<0.05$) (more statistical details are available in supplementary material Table S2).

malformations (Fig. 3C,D), and vertebral curvatures such as scoliosis, lordosis and kyphosis (Fig. 3I,J). Structures such as haemal and neural spines and arches were some of the most affected structures across treatments (Fig. 3C–G).

Future ocean warming and high $p\text{CO}_2$ conditions had a significant effect on the incidence of skeletal deformities in *S. senegalensis* larvae (Figs 4, 5; see also supplementary material Table S3). Rising temperature and CO_2 levels increased the frequency of total skeletal deformities (Fig. 4A), from $70.9\pm2.6\%$ at the present-day scenario to $93.2\pm2.7\%$ under the future conditions ($P<0.05$), an increase of 31.5%. No cranium or pectoral fin deformities were observed under

Table 1. Thermal sensitivity (Q_{10}) between 18 and 22°C of 0 and 30 days post hatching (dph) *Solea senegalensis* larvae at normocapnia (pH=8.0) and hypercapnia (pH=7.5)

Developmental stage	pH	Q_{10}
0 dph	7.5	1.89
	8.0	2.62
30 dph	7.5	2.77
	8.0	2.79

control temperature and $p\text{CO}_2$ rearing conditions. Under the future scenario, caudal vertebra was the most affected region (Fig. 4D), followed by cranium (Fig. 4B), caudal fin (Fig. 4E), abdominal vertebra (Fig. 4C), pelvic fin (Fig. 4H), dorsal fin (Fig. 4F), and finally the pectoral fins (Fig. 4G). In what concerns severe skeletal deformities, $p\text{CO}_2$ was the main factor contributing to the higher proportion of deformities observed in the future scenario (Fig. 5). Under present-day conditions, less than 1.9% of the larvae presented severe vertebral curvatures such as scoliosis (Fig. 5B) or lordosis (Fig. 5C), and no kyphotic larvae were observed (Fig. 5D). In contrast, all types of severe anomalies significantly increased ($P<0.05$) with future environmental predictions, especially with high $p\text{CO}_2$. The interaction factor between temperature and $p\text{CO}_2$ did not have a significant effect ($P>0.05$) on the incidence of skeletal deformities (including the severe ones), except for abdominal vertebra and dorsal fin deformities.

Otolith size was also greatly affected by future warming and hypercapnia conditions (Fig. 6; see also supplementary material Table S1). *Solea senegalensis* larvae experienced a 109.3% increase in otolith area with increasing temperature and $p\text{CO}_2$ ($P<0.05$). Otolith area increased from $1063.6\pm398.8 \text{ mm}^2$ under the present-day conditions to $1994.5\pm234.5 \text{ mm}^2$ under warming, and then to $2226.2\pm187.0 \text{ mm}^2$ under the combined effect of rising temperature and $p\text{CO}_2$. However, the interaction of both factors was not significant ($P>0.05$).

DISCUSSION

The future predictions of ocean warming and acidification were revealed to have a negative impact on several aspects of the early ontogeny of the environmentally resilient flatfish *S. senegalensis*. Despite the short embryonic development time of this species (less than 2 days), the warming experienced during egg incubation was enough to elicit a negative effect on hatching success. Hatching rates decreased 16.7 percentage points with warming and acidification, in comparison to the present-day conditions. Moreover, the high temperature and $p\text{CO}_2$ levels had a further negative effect on larval survival, representing a decrease of 28.4 percentage points in relation to the present scenario.

As expected, larval growth greatly increased with warming. Increased temperature was responsible for increasing length by 46.5–48.6%. Nevertheless, it is important to keep in mind that this increment does not reflect differences in size at a specific stage of development, as development is accelerated at higher temperatures. In contrast, larval growth decreased under high $p\text{CO}_2$ levels. Contrary to some studies that have shown that larvae can become bigger under high $p\text{CO}_2$ conditions (Munday et al., 2009a; Hurst et al., 2012; Hurst et al., 2013), *S. senegalensis* larvae became almost 25% smaller with increasing $p\text{CO}_2$.

An almost identical trend was observed for larval metabolic rates and thermal tolerance limits. While temperature had a positive effect on OCR (within normal Q_{10} values) and thermal tolerance limits, hypercapnic conditions triggered a significant reduction in such

Table 2. Types of skeletal deformities considered in this study (adapted from Wagemans et al., 1998; Gavaia et al., 2002; Dionísio et al., 2012)

Affected area	Types of skeletal deformities	Description
Cranium	Jaw deformities Ocular migration deformities Deformed opercle	Malformed and/or reduced maxillary, premaxillary, angular and/or dentary bones Incomplete or non-existent ocular migration Deformed opercular, ceratobranchial and ceratohyal bones
Abdominal vertebra	Vertebral body malformation Vertebral fusion Vertebral compression Malformed neural and/or haemal arch Malformed neural and/or haemal spine Malformed parapophysis Scoliosis Lordosis Kyphosis	Torsion and/or malformation of one or more vertebrae Partial or total fusion of two or more vertebrae Partial or total compression of two or more vertebrae Deformed, absent or fused Deformed, absent or fused Deformed, absent, fused or supernumerary Side-to-side vertebral curvature Excessive inward vertebral curvature Excessive outward vertebral curvature
Caudal vertebra	Vertebral body malformation Vertebral fusion Vertebral compression Malformed neural and/or haemal arch Malformed neural and/or haemal spine Scoliosis Lordosis	Torsion and/or malformation of one or more vertebrae Partial or total fusion of two or more vertebrae Partial or total compression of two or more vertebrae Deformed, absent, asymmetric or fused Deformed, absent, asymmetric or fused Side-to-side vertebral curvature Excessive inward vertebral curvature
Caudal fin complex	Malformed hypural Malformed epural Malformed parahypural Malformed fin rays	Deformed, absent, asymmetric, fused or supernumerary Deformed, absent, asymmetric, fused or supernumerary Deformed, absent, asymmetric, fused or supernumerary Deformed, absent, asymmetric, fused or supernumerary
Dorsal fin	Malformed fin rays Malformed pterygiophores	Deformed, absent, asymmetric, fused or supernumerary Deformed, absent, fused or supernumerary
Pectoral/pelvic fin	Malformed fin rays	Deformed, absent, asymmetric, fused or supernumerary

physiological parameters. Additionally, and as expected, mass-specific metabolic rates decreased with development, while thermal tolerance limits revealed an opposite ontogenetic trend, i.e. older larvae revealed higher thermal tolerance limits than newly hatched ones. We presume that exposure to higher $p\text{CO}_2$ might have impaired acid–base balance regulation, which directly affects the efficiency of cellular activities (Pörtner et al., 2005; Perry and Gilmour, 2006) and may cause deleterious effects on larval physiology and growth.

Faster growth at higher temperatures could have some advantages, because slower growing larvae are potentially more vulnerable to predators and may thus experience greater mortality (Anderson, 1988). Nevertheless, growth enhancement with temperature might also present some disadvantages, because faster larval growth was accompanied by an increase in the incidence of skeletal deformities. Indeed, temperature is known to be one of the most important environmental factors that can induce morphological deformities during fish development (Aritaki and Seikai, 2004; Georgakopoulou et al., 2010; Dionísio et al., 2012). Additionally, pH may also affect the prevalence of fish skeletal deformities (Lall and Lewis-McCrea, 2007). Although fish skeleton is predominantly composed of calcium phosphate (in the form of hydroxyapatite and cartilaginous material) (Lall and Lewis-McCrea, 2007), additional buffering of tissue pH with bicarbonate and non-bicarbonate ions is expected under acidified conditions, which may interfere with larval skeletal development. In this study, the future warming and high $p\text{CO}_2$ scenario was responsible for increasing the incidence of total skeletal deformities by 22.2 percentage points, affecting 93.1% of the larvae. Moreover, high $p\text{CO}_2$ was the main factor responsible for the increase of severe skeletal deformities in flatfish larvae. Under the present-day conditions, less than 1.9% of the larvae presented vertebral curvature deformities such as scoliosis or lordosis, and no kyphotic larvae were observed. In contrast, more than 50% of the larvae under the future environmental scenario presented vertebral

curvature deformities. These findings, however, are in disagreement with a recent study that found no effects of CO_2 on the skeletal development of a reef fish (Munday et al., 2011b).

However, the higher incidence of malformations under the future scenario should be carefully interpreted. The high percentage of skeletal deformities found in *S. senegalensis* under control temperature and $p\text{CO}_2$ conditions ($70.9 \pm 2.7\%$), although similar to the values commonly found for this species under intensive rearing conditions (Fernández et al., 2009; Dionísio et al., 2012), may indicate that fish were potentially stressed in captivity and would, therefore, be more susceptible to the negative effects of higher temperature and CO_2 levels. Nevertheless, this fact does not exclude the amplifying effect that warming and hypercapnia had on the incidence of skeletal deformities. Even though the increase may be overestimated, the higher rate of malformations in captive larvae under high temperature and $p\text{CO}_2$ conditions may provide an insight into how future warming and acidification may impact the development of wild flatfish larvae and their future performance in a changing ocean.

Skeletal deformities may impair the ecophysiological performance of fish larvae in many different ways. Vertebral curvatures and fin deformities may affect larval swimming behavior, feeding efficiency and the capacity to maintain their position in a current (Powell et al., 2009). Additionally, larvae with cranium deformities, such as ocular migration anomalies, probably will have their capability to feed, attack prey and avoid predators affected. Larvae with operculum deformities may increase gill's susceptibility to fungus, bacteria and amoebic parasitic infections (Powell et al., 2008) and, as a result, their swimming and cardiovascular performance might be compromised (Powell et al., 2008; Lijalad and Powell, 2009; Powell et al., 2009). Additionally, fish with dental, premaxillary or maxillary deformities cannot adduct their mandible and, besides having potential feeding restrictions, the buccal-opercular pumping of water across gills is also likely to be impaired and compromised (Lijalad and Powell, 2009).

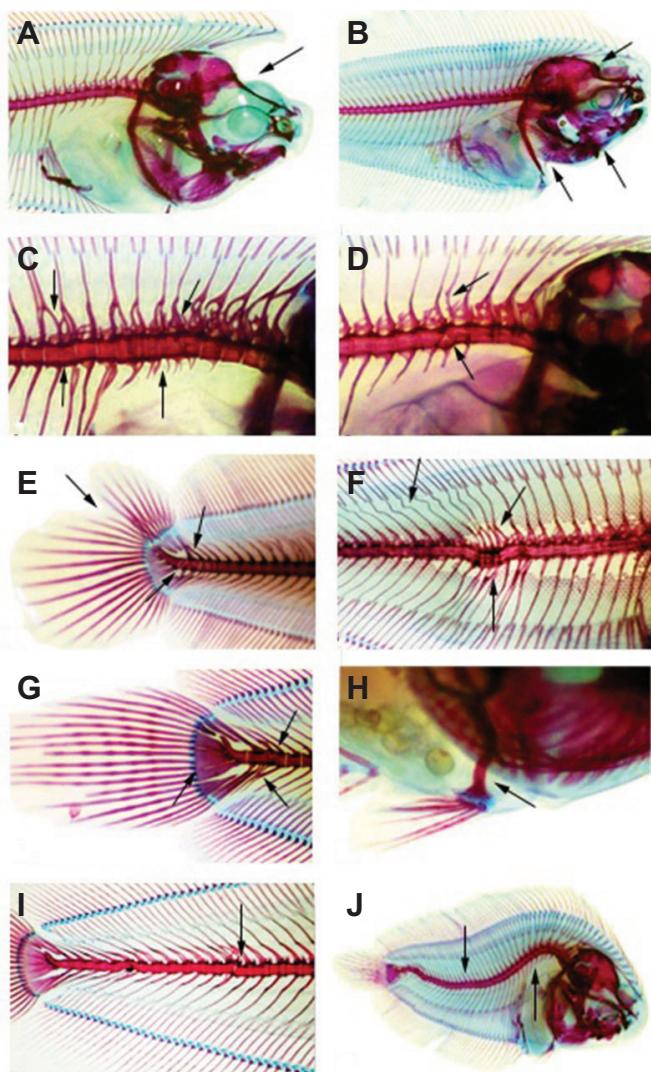


Fig. 3. Skeletal deformities of 30 dph *Solea senegalensis* larvae under the effects of ocean warming and acidification. (A) Cranium deformity, ocular migration anomaly; (B) opercle and cranium deformity; (C) vertebra fusion and compression, deformed spines, arches and parapophysis; (D) vertebra fusion and deformed spines and arches; (E) vertebra fusion, urostyle fusion and caudal fin complex anomalies such as modified neural and hemal spine, hypural and fin rays; (F) vertebra fusion and compression, deformed spines and arches; (G) vertebral fusion, deformed hypural and modified hemal spines; (H) pelvis fin deformity; (I) scoliosis; (J) lordosis and kyphosis.

In addition to skeletal deformities, *S. senegalensis* larvae under this future climate change scenario will also be affected by changes in otolith size. *Solea senegalensis* larvae experienced a 109.3% increase in otolith area with rising temperature and $p\text{CO}_2$. Although otoliths are calcified structures composed of aragonite–protein bilayers, recent studies revealed that pH regulation in otolith endolymph may lead to increased precipitation of calcium carbonate in otoliths of fingerlings exposed to elevated CO_2 (Checkley et al., 2009; Munday et al., 2011a; Bignami et al., 2013). However, this is not a rule among fishes. In at least one coral reef fish species, otolith size was not affected by exposure to elevated $p\text{CO}_2$ (Munday et al., 2011b). Otoliths are used by fish for orientation, perception and acceleration, and to maintain postural equilibrium. Thus, changes in otolith size may have implications for their ecological performance,

behavior and individual fitness (Gagliano et al., 2008; Bignami et al., 2013).

In conclusion, the results presented in our study provide comprehensive insight into the combined effects of ocean warming and hypercapnia conditions on *S. senegalensis* larval development. Fish larval stages represent a critical life phase for species' ecological success. Therefore, climate-change-related impairments in metabolism, thermal tolerance, growth, skeletal development and survival may lead to substantial declines in adult populations, putting in jeopardy the species' persistence under a climate change scenario.

MATERIALS AND METHODS

Egg collection and incubation

Solea senegalensis eggs were obtained from a wild-caught broodstock of four females and two males, under natural spawning conditions at Instituto Português do Mar e da Atmosfera (IPMA), Centro Regional de Investigação Pesqueira do Sul (CRIPSul, Olhão, Portugal), during June 2012. After collection, eggs were transported and immediately transferred, under environmentally controlled conditions, to the aquaculture facilities in Laboratório Marítimo da Guia (Cascais, Portugal). To estimate the potential physiological responses of early life stages to climate change, *S. senegalensis* eggs and larvae were acclimated for 1 month at: (1) 18°C – control temperature, the mean sea surface temperature in summer (sSST) – and normocapnia (0.04% CO_2 , $p\text{CO}_2$ =~400 μatm , pH=8.0); (2) 18°C and hypercapnia (0.16% CO_2 , $p\text{CO}_2$ =~1600 μatm , $\Delta\text{pH}=0.5$, pH=7.5); (3) 22°C – the future sSST warming scenario for the western coast of Portugal in 2100 [+4°C above the average sSST (Meehl et al., 2007)] – and normocapnia; and (4) 22°C and hypercapnia. Prior to releasing the eggs in the rearing tanks, a 2 h thermal and chemical acclimation was performed.

Eggs and larvae were reared in 12 individual recirculating systems (i.e. three systems per treatment), filled with filtered (series of 20, 10, 5 and 0.35 μm) and UV-irradiated natural seawater. Each system comprised a 19 l cylindrical shaped tank (larval rearing tank) connected to a 100 l sump. All rearing tanks were placed inside 400 l water bath tanks (see supplementary material Fig. S1), where temperatures (18.0 ± 0.2 and $22.0\pm0.2^\circ\text{C}$) were maintained and controlled via seawater chillers (HC-1000A, Hailea, Guangdong, China), in order to ensure thermo-controlled conditions.

The photoperiod was set at 14 h:10 h light:dark. Water filtration was performed through mechanical (glass wool), physical (protein skimmer, Schuran, Jülich, Germany) and biological (ouriço® bioballs, Fernando Ribeiro, Portugal) filters, as well as UV sterilization (TMC, Chorleywood, UK). Throughout the experiment, ammonia and nitrite levels were monitored daily and kept below detectable levels. Temperatures were controlled via seawater chillers (Frimar, Fernando Ribeiro, Portugal), while pH was adjusted automatically via a Profilux system (GHL, Kaiserslautern, Germany) connected to pH probes (WaterTech pH 201S) in the rearing tanks and to a standard solenoid valve system connected to a CO_2 tank. Any seawater pH modifications initiated CO_2 addition (if the pH increased) or CO_2 filtered air injection (if the pH decreased), until pH returned to the set value. Additionally, temperature and pH were controlled daily using a digital thermometer (Ebro thermometer TFX430) and a portable pH meter (SevenGo pro™ SG8, Mettler Toledo). Mean values were 18.0 ± 0.2 and $22.0\pm0.2^\circ\text{C}$ for temperature and 8.02 ± 0.05 and 7.51 ± 0.05 for pH. Salinity was kept at 35.4 ± 0.4 . Seawater carbonate system speciation (Table 3) was calculated weekly from total alkalinity [determined according to Sarazin (Sarazin et al., 1999)] and pH measurements. Bicarbonate and $p\text{CO}_2$ values were calculated using the CO2SYS program (Lewis and Wallace, 1998), with dissociation constants from Mehrbach et al. (Mehrbach et al., 1973) as refitted by Dickson and Millero (Dickson and Millero, 1987).

Larval rearing

Newly hatched larvae were randomly placed into rearing tanks (19 l volume each) at a stocking density of 70 larvae per liter. All larvae were reared until 30 dph under the different experimental conditions. The feeding schedule was based on larval development under each set of experimental conditions. Larvae opened their mouth at approximately 2 dph and started to feed on

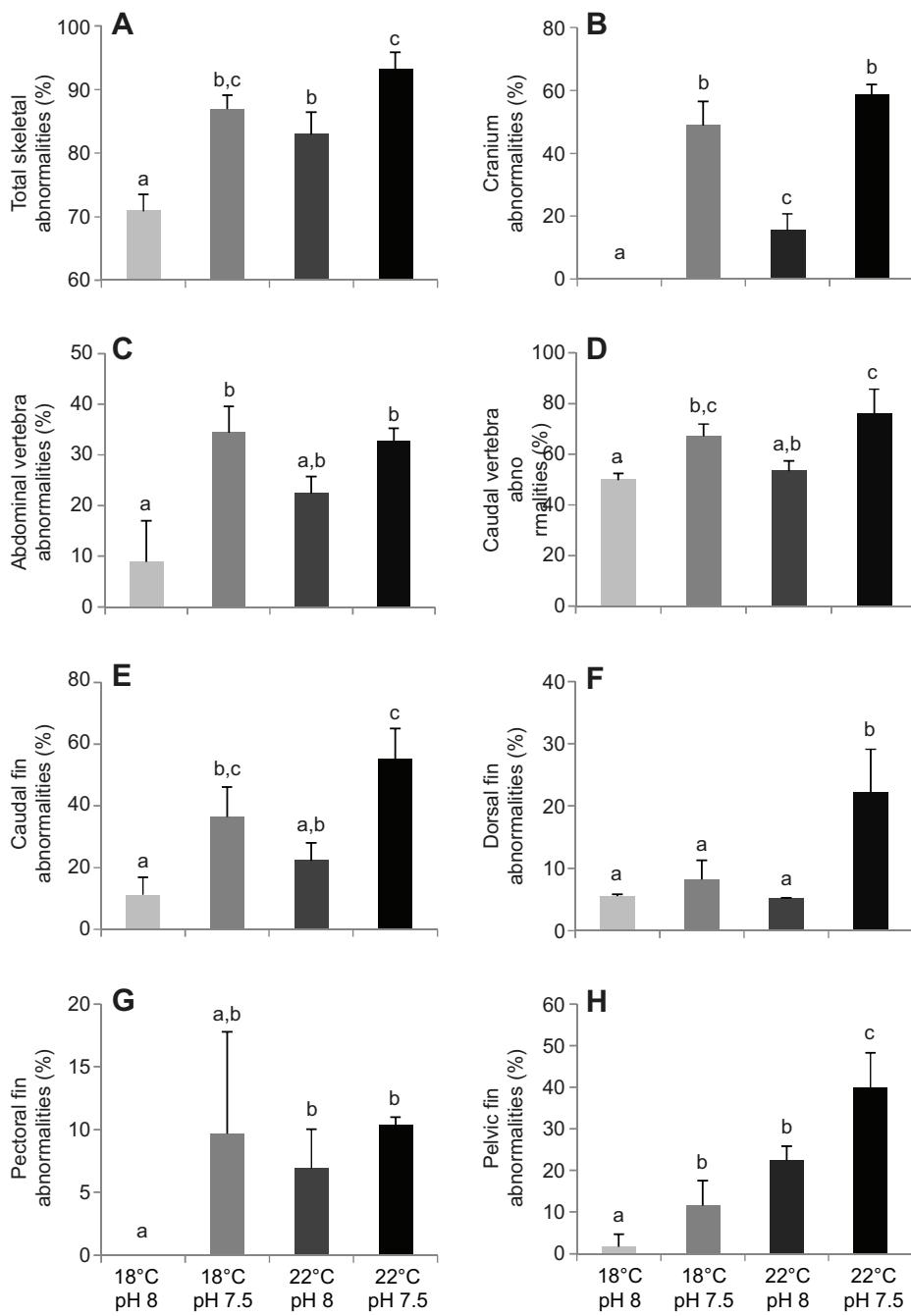


Fig. 4. Incidence of skeletal deformities in *Solea senegalensis* larvae under the effects of ocean warming and acidification. (A) Total skeletal deformities of 30 dph larvae at different temperature and pH scenarios, which include deformities in the (B) cranium, (C) abdominal vertebra, (D) caudal vertebra, (E) caudal fin complex, (F) dorsal fin, (G) pectoral fin and (H) pelvic fin. Values are given as means \pm s.d. ($n=60$). Different letters represent significant differences between the different climate change scenarios ($P<0.05$) (more statistical details are available in supplementary material Table S3).

rotifers, *Brachionus plicatilis*, at a density of 5 to 10 rotifers ml⁻¹. Live enriched (AlgaMac-3050) *Artemia* metanauplii were introduced at 5 dph and their proportion was gradually increased from 0.5 to 12 metanauplii ml⁻¹, becoming the only prey offered at 8 dph. Frozen metanauplii were also introduced as feed after larval settlement.

Hatching success, larval growth and survival

Hatching success was analyzed in small rearing boxes placed inside the rearing tanks (one per rearing system). In the beginning of the experiment, a total of 10 eggs (per box) were randomly placed inside each of the 12 boxes (three per treatment), and these were followed throughout the embryonic development. The hatching success was calculated as the percentage of eggs that hatched to normal larvae.

At 0 and 30 dph, 20 larvae per tank (60 larvae per treatment) were randomly sampled and their standard length was measured from the anterior

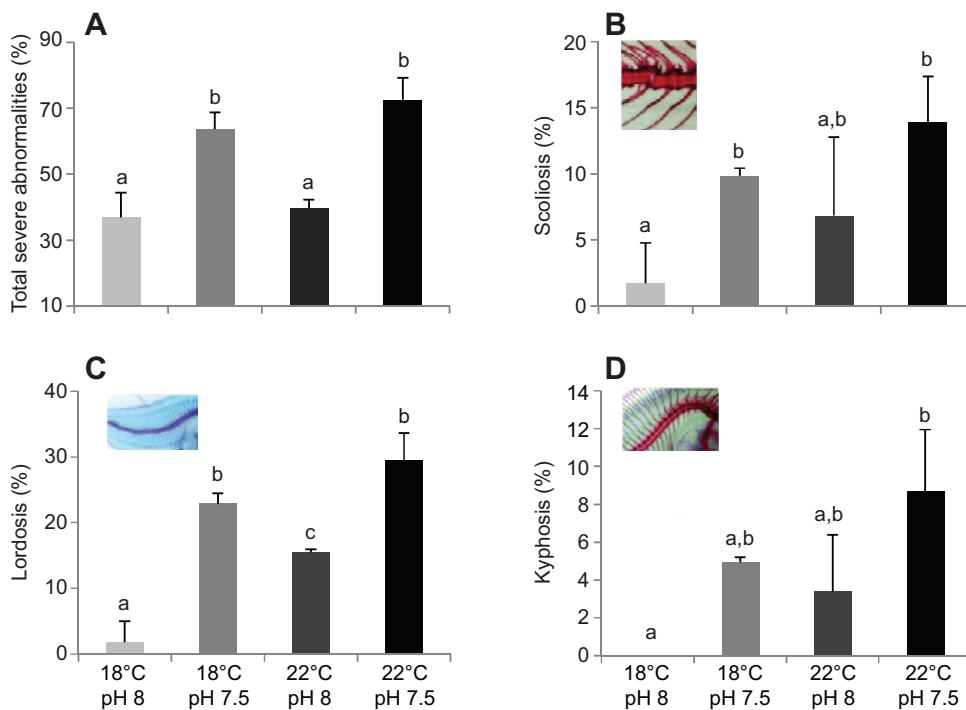
extremity to the urostyle flexion, by means of stereoscopic microscope observations (Leica S6D, Leica Microsystems). The standard length of newly hatched larvae was 2.57 ± 0.13 mm. The specific embryonic growth rate (SGR) was calculated as:

$$\text{SGR} = \frac{[\ln \text{embryo size } (T_2) - \ln \text{embryo size } (T_1)]}{\text{number of days elapsed between } T_1 \text{ and } T_2} \times 100. \quad (1)$$

The survival rate was calculated as the percentage of surviving fish by the end of the experiment, with respect to the number of larvae at the beginning of the trial minus those individuals removed for sampling.

Oxygen consumption rates, thermal sensitivity and thermal tolerance limits

Oxygen consumption measurements were determined according to previously established methods (Pimentel et al., 2012; Rosa et al., 2012). Nine newly



hatched (0 dph) and nine 30 dph larvae were incubated at each of the four treatment conditions, in sealed water-jacketed respirometry chambers (RC300 Respiration Cell, Strathkelvin Instruments Limited, North Lanarkshire, UK) containing 0.35-μm-filtered and UV-irradiated seawater mixed with antibiotics (50 mg l⁻¹ streptomycin), in order to avoid bacterial respiration. Water volumes were adjusted in relation to animal mass (up to 10 ml) in order to minimize locomotion and stress but still allow for spontaneous and routine activity of the hatchlings. Controls (blanks) were used to correct for possible bacterial respiratory activity. Respiration chambers were immersed in water baths (Lauda, Lauda-Königshofen, Germany) to control temperature. Oxygen concentrations were recorded with Clarke-type O₂ electrodes connected to a multi-channel oxygen interface (Model 928, Strathkelvin Instruments). The duration of respiratory runs varied between 3 and 6 h. Thermal sensitivity (Q_{10}) was determined using the standard equation:

$$Q_{10} = \frac{R(T_2)^{\frac{10}{(T_2-T_1)}}}{R(T_1)}, \quad (2)$$

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

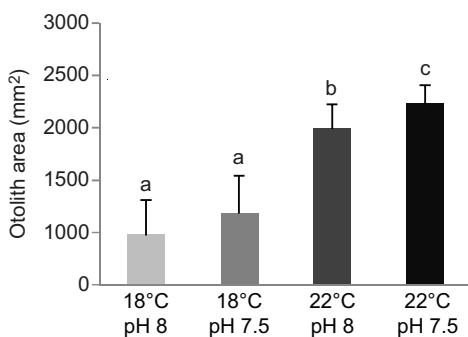


Fig. 6. Effect of ocean warming and acidification on otolith size of 30 dph Solea senegalensis larvae. Otolith area at different temperature and pH scenarios. Values are given as means ± s.d. (n=60). Different letters represent significant differences between the different climate scenarios ($P<0.05$) (more statistical details are available in supplementary material Table S1).

Fig. 5. Incidence of severe skeletal deformities in Solea senegalensis larvae under the effect of ocean warming and acidification. (A) Total severe skeletal deformities and severe vertebral curvatures, such as (B) scoliosis, (C) lordosis and (D) kyphosis of 30 dph larvae at different temperature and pH scenarios. Values are given as means ± s.d. (n=60). Different letters represent significant differences between the different climate scenarios ($P<0.05$) (more statistical details are available in supplementary material Table S3).

Upper thermal tolerance limits were determined based on previously established methods (Stillman and Somero, 2000). In brief, 0 and 30 dph larvae were incubated in glass containers with ~100 ml of 0.35-μm-filtered and UV-irradiated seawater collected from the rearing tanks. Each container was stocked with 20 specimens, and a total of three containers were used per experimental treatment. These glass containers were suspended in a temperature-regulated water bath that was controlled to the nearest 0.1°C. Water bath temperature was set to the acclimation temperature and maintained for 30 min. Thereafter, temperature was increased at a rate of 1°C 30 min⁻¹. Seawater was aerated by means of an air stone and the temperature in each container was checked with thermocouple probes. Every 30 min, if no responsiveness was noticed, the specimen was considered to be dead. The percentage of live individuals at each temperature was calculated, and then transformed by the arcsine square root function and expressed in radians. Linear regression analysis was then used to find the slope of the line, and the temperature at which 50% of the organisms had died (0.785 rad) was calculated. This was used as a measure of upper thermal tolerance limits and referred to as the LT₅₀. Critical thermal maximum (CT_{max}) was calculated using the equation:

$$CT_{\text{max}} = \frac{\sum T_{\text{end-point}}}{n}, \quad (3)$$

where $T_{\text{end-point}}$ is the temperature at which the end-point was reached for each individual (1 to n), and n is the number of individuals in the sample.

Skeletal deformities and otolith morphometrics

To identify and quantify larval skeletal deformities, 20 larvae per rearing tank (60 larvae per treatment) were randomly sampled and fixed in 4% (v/v) buffered paraformaldehyde for 24 h and then transferred to 70% ethanol until double stained. Larvae were stained for bone and cartilage using a modification of the method described by Walker and Kimmel (Walker and Kimmel, 2007), and observed under a stereoscopic microscope (Leica S6D, Leica Microsystems) in order to identify skeletal deformities. Skeletal deformities were defined according to previously established methods (Wagemans et al., 1998; Gavaia et al., 2002; Deschamps et al., 2008; Fernandez et al., 2009; Dionísio et al., 2012). Deformities were divided into several categories according to the affected structure (e.g. cranium, abdominal vertebra, caudal vertebra, caudal fin, dorsal fin, pectoral fin and pelvic fin), and are described in Table 2. Skeletal deformities such as scoliosis, lordosis, kyphosis, multiple vertebral fusions or more than three anomalies per

Table 3. Seawater carbonate chemistry data for the different climate change scenarios

Temperature (°C)	pH (total scale)	A_T ($\mu\text{mol kg}^{-1}$ SW)	C_T ($\mu\text{mol kg}^{-1}$ SW)	$p\text{CO}_2$ (µatm)	HCO_3^- ($\mu\text{mol kg}^{-1}$)	Ω_{arag}
22.02±0.42	8.03±0.05	2335.74±89.09	2148.20±81.43	424.53±19.97	1985.25±75.28	2.24±0.08
22.12±1.01	7.51±0.05	2317.40±36.40	2314.73±36.72	1654.20±49.06	2194.88±34.84	0.78±0.01
18.20±0.40	8.02±0.04	2305.70±80.54	2141.80±76.78	400.00±66.71	1993.35±72.21	1.95±0.07
18.15±0.29	7.50±0.03	2281.07±61.89	2290.90±62.73	1607.90±24.78	2173.55±59.50	0.67±0.02

Total carbon (C_T), carbon dioxide partial pressure ($p\text{CO}_2$), bicarbonate concentration (HCO_3^-) and aragonite saturation state of seawater (Ω_{arag}) were calculated with CO2SYS using salinity, temperature, pH and total alkalinity (A_T). Values are means ± s.d. SW, seawater.

individual were considered severe deformities. Skeletal deformities were quantified as the percentage of fish exhibiting a specific deformity.

In order to analyze otolith area, 20 larvae per rearing tank (60 larvae per treatment) were randomly selected, measured and preserved in absolute ethanol. The left and right sagittal otoliths of each individual were removed and photographed under a stereoscopic microscope (Leica S6D, Leica Microsystems). Otolith area was measured using the ImageJ program. Otolith area was calculated as the mean of the right and left otoliths, and normalized to fish length.

Statistical analysis

ANOVA was used to test for significant differences between the tanks of each experimental treatment. As no differences were found between tanks, all of the samples from the same treatment were pooled and analyzed together. Two-way ANOVAs were then conducted in order to detect significant differences in hatching success, larval survival, standard length, SGR, skeletal deformities and otolith size between temperature and $p\text{CO}_2$ treatments. Three-way ANOVA were applied to detect significant differences in OCR, LT_{50} and CT_{\max} between temperature and $p\text{CO}_2$ treatments and development stage (0 and 30 dph). Subsequently, *post hoc* Tukey's honest significant difference tests were performed. All statistical analyses were performed using a significance level of 0.05, using Statistica 10.0 software (StatSoft Inc., Tulsa, OK, USA).

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Competing interests

The authors declare no competing financial interests.

Author contributions

R.R. designed the experiment; M.S.P. and F.F. performed the experiment; M.S.P., F.F., G.D., T.R., P.P., J.M. and R.R. analyzed the data; M.S.P., F.F. and R.R. wrote the main paper. All authors discussed the results and their implications, and commented on the manuscript at all stages.

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Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.092635/-DC1>

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CHAPTER 3

Foraging behaviour, swimming performance and malformations of early stages of commercially important fishes under ocean acidification and warming

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In review in *Climatic Change*

Foraging behaviour, swimming performance and malformations of early stages of commercially important fishes under ocean acidification and warming

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Abstract

Early life stages of many marine organisms are being challenged by climate change, but little is known about their capacity to tolerate future ocean conditions. Here we investigated a comprehensive set of biological responses of larvae of two commercially important teleost fishes, *Sparus aurata* (gilthead seabream) and *Argyrosomus regius* (meagre), after exposure to future predictions of ocean warming (+4 °C) and acidification ($\Delta\text{pH}=0.5$). The combined effect of warming and hypercapnia elicited a decrease in the hatching success (by 26.4 and 14.3% for *S. aurata* and *A. regius*, respectively) and larval survival (by half) in both species. A significant effect of hypercapnia was also found for larval growth. However, while *S. aurata* growth was reduced (24.8-36.4% lower), *A. regius* growth slightly increased (3.2-12.9% higher) under such condition. Under acidification, larvae of both species spent less time swimming, and displayed reduced attack and capture rates of prey. The impact of warming on these behavioural traits was opposite but less evident. While not studied in *A. regius*, the incidence of body malformations in *S. aurata* larvae increased significantly (more than tripled) under warmer and hypercapnic conditions. These morphological impairments and behavioural changes are expected to affect larval performance and recruitment success, and further influence the abundance of fish stocks and the population structure of these commercially important fish species. However, given the pace of ocean climate change, it is important not to forget that species may have the opportunity to acclimate and adapt.

Keywords: Ocean climate change, fish early stages, survival and growth, malformations, behaviour, ecophysiology

Introduction

Atmospheric CO₂ levels are rising at an unprecedented rate. The continuous absorption of atmospheric CO₂ by oceans is causing a decline in ocean's pH, which is expected to decrease 0.4-0.5 units by the year 2100. In parallel, sea surface temperature is expected to rise up to 4 °C by the end of the century (Collins et al. 2013). Ocean warming and acidification represent a major threat to many marine organisms by affecting their acid-base balance, metabolism, growth and behaviour (Munday et al. 2011; Pörtner et al. 2004) in ways that often compromise species fitness and survival (Wittmann and Pörtner 2013).

Fishes were thought to be quite resilient to exposure to elevated CO₂, given their strong ability to regulate acid-base balance by bicarbonate accumulation and ion exchange across the gills (Melzner et al. 2009). Nevertheless, fish early life stages have shown to be more susceptible to elevated CO₂ than adult fish (reviewed by Pörtner et al. 2005). Several studies have reported direct effects of elevated *p*CO₂ on survival, growth, metabolism, behaviour, otoliths and skeletal development of marine fish larvae (Baumann et al. 2012; Frommel et al. 2014; Munday et al. 2011; Pimentel et al. 2014). Other studies have found no significant effects of increasing *p*CO₂ on fish larvae (Harvey et al. 2013; Hurst et al. 2013; Maneja et al. 2013b), suggesting species-specific responses to changing ocean conditions.

To date, very few studies have investigated the susceptibility of early stages of commercially important fish species to climate-driven changes, including codfish (Frommel et al. 2014) and yellowfin tuna (Bromhead et al. 2015). Given the importance of larval growth and survival rates to the year-class success in marine fish populations (Peck et al. 2012), deleterious effects of climate-driven changes in *p*CO₂ and temperature may have profound consequences on the distribution and abundance of marine fish stocks (Pörtner and Peck 2010).

Here we analysed the effects of ocean warming (+4 °C) and acidification ($\Delta\text{pH}=0.5$) on the development and behaviour of early life stages of two commercially important fish species in the NE Atlantic Ocean, namely *Sparus aurata* and *Argyrosomus regius*.

Methods

Egg collection

Eggs of *S. aurata* were collected from the hatchery Maresa - Mariscos de Estero, Spain, in November 2013. Eggs of *A. regius* were obtained from Instituto Português do Mar e da Atmosfera (IPMA) - Centro Regional de Investigação Pesqueira do Sul (CRIPSul), Olhão, Portugal, in May 2014. Eggs of both species were collected immediately after spawning and transferred to the aquaculture facilities in Laboratório Marítimo da Guia, Cascais, Portugal. At approximately 5 hours after spawning, eggs were acclimated to the different experimental conditions.

Egg incubation and larval rearing

Following an acclimation period of about 2 h, *S. aurata* and *A. regius* eggs were exposed to four different treatments, a cross-factor design of two temperatures and two $p\text{CO}_2$ levels: **(1)** control temperature and normocapnia ($p\text{CO}_2 \sim 350 \mu\text{atm}$, $\text{pH}=8.0$); **(2)** control temperature and hypercapnia ($p\text{CO}_2 \sim 1400 \mu\text{atm}$, $\text{pH}=7.5$, $\Delta\text{pH}=0.5$); **(3)** the expected warming scenario ($+4^\circ\text{C}$) and normocapnia; and **(4)** warming and hypercapnia. Control temperatures represented the average temperature during the spawning season of *S. aurata* (18°C ; Arias 1980) and *A. regius* (20°C ; Quéro and Vayne 1987).

For each species and treatment, eggs and larvae were reared in 3 independent recirculating systems (12 per species in total), each composed by a 19-L cylindrical rearing tank connected to a 100-L sump. To ensure an accurate water temperature in each experimental treatment, the rearing tanks were placed inside 400-L water bath tanks. All rearing systems were filled with filtered ($1 \mu\text{m}$) and UV-irradiated seawater (salinity 35). Temperatures were kept stable via seawater chiller systems. pH was automatically adjusted via solenoid valves, by injecting a certified CO_2 gas mixture into the water or by aerating the water with CO_2 filtered air (by using CO_2 scrubbers with soda lime). Salinity, temperature and pH levels were also manually monitored daily. Total alkalinity was determined according to Sarazin et al. (1999). The seawater carbonate chemistry (Table 1) was calculated using the CO2SYS software (Lewis and Wallace 1998). Ammonia and nitrites were monitored regularly and maintained within recommended levels.

Table 1. Seawater carbonate chemistry data for *Sparus aurata* and *Argyrosomus regius* larvae under different climate change scenarios. Total carbon (C_T), carbon dioxide partial pressure (pCO_2), bicarbonate concentration (HCO_3^-) and aragonite saturation state of seawater (Ω_{arag}) were calculated with CO2SYS using salinity, temperature, pH and total alkalinity (A_T). Values are means \pm SD.

Temperature (°C)	pH (Total scale)	A_T [$\mu\text{mol kg}^{-1}$ SW]	C_T [$\mu\text{mol kg}^{-1}$ SW]	pCO_2 [μatm]	HCO_3^- [$\mu\text{mol kg}^{-1}$]	Ω_{arag}
<i>Sparus aurata</i>						
20.4 \pm 0.4	8.07 \pm 0.08	2341.7 \pm 81.7	2140.3 \pm 87.8	354.5 \pm 75.1	1830.7 \pm 90.9	3.4 \pm 0.4
20.3 \pm 0.4	7.52 \pm 0.02	2325.7 \pm 59.2	2273.4 \pm 56.9	1489.0 \pm 76.3	2156.2 \pm 54.1	1.1 \pm 0.1
24.3 \pm 0.3	8.08 \pm 0.08	2315.0 \pm 90.3	1965.2 \pm 75.1	352.2 \pm 61.0	1727.6 \pm 86.3	3.6 \pm 0.4
24.0 \pm 0.3	7.52 \pm 0.05	2299.6 \pm 86.8	2230.3 \pm 85.2	1493.5 \pm 76.6	2111.1 \pm 99.6	1.2 \pm 0.1
<i>Argyrosomus regius</i>						
18.4 \pm 0.3	8.09 \pm 0.07	2331.7 \pm 67.0	2055.2 \pm 60.5	342.3 \pm 69.5	1836.2 \pm 70.2	3.2 \pm 0.6
18.2 \pm 0.3	7.53 \pm 0.04	2318.6 \pm 50.7	2284.0 \pm 57.0	1484.7 \pm 85.8	2169.1 \pm 54.2	1.0 \pm 0.1
22.3 \pm 0.3	8.09 \pm 0.07	2308.3 \pm 78.8	1970.5 \pm 80.2	337.0 \pm 73.4	1736.9 \pm 98.7	3.5 \pm 0.5
22.1 \pm 0.4	7.53 \pm 0.05	2321.0 \pm 93.2	2292.2 \pm 83.2	1473.0 \pm 92.0	2137.0 \pm 99.5	1.2 \pm 0.1

For the embryonic development experiment, 10 eggs were randomly placed inside a small rearing box in each rearing tank, and followed for approximately 43 hours until hatching. The remaining eggs were distributed in egg-incubation tanks and further used for the larval experiment. After hatching, larvae were carefully counted and transferred to the rearing tanks. *S. aurata* larvae were randomly distributed at a density of 70 larvae L⁻¹ and reared until 15 days post-hatch (dph). Larvae were fed on rotifers (*Brachionus plicatilis*) at an increasing density of 5 to 10 rot mL⁻¹ between 2 and 15 dph, and *Artemia* nauplii (0.2-2 art mL⁻¹) from 10 to 15 dph [adapted from Fernández et al. (2008)]. *A. regius* larvae were reared at a density of 45 larvae L⁻¹ for 10 days. Larvae started to feed on rotifers (from 5 to 10 rot mL⁻¹) between 2 and 10 dph, and *Artemia* nauplii (0.2-2 art mL⁻¹) was gradually introduced at 6 dph until the end of the experiment [based on Pousão-Ferreira et al. (2013)]. Both rotifers and *Artemia* nauplii were enriched with Red Pepper. At the end of each day, prey availability in each rearing tank was checked to ensure that prey density was never a limiting factor regardless of the treatment. The light regime in both experiments was 14 L:10 D.

Hatching success, survival and growth

The hatching success and larval survival were determined, per rearing tank, based on the number of surviving larvae at hatching and at the end of the experiment, respectively. In each tank, the standard length at hatching and at the end of the experiment (i.e., 15 dph and 10 dph for *S. aurata* and *A. regius*, respectively) was measured for 4 individuals using a dissecting microscope. The somatic growth length (SGL) was calculated as the difference between the mean length at hatching and the length of each larva at the end of the experiment divided by the time elapsed.

Body malformations

At the end of the experiment, 20 *S. aurata* larvae per tank were sampled and fixed in 4% buffered paraformaldehyde for 24 h, and then transferred to 70% ethanol. Larvae were observed under a microscope to identify and quantify body structure malformations and/or axial deviations, based on Boglione et al. (2001). Malformations were classified according to the affected area (cranium, abdominal and caudal region). Cranium malformations included asymmetric eye, deformed meckel's cartilage in the jaw and deformed ceratobranchial in the opercle. Abdominal and caudal malformations included abnormal body curvatures such as side-to-side, excessive inward and outward curvatures, and abnormal urostyle flexion. Malformations were quantified as the percentage of fish exhibiting a specific deformity. The incidence of body malformations in *A. regius* larvae was not assessed.

Behavioural patterns

Behavioural observations of *S. aurata* and *A. regius* larvae were conducted at the end of both experiments. A preliminary study was performed to establish the ethogram for both species (Table 2). Swimming (S) and spin (Sp) behaviours were recorded as time variables, whereas miss (M), attack (A) and capture (C) behaviours were recorded as frequency variables. The capture success was calculated as the fraction of successful attack events (based on Drost 1987). The behavioural patterns were analysed through direct observation by a blind observer, using the focal animal technique. Four larvae per rearing tank (12 per

treatment) were randomly selected and the behaviour of each larva was analysed inside the rearing tanks during 1 minute, 30 min after feeding.

Table 2. Ethogram of activity and foraging patterns of *Sparus aurata* and *Argyrosomus regius* larvae.

Category	Subcategory	Description
Activity	Swimming (S)	Forward movement of the larva through the water column using the posterior area of the body.
	Spin (Sp)	Erratic movement of the larvae with irregular and swirling movements, spinning around itself.
Foraging	Capture (C)	Larva bites and ingests prey. The movement towards the prey is accomplished by a posterior thrust of the tail.
	Miss (M)	Larva fails to capture prey after an attack.
	Attack (A)	Sum of miss (M) and capture (C) behaviours.

Statistical analyses

Experiments led to data expressed as (1) proportions (hatching success, survival success, and malformations), (2) counts (number of observed behaviours), and (3) positive quantities (measures of lengths and growths). All data were analyzed via generalized linear mixed models (GLMM, e.g. Zuur et al. 2009). The distributional family considered was binomial (logit link function), Poisson (logit link function) and Gaussian (identity link function) for proportions, counts and positive quantities, respectively. Model's residuals were checked for departures from the assumed distributions and no significant deviations were found. The mixed model component was introduced to respect the properties of the experimental design, i.e., box/tank were always included as a random effect to account for possible dependency within tanks. Following the recommendation from Barr et al. (2013), we kept the random effects in the model irrespectively of the amount of variation it explained. All models considered included the same 2-level fixed effects, the experimental treatments, temperature and pH, as well as their all second order interaction. For body malformations, behaviour and survival, which response

could be conditional on the rearing time, we did not include species as an explanatory variable in the model to avoid confounding between effects by species and rearing time. For variables not dependent on rearing time, we considered species as an additional fixed effect, as well as the corresponding three second order interactions. The most parsimonious models were selected based on akaike information criterion and used for inference. This potentially allowed to borrow strength across species to find significant treatment effects. All statistical analyses were implemented in R (R Core Team, 2014), using the hglm package (Ronnegard et al. 2010). Effect sizes, odds ratios and confidence limits are presented, allowing a more informative discussion of the results.

Results

Hatching success and survival

Regarding hatching success (Fig. 1A,B), the most parsimonious model just included the main effects of temperature ($\beta=-0.721$, SE=0.272, $p=0.008$) and $p\text{CO}_2$ ($\beta=-0.599$, SE=0.271, $p=0.027$). Neither the effect of species ($p=0.579$) nor the interaction between both factors ($p=0.981$) had a significant effect. The odds of hatching under warming were only 0.49 (95% CI: 0.29, 0.83) times the odds of hatching under control temperature, while the odds of hatching under hypercapnia were only 0.55 (95% CI: 0.32, 0.93) times the odds of hatching under normocapnia. Specifically, the hatching success of *S. aurata* decreased from $88.3\pm7.6\%$ in the control to $65.0\pm10.0\%$ in the future scenario, while the hatching success of *A. regius* decreased from $93.3\pm5.7\%$ in the control to $80.0\pm10.0\%$ in the future scenario.

In terms of survival, a model was considered for each species. Regarding *S. aurata* (Fig. 1C), the main effects of temperature ($\beta=-1.237$, SE=0.130, $p<0.001$) and $p\text{CO}_2$ ($\beta=-0.313$, SE=0.130, $p=0.016$) were significant, with survival being lower under higher temperatures and hypercapnia. The odds of survival under warming were only 0.29 (95% CI: 0.22, 0.37) times the odds of survival under control temperature, while the odds of survival under hypercapnia were 0.73 (95% CI: 0.57, 0.94) times the odds of survival under normocapnia. However, the interaction between temperature and $p\text{CO}_2$ did not have a significant effect

($p=0.414$). Survival rates of this species decreased from $43.3\pm2.8\%$ under control conditions to $20.8\pm2.9\%$ under the future scenario. Regarding *A. regius* (Fig. 1D), the main effects of temperature ($\beta=-1.015$, $SE=0.113$, $p<0.001$) and pCO_2 ($\beta=-0.301$, $SE=0.113$, $p=0.008$) were also significant, but not the interaction between them ($p=0.236$). The odds of survival under warming were only 0.36 (95% CI: 0.29, 0.45) times the odds of survival under control temperature, while the odds of survival under hypercapnia were 0.74 (95% CI: 0.59, 0.92) times the odds of survival under normocapnia. The survival of this species decreased from $40.0\pm10.0\%$ under control to $20.0\pm5.0\%$ under future conditions.

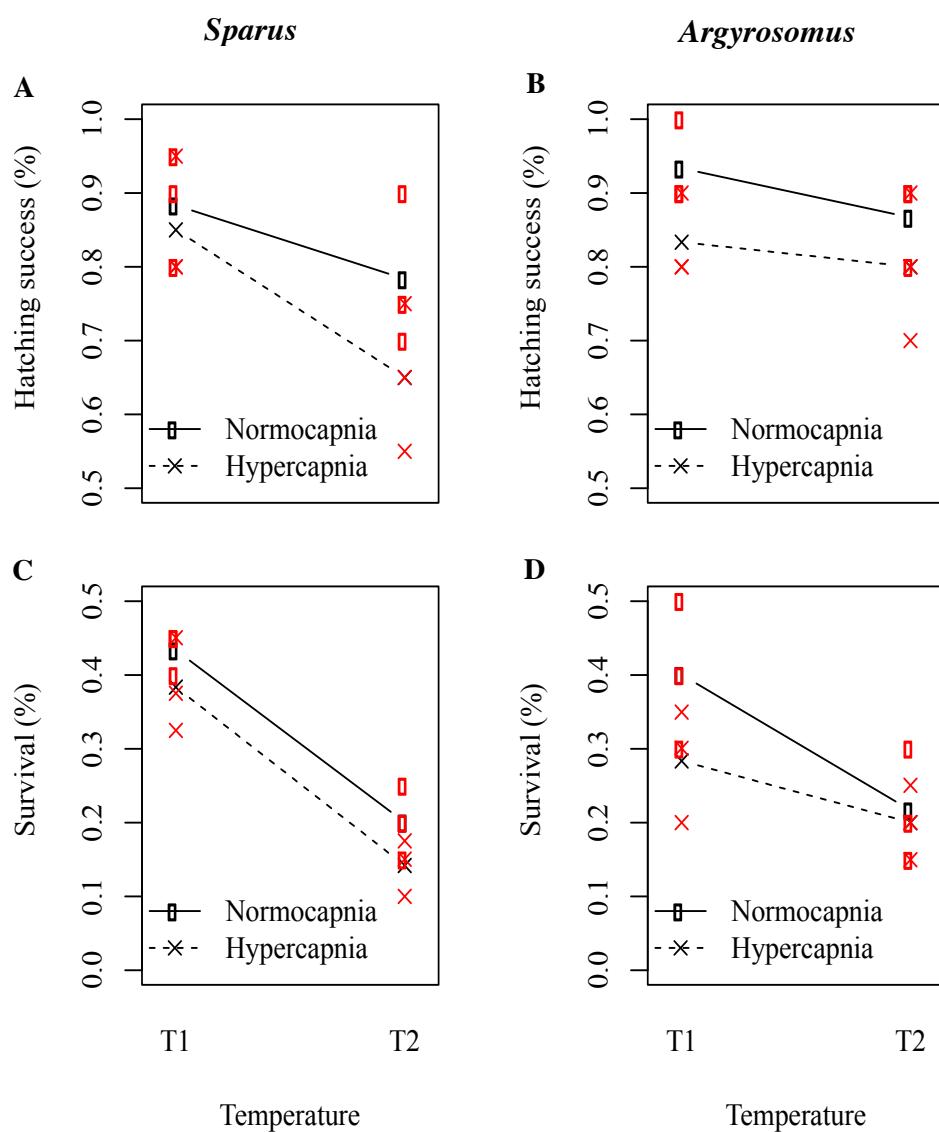


Fig. 1. Effect of ocean warming and acidification on the survival of early stages of *Sparus aurata* and *Argyrosomus regius*. Hatching success of *S. aurata*

(A) and *A. regius* **(B)**, and survival of 15 dph *S. aurata* larvae **(C)** and 10 dph *A. regius* larvae **(D)** under different temperature and pH scenarios. T1 represents control temperature and T2 represents warming temperature. Open circles represent normocapnia and crosses hypercapnia conditions. Red dots represent each point of observation (n=12; p<0.05).

Length and growth

The mean standard length of newly-hatched larvae (at 0 dph) seems to be independent of the treatments (Fig. 2A,B), with no terms found to be significant ($p=0.284$, $p=0.982$ and $p=0.393$ for temperature, $p\text{CO}_2$ and the interaction between both factors, respectively). Size at hatching ranged between 2.6 ± 0.2 to 2.7 ± 0.3 mm for *S. aurata*, and between 2.5 ± 0.5 and 2.7 ± 0.3 mm for *A. regius*. Considering growth (Fig. 2C,D), the main effect of $p\text{CO}_2$ was significant ($\beta=-0.028$, $SE=0.008$, $p=0.009$), as well as the interaction between $p\text{CO}_2$ and species ($\beta=0.037$, $SE=0.012$, $p=0.003$). However, neither temperature ($p=0.098$) nor species ($p=0.107$) had a significant effect. SGL was higher for *A. regius*, with values ranging from 0.09 to 0.11 mm day $^{-1}$, while for *S. aurata* it ranged from 0.06 to 0.09 mm day $^{-1}$. The significant interaction between $p\text{CO}_2$ and species arises from the fact that growth was higher under normocapnia for *S. aurata* but higher under hypercapnia for *A. regius*.

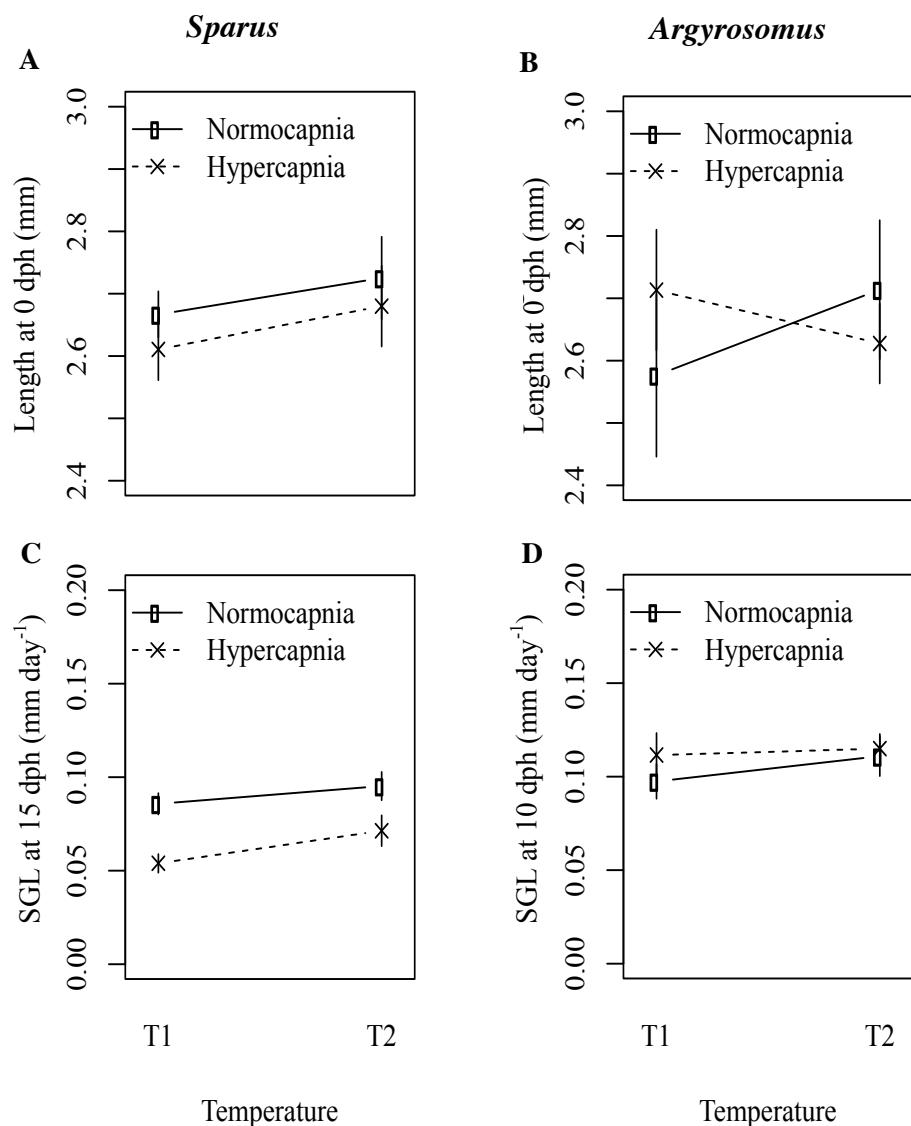


Fig. 2. Effect of ocean warming and acidification on the growth of *Sparus aurata* and *Argyrosomus regius* larvae. Standard length of *S. aurata* (A) and *A. regius* (B), and somatic growth length (SGL) of 15 dph *S. aurata* (E) and 10 dph *A. regius* (F) under different temperature and pH scenarios. T1 represents control temperature and pH, and T2 represents warming temperature and acidification. Open circles represent normocapnia and crosses represent hypercapnia conditions. Values represent means \pm SE ($n=12$; $p<0.05$).

Body malformations

Malformations were only assessed for *S. aurata* (Figs. 3 and 4). Regarding total malformations (Fig. 4A), both the main effects temperature ($\beta=0.946$, $SE=0.328$, $p=0.004$) and $p\text{CO}_2$ ($\beta=1.730$, $SE=0.332$, $p<0.01$) were significant, but not the

interaction between both factors ($p=0.287$). The odds of total malformations under warming were 2.57 (95% CI: 1.35, 4.90) times the odds of malformations under control temperature, while the odds under hypercapnia were 5.64 (95% CI: 2.95, 10.80) times the odds under normocapnia. The incidence of malformations increased from $0.25\pm0.13\%$ under control conditions to $0.83\pm0.10\%$ under the warmer and acidified scenario.



Fig. 3. Malformations observed in *Sparus aurata* larvae. Abdominal axial deviations: excessive outward curvature (A), excessive inward curvature (B), deformed curvature (C) and side-to-side curvature (D). Caudal axial deviations: abnormal urostyle flexion (E), and excessive outward curvature (F). Cranium malformations: in the eye (G, H), jaw (C, G, H), and opercle (B, C).

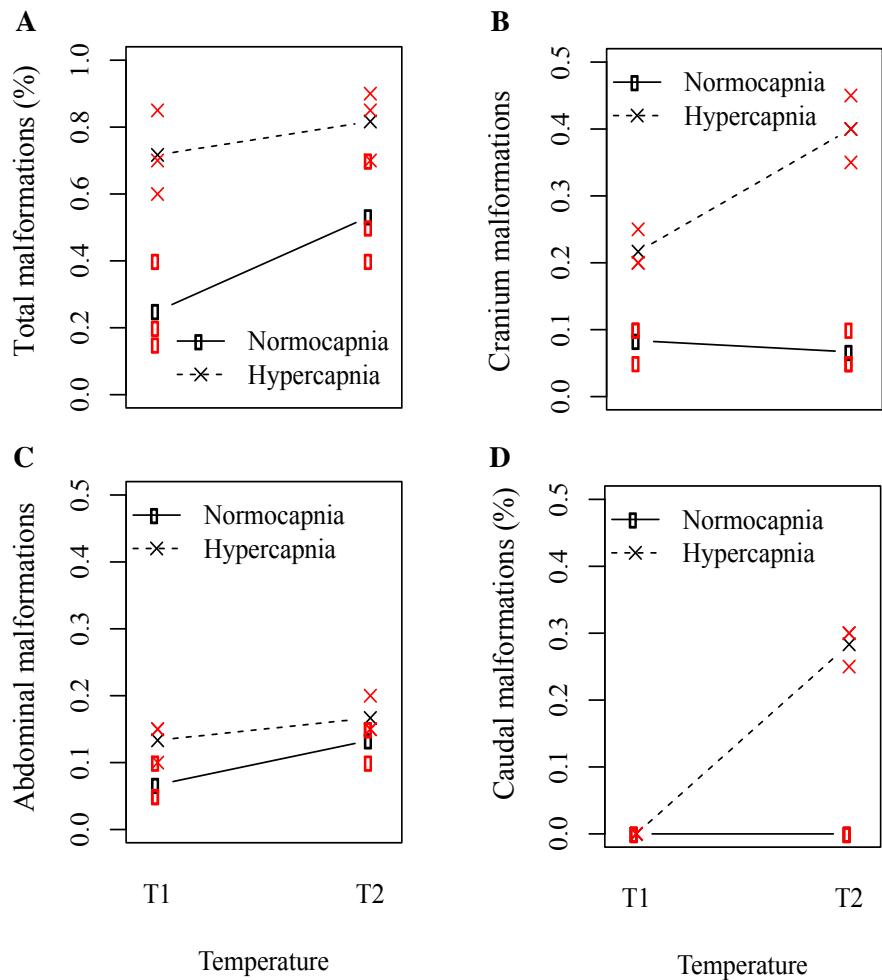


Fig. 4. Effect of ocean warming and acidification on the incidence of malformations in *Sparus aurata* larvae. Total malformations (A), cranium malformations (B), and axial deviations in the caudal (C) and abdominal regions (D) of *S. aurata* larvae under different temperature and pH scenarios. T1 represents control temperature and T2 represents warming temperature. Open circles represent normocapnia and crosses hypercapnia conditions. Red dots represent each point of observation ($n=60$; $p<0.05$).

The only term included in the cranium malformations model was $p\text{CO}_2$ ($\beta=1.704$ SE=4.272, $p<0.001$), with a higher proportion of malformations observed under hypercapnia (Fig. 4B). No significant effect was observed for temperature ($p=0.729$) or the interaction between both factors ($p=0.166$). The odds under hypercapnia were 5.5 (95% CI: 2.52, 12.02) times the odds under normocapnia. Under elevated $p\text{CO}_2$, the incidence of cranium malformations increased significantly from 0.08 ± 0.03 to $0.22\pm0.03\%$ under control temperature, and from 0.07 ± 0.03 to $0.40\pm0.05\%$ under warming. With respect to abdominal

malformations (Fig. 4C), none of the variables was significant ($p=0.232$, $p=0.232$ and $p=0.539$ for temperature, $p\text{CO}_2$ and the interaction between both factors, respectively). For caudal malformations (Fig. 4D), there was not enough information to build a model, since this type of malformation was only observed in one of the four treatments. However, a great proportion ($26.7\pm2.9\%$) of the fish in the warmer and acidified scenario presented this malformation.

Behavioural patterns

In terms of behaviour (Fig. 5), a model was considered for each species. For *S. aurata*, swimming (Fig. 5A) was only significantly affected by $p\text{CO}_2$ ($\beta=-18.333$, $SE=2.590$, $p<0.001$), and not by temperature ($p=0.164$) or the interaction between both factors ($p=0.243$). Swimming duration significantly decreased with $p\text{CO}_2$ from 40.1 ± 5.6 to 30.1 ± 5.8 sec at normal temperature, and from 49.7 ± 4.4 to 37.9 ± 5.1 sec at the warming condition. For attack (Fig. 5C), both temperature ($\beta=0.352$, $SE=0.163$, $p=0.031$) and $p\text{CO}_2$ ($\beta=-1.214$, $SE=0.261$, $p<0.001$) were found significant, as well as the corresponding interaction ($\beta=0.862$, $SE=0.308$, $p=0.005$). More attacks happened under higher temperatures, but this increase was more pronounced under hypercapnia than normocapnia. The capture success (Fig. 5E) was also significantly affected by $p\text{CO}_2$ ($\beta=-1.466$, $SE=0.320$, $p=0.000$) and by the interaction between both factors ($\beta=0.802$, $SE=0.382$, $p=0.036$), but not by temperature ($p=0.145$). The capture success of this species decreased significantly under hypercapnic conditions, from 4.3 ± 1.7 to $1.0\pm0.4\%$ under control temperature, and from 5.7 ± 1.4 to $3.0\pm0.9\%$ at warmer temperatures. For spin behaviour (Fig. 5G), there was not enough information to build the model because this behaviour was only observed in one of the four treatments.

Regarding *A. regius*, swimming (Fig. 5B) was significantly affected by temperature ($\beta=8.750$, $SE=1.817$, $p=0.001$) and $p\text{CO}_2$ ($\beta=-10.917$, $SE=1.817$, $p=0.000$), but not by the interaction between them ($p=0.627$). Warmer temperature significantly increased the time larvae spent swimming from 40.1 ± 5.6 to 49.7 ± 4.4 sec under normocapnia, and from 30.1 ± 5.8 to 37.9 ± 5.1 sec under hypercapnia.

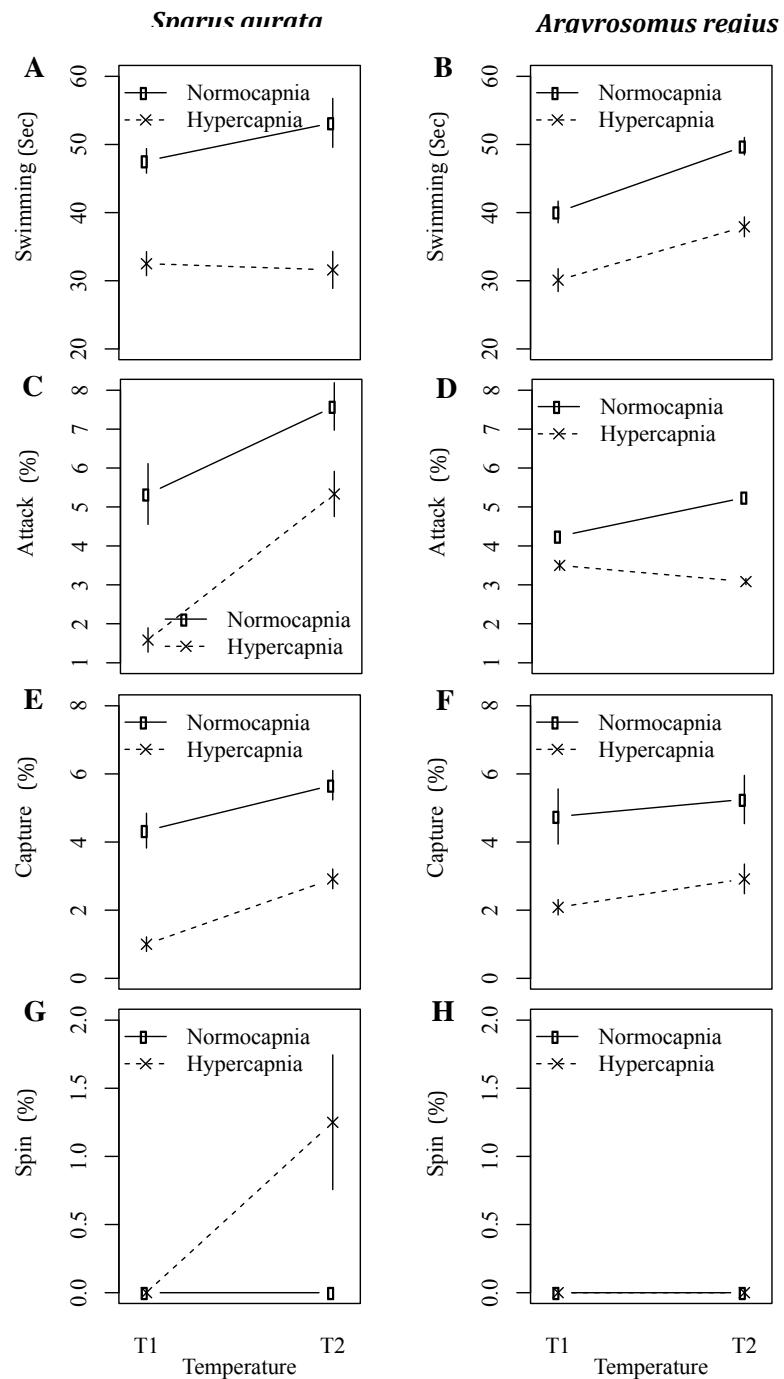


Fig. 5 - Effect of ocean warming and acidification on the behaviour of *Sparus aurata* and *Argyrosomus regius* larvae. Time spent swimming (A, B), and the occurrence of attack (C, D), capture (E, F) and spin (G, H) behaviours of 15 dph *S. aurata* and 10 dph *A. regius* larvae under different temperature and pH scenarios. T1 represents control temperature and T2 represents warming temperature. Open circles represent normocapnia and crosses hypercapnia conditions. Values represent means \pm SE ($n=12$; $p<0.05$).

In contrast, swimming duration significantly decreased with $p\text{CO}_2$, from 40.1 ± 5.6 to 30.1 ± 5.8 sec at normal temperature, and from 49.7 ± 4.4 to 37.9 ± 5.1 sec at the warming condition. The attack (Fig. 5D) and capture rates (Fig. 5F) were only significantly affected by $p\text{CO}_2$ ($\beta = -0.367$, $\text{SE} = 0.146$, $p = 0.012$ and $\beta = -0.693$, $\text{SE} = 0.158$, $p < 0.001$, respectively). No significant effect of temperature ($p = 0.250$) or interaction ($p = 0.459$) were detected. Attack rates decreased significantly from 5.3 ± 2.7 to $1.6 \pm 0.9\%$ at control temperature, and from 7.6 ± 2.1 to $5.3 \pm 2.0\%$ at warming. The capture success also decreased significantly from 4.8 ± 2.7 to $2.1 \pm 0.8\%$ under present-day temperature, and from 5.2 ± 2.4 to $2.9 \pm 1.4\%$ under warming.

Discussion

In the present study, we showed that early life stages of *S. aurata* and *A. regius* were quite sensitive to future ocean conditions. Both warming and acidification lowered significantly the hatching rates of both species. Hatching success showed to be 2 times higher under the control temperature than under warming, and approximately twice as higher under normocapnia than under hypercapnia. Such losses can have further severe repercussions for species persistence in tomorrow's ocean. Indeed, survival rates of both species (15 dph *S. aurata* and 10 dph *A. regius* larvae) decreased significantly with warming and acidification. Survival rates were approximately 3 times higher under the control temperature than when compared to warming, and nearly 1.4 times higher under normocapnia than under hypercapnia.

Hypercapnia also had a significant effect on larval growth, but it differed between species. While the SGL of *S. aurata* showed a 24.8-36.4% decrease under hypercapnic conditions, a slightly increase (3.2-12.9%) was observed in *A. regius*. The former results may suggest a weak control and maintenance of internal pH on *S. aurata* and a consequent decrease in protein biosynthesis (Langenbuch and Pörtner 2003). In this species, the energy budget may have been allocated away from non-essential processes, such as growth, towards maintenance (Pörtner and Peck 2010). The present difference observed between species reinforces the

absence of consensus among studies on the effect of ocean acidification on the size and growth of marine fish larvae. While some studies have reported decreased size and growth under high $p\text{CO}_2$ levels (Baumann et al. 2012; Frommel et al. 2014; Pimentel et al. 2014), others indicate that larvae may grow equally well or even faster under high $p\text{CO}_2$ conditions (Hurst et al. 2013; Hurst et al. 2012). If the impact on growth is truly species-specific, then ocean acidification and warming may have a complex impact on the dynamics of marine food webs, since larval growth and body size may mediate susceptibility to predation mortality (Anderson 1988). Nonetheless, we can also argue that such contradictory findings may be the result of experiments being carried out at temperatures with unclear positioning on thermal performance curves, or possibly due to experimental rearing artefacts (e.g. different feeding regimes). It is worth noting that our findings on survival and growth under present-day conditions were quite similar to those found in the literature for these species under intensive rearing conditions (Papandroulakis et al. 2000; Roo et al. 2010).

Ocean warming and acidification also had a significant effect on the incidence of malformations. At present-day conditions, the formation pattern of the axial skeleton elements in *S. aurata* was similar to that reported for other teleost larvae (Sfakianakis et al. 2004). Under the combined effect of hypercapnia and warming, the incidence of malformations greatly increased. Total malformations were approximately 3 times higher under warming than when compared to control temperature, and nearly 6 times higher under hypercapnia than under normocapnia. Cranium malformations also increased significantly with ocean acidification. Under the future scenario, the occurrence of this malformation was 31.7 percentage points higher than in present-day conditions. Although there was not enough information to build a model for caudal axial deviations, it has to be noticed that almost 30% of the fish presented this malformation when exposed to the combined effect of warming and acidification. Other studies also found greater incidence of abnormal development in fish larvae under elevated temperature and/or $p\text{CO}_2$ (Baumann et al. 2012; Pimentel et al. 2014). During early development, axial deviations may result from defective development of the notochord and perinotochordal connective sheet (Sanatamaría et al. 2005), which may in turn lead to further skeletal malformations in the vertebral column such as

lordosis, scoliosis and kyphosis. We argue that such malformations may affect the larval capacity to maintain the position in the water column, and further compromise their swimming, foraging and predator avoidance (Powell et al. 2009).

In the present study, larval behaviour was affected by future ocean conditions. Temperature increased the time *A. regius* larvae spent swimming, but did not affect *S. aurata* swimming. In contrast, hypercapnia decreased the time spent swimming in both species. Interestingly, *S. aurata* larvae showed an erratic “spin” movement only at higher temperatures and $p\text{CO}_2$ levels. Even though some previous studies have found no effect of ocean acidification on fish swimming behaviour (e.g. Maneja et al. 2013a), others have reported significant changes in this behaviour under such environmental conditions (e.g. Dixson et al. 2010; Munday et al. 2010). Reduced swimming skills and the occurrence of erratic movements by the larvae in the wild may increase their vulnerable to predation. Moreover, hypercapnia also decreased the attack and capture rates of prey. The lower capture success of prey will most certainly impact their growth and development, and further affect larval performance, survival and recruitment rates (Stanley 2009). It is however important to keep in mind that some bias may potentially arise from the effects that ocean climate change may have on live prey and larvae-prey interaction, which may directly affect food availability and fish larval behaviour.

In conclusion, the biological responses of *S. aurata* and *A. regius* larvae presented in the present study may provide an insight of how future warming and acidification may impact the development of wild fish larvae and their fitness in a changing ocean. However, given the time frame in which ocean warming and acidification are expected to occur, it is important not to forget that there will be an opportunity for acclimatization and adaptation. Although the mechanisms for adaptation remain poorly known, some studies have already shown that parental (transgenerational) acclimation can modify the response of fish larvae to climate change conditions (e.g. Schade et al. 2014; Welch et al. 2014). It is therefore expected that such processes can moderate the negative impacts of future ocean conditions on *S. aurata* and *A. regius* larvae. Future efforts should focus on how these environmental factors may affect commercially important fish species at

higher levels of organization (e.g. at a population-level) in a way to help managers and policy-makers to take proactive measures.

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Author contributions

R.R. and M.S.P. designed the experiment; M.S.P. and G.D. performed the experiments; M.S.P., F.F., T.M., R.B., G.D., J.M., P.P.F, and R.R. analysed the data; M.S.P., F.F., T.M., R.B., G.D., A.M.F., J.M., M.P., H.P., E.J.G. and R.R. wrote the main paper. All authors discussed the results and their implications, and commented on the manuscript at all stages.

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CHAPTER 4

Impact of ocean acidification in the metabolism and swimming behavior of the dolphinfish (*Coryphaena hippurus*) early larvae

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Impact of ocean acidification in the metabolism and swimming behavior of the dolphinfish (*Coryphaena hippurus*) early larvae

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Abstract Since the industrial revolution, $[CO_2]_{atm}$ has increased from 280 μatm to levels now exceeding 380 μatm and is expected to rise to 730–1,020 μatm by the end of this century. The consequent changes in the ocean's chemistry (e.g., lower pH and availability of the carbonate ions) are expected to pose particular problems for marine organisms, especially in the more vulnerable early life stages. The aim of this study was to investigate how the future predictions of ocean acidification may compromise the metabolism and swimming capabilities of the recently hatched larvae of the tropical dolphinfish (*Coryphaena hippurus*). Here, we show that the future environmental hypercapnia ($\Delta\text{pH } 0.5$; 0.16 % CO_2 , ~1,600 μatm) significantly ($p < 0.05$) reduced oxygen consumption rate up to 17 %. Moreover, the swimming duration and orientation frequency also decreased with increasing pCO_2 (50 and 62.5 %, respectively). We argue that these hypercapnia-driven metabolic and locomotory challenges may potentially influence recruitment, dispersal success, and the population dynamics of this circumtropical oceanic top predator.

Introduction

The atmospheric concentration of carbon dioxide (CO_2) has increased nearly 40 % from preindustrial levels of

280 μatm to present-day levels (~380 μatm), and it is expected to rise up to 730–1,020 μatm by the end of the century (Meehl et al. 2007). As the world's ocean represents a major CO_2 sink, the continuous CO_2 uptake by the ocean will change the seawater chemistry and consequently will lead to an estimated drop in oceans pH of 0.4–0.5 units (Caldeira and Wickett 2005). The expected changes in ocean chemistry will challenge many marine organisms and is predicted to negatively impact marine ecosystems (Talmage and Gobler 2010). Ocean acidification is considered a major threat to many marine organisms as it can lead to disturbances in their acid–base balance, protein biosynthesis, and metabolism (Portner et al. 2004; Rosa et al. 2013). Consequently, elevated CO_2 can be particularly detrimental to survival, growth (Byrne 2011; Baumann et al. 2012), and to behavioral ecology of several marine species (Munday et al. 2009; Dixson et al. 2010; Simpson et al. 2011; Domenici et al. 2012; Ferrari et al. 2012). Exposure to elevated environmental CO_2 affects particularly marine organisms with exoskeletons made from calcium carbonate, because the availability of the carbonate ions required for calcification processes decreases (Fabry et al. 2008; Talmage and Gobler 2010). Although fish have evolved the capacity to accumulate bicarbonate and exchange ions across gills within hypercapnic conditions (Portner et al. 2005; Ishimatsu et al. 2008), newly hatched marine fish larvae often lack this osmoregulatory capacity and the ability to effectively regulate internal pH (Perry and Gilmour 2006; Baumann et al. 2012; Frommel et al. 2012).

Early stages are expected to be the most vulnerable to these new climate change-related conditions, and their eventual inability to cope and adapt may constitute a bottleneck for species persistence in a changing ocean (Rosa et al. 2012). Nevertheless, ocean acidification studies on larval fish performance are at present scarce or have

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reported negligible effects of ocean acidification on larval fish (Franke and Clemmesen 2011; Frommel et al. 2012). Here, we investigated the effects of ocean acidification on the metabolism and behavior patterns of the early life stages of the dolphinfish, *Coryphaena hippurus*. It is a highly migratory epipelagic fish (also known as mahi mahi) that is distributed in the world's tropical and subtropical regions (Beardsley 1967), and constitutes an important marine resource, supporting commercial and sport fisheries throughout its range (Oxenford 1999).

Materials and methods

Egg collection

Coryphaena hippurus eggs were collected in June 2013 at the University of Miami Experimental Hatchery (UMEH). More specifically, the recently spawned eggs were immediately transferred (under the supervision of Dr. Daniel Benetti—for more details, see in Rodrigues et al. 2013) to the aquaculture facilities in Laboratório Marítimo da Guia, Cascais. *C. hippurus* recently hatch larvae were exposed to increased $p\text{CO}_2$ (ΔpH 0.5; 0.16 % CO_2 , ~1,600 μatm). *C. hippurus* is a circumtropical oceanic pelagic species that is generally distributed in waters of the Atlantic, Pacific, and Indian Oceans (Castro et al. 1999), and known to inhabit areas of coastal upwelling. Organisms inhabiting such regions are commonly exposed to seasonal high $p\text{CO}_2$ events (>500 μatm ; Perez et al. 1999) due to the emergence of deep hypercapnic water masses. Consequently, in these regions, the future $p\text{CO}_2$ levels are expected to exceed the forecasted 1,000–1,200 μatm (ΔpH 0.4–0.5) for 2,100 (Meehl et al. 2007).

The systems were filled with filtered (series 20, 10, 5, and 1 μm) and UV-sterilized seawater, and tanks were illuminated with a photoperiod of 14-h light/10-h dark. Water quality was ensured using wet-dry filters (bioballs), protein skimmers (Schuran, Jülich, Germany), and 30 W UV sterilizers (TMC, Chorleywood, UK). Ammonia and nitrite were monitored regularly and kept below detectable levels. Salinity throughout the experiment was of 35.33 ± 0.19 , and temperatures of 26.0 ± 0.2 °C were controlled via Heilea chillers (Guangdong, China). Additionally, pH was

manually controlled (daily) showing average values in the range of 8.02 ± 0.05 and 7.51 ± 0.05 , respectively. pH was adjusted automatically via the Profilux system (Kaiserslautern, Germany) as described in Rosa et al. (2013, in press). Seawater carbonate system speciation was weekly calculated from total alkalinity according to previously established methods (Sarazin et al. 1999) (spectrophotometrically at 595 nm) and pH measurements (Table 1). Bicarbonate and $p\text{CO}_2$ values were calculated using the CO2SYS software (Lewis and Wallace 1998).

Larvae rearing

Eggs and larvae were reared in twelve recirculating (19 L each) seawater systems. *Coryphaena hippurus* newly hatched larvae were reared in a CO_2 system that comprises twelve recirculating (19 L each) seawater systems. Larvae were randomly individualized into each replicate (19 L each) at a density of 10 larvae per litter (Benetti et al. 2003; Bignami 2013) and were reared at 26 °C (the optimal spawning temperature, Benetti et al. 1995) under two different $p\text{CO}_2$ conditions, namely normocapnia (26 °C, $p\text{CO}_2 = \sim 400 \mu\text{atm}$) and future environmental hypercapnia (26 °C, $p\text{CO}_2 = \sim 1,600 \mu\text{atm}$). Feeding schedule was based on previous studies (Benetti et al. 2003; Bignami 2013). Larvae opened the mouth around 2 days post-hatching (dph) and started to feed on rotifers (*Branchionus plicatilis*) and copepods (*Acartia granii*). Two experiments were run to evaluate the potential effects of exposure to hypercapnic conditions on metabolism and behavior of tropical fish early life stages.

Oxygen consumption rates

Mass-specific oxygen consumption measurements were taken according to the previously established methods (Pimentel et al. 2012; Rosa et al. 2012, 2013). Eight larvae with 3 days post-hatching (dph) were incubated individually in sealed water-jacketed respirometry chambers (RC300 Respiration cell, Strathkelvin, North Lanarkshire, Scotland) containing 1- μm filtered and UV-irradiated natural seawater mixed with antibiotics (50 mg L^{-1} streptomycin) to avoid bacterial respiration. The larval size and weight varied around 2.4 ± 0.2 mm and 0.65 ± 0.12 mg, respectively, for the larvae reared at normal $p\text{CO}_2$; it

Table 1 Seawater carbonate chemistry data for the different climate change scenarios

Temperature (°C)	pH (total scale)	A_T ($\mu\text{mol kg}^{-1}$ SW)	C_T ($\mu\text{mol/kg}^{-1}$ SW)	$p\text{CO}_2$ (μatm)	HCO_3^- ($\mu\text{mol kg}^{-1}$)	Ω_{arag}
26.05 ± 0.42	8.04 ± 0.03	2333.33 ± 89.09	2048.19 ± 81.43	457.23 ± 22.36	1830.02 ± 89.50	3.27 ± 0.16
26.12 ± 1.01	7.54 ± 0.05	2299.67 ± 81.02	2237.09 ± 79.98	1671.69 ± 59.77	2115.79 ± 75.69	1.19 ± 0.04

Total carbon (C_T), carbon dioxide partial pressure ($p\text{CO}_2$), bicarbonate concentration (HCO_3^-), and aragonite saturation state of seawater (Ω_{arag}) were calculated with CO2SYS using salinity, temperature, pH, and total alkalinity (A_T). Values are mean \pm SD

varied around 2.5 ± 0.1 mm and 0.63 ± 0.10 mg for the ones reared at hypercapnic condition. Water volumes were adjusted in relation to animal mass (up to 10 mL) in order to minimize locomotion and stress but still allow for spontaneous and routine activity rates of larvae. Controls (blanks) were used to correct for possible bacterial respiratory activity. Respiration chambers were immersed in water baths (Lauda, Lauda-Königshofen, Germany) to control temperature. Oxygen concentrations were recorded with Clarke-type O₂ electrodes connected to a multi-channel oxygen interface (Model 928, Strathkelvin, North Lanarkshire, Scotland). After an acclimatization period of about 2 h, the duration of respiratory runs varied from 3 to 4 h.

Behavioral patterns

Behavioral patterns of *Coryphaena hippurus* were analyzed by using the focal animal technique (Altman 1974; Martin and Bateson 1993; Tojeira et al. 2012). The observations were performed 30 min after feeding, for a total of 10 larvae (with same size) per treatment. A preliminary study was performed in order to establish the ethogram of *C. hippurus* 3 dph larvae. The catalogue of behaviors (ethogram) exhibited by *C. hippurus* larvae was categorized into two groups: locomotory and non-directed patterns. The locomotory category was then divided into (1) swimming duration (*S*)—duration of larvae movements per minute, (2) active larvae (*A*)—larvae that exhibit a forward movement through the water column accomplished by caudal fin action within a minute, and the non-directed category into (3) orientation (*O*)—number of times that larvae, in a minute, assumes a vertical body position in water column, with head toward the bottom of the rearing tanks. Behaviors (1) and (3) were recorded as time variables, whereas behavior (2) was recorded as frequency variable.

Statistical analysis

The effect of pH on metabolism and behavior was evaluated using a one-way ANOVA, followed by Tukey's post hoc test. Previously, normality and homogeneity of variances were verified by Kolmogorov-Smirnov and Bartlett tests, respectively. All statistical analyses were performed for a significance level of 0.05, using Statistica 10.0 software.

Results and discussion

Coryphaena hippurus early larval stages were found to be particularly sensitive to ocean acidification. Despite the short embryonic development time of *C. hippurus* (less than 2 days), egg incubation under short-term acidified conditions was enough to elicit a negative impact on larvae metabolism and swimming behavior.

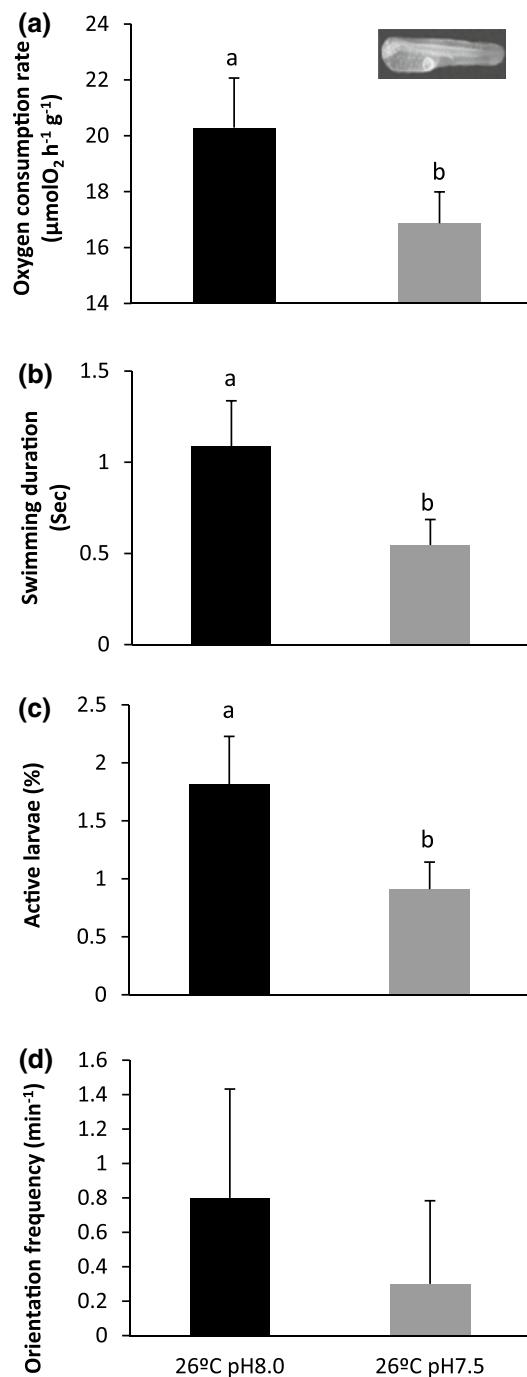


Fig. 1 Impact of ocean acidification on the metabolism and swimming behavior of *Coryphaena hippurus* recently hatched larvae. Oxygen consumption rate ($\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ ww, $n = 5$) (a), swimming duration (sec, $n = 10$) (b), percentage of active larvae (%), $n = 10$ (c), and vertical orientation (min^{-1} , $n = 10$) (d) of larvae at different $p\text{CO}_2$ scenarios. Values are given in mean \pm SD. Different letters represent significant differences ($p < 0.05$)

In fact, oxygen consumption rates (OCR) were significantly affected by future hypercapnic conditions ($p < 0.05$; Fig. 1a). The metabolism of *C. hippurus* 3 dph larvae

decreased 16.86 % with increasing $p\text{CO}_2$, from 20.3 ± 1.8 (26°C , $p\text{CO}_2 = \sim 400 \mu\text{atm}$) to $16.9 \pm 1.1 \mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ (26°C , $p\text{CO}_2 = \sim 1,600 \mu\text{atm}$). Under longer time frames, this metabolic depression may cause a reduction in protein synthesis and growth (Hochachka and Somero 2002; Storey and Storey 2004). Additionally, slower growing larvae are potentially more vulnerable to predators and may thus experience greater mortalities (Anderson 1988).

Fish larvae in order to maintain a vertical orientation in water column use periodic bouts of oriented swimming (Hurst et al. 2009), but abiotic factors, such as temperature and salinity, are known to affect the buoyancy and metabolic efficiencies of the larvae inducing behavioral mitigation (Hurst et al. 2009). Here, we show that, besides metabolic depression (Fig. 1a), there were also impairments in the swimming activity under environmental hypercapnia (Fig. 1b, c). In fact, swimming duration (S) of 3 dph larvae was significantly affected by near-future $p\text{CO}_2$ conditions ($p < 0.05$; Fig. 1b), decreasing from 1.08 ± 0.33 (26°C , $p\text{CO}_2 = \sim 400 \mu\text{atm}$) to 0.54 ± 0.20 s (26°C , $p\text{CO}_2 = \sim 1,600 \mu\text{atm}$). Concomitantly, the frequency of active larvae decreased 50 % under hypercapnic condition ($p < 0.05$; Fig. 1c), from 1.8 ± 0.54 (26°C , $p\text{CO}_2 = \sim 400 \mu\text{atm}$) to 0.9 ± 0.33 % (26°C , $p\text{CO}_2 = \sim 1,600 \mu\text{atm}$). The vertical orientation frequency (min^{-1}) followed a similar (albeit not significant) trend with a decrease of 62.5 % from normocapnia to hypercapnic conditions (0.8 ± 0.63 and $0.3 \pm 0.48 \text{ min}^{-1}$, respectively) ($p > 0.05$; Fig. 1d). Recent studies have also shown significant changes in fish swimming behavior under acidified conditions (Dixson et al. 2010; Munday et al. 2010), nevertheless others reported the opposite effect (Maneja et al. 2013). One should keep in mind that simple behavior deviations may greatly influence larvae growth; feeding; and predation rate, survival, and recruitment (Leis 2006; Vikebø et al. 2007; Stanley 2009).

The hypercapnia-related metabolic and locomotory challenges may potentially influence dolphinfish recruitment and dispersal success, which may consequently affect the global circumtropical distribution and population dynamics of this top oceanic predator under this future climate scenario. Therefore, it is challenging but important to scale up these physiological impairments of early life stages to potential population-level consequences for a species. Moreover, ocean acidification will be accompanied by warming in large expanses of the oceans and it will be of great importance to assess/predict how the synergistic effects will influence early life stages of this apex fish predator.

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CHAPTER 5

Metabolic potential of fish early stages with different life strategies and locomotory abilities under ocean warming and acidification

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**Metabolic potential of fish early stages with different life strategies and
locomotory abilities under ocean warming and acidification**

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Abstract

Until now the effects of future ocean climate conditions in the activity of key metabolic enzymes of fish early life stages are poorly known. Here we investigate the combined effects of warming ($+4^{\circ}\text{C}$) and acidification ($\Delta\text{pH}=0.5$ units) on the oxygen consumption, heart rates and on the metabolic enzymatic machinery of fish larvae with different life strategies and locomotory abilities, namely seabream, *Sparus aurata* (pelagic continuous-swimmers) and Senegalese sole, *Solea senegalensis* (benthonic poor-swimmers). Both oxygen consumption and heart rates showed to be positively affected by temperature and negatively affected by hypercapnia. While the metabolism of seabream was higher than of the sole, no significant differences in heart rates were found between both species. As expected the faster swimming fish species with higher metabolic demands revealed a stronger aerobic capacity, as suggested by the higher citrate synthase (CS) and lactate dehydrogenase (LDH) activities. Both species also differed significantly in β -hydroxyacyl CoA dehydrogenase (HOAD) activity, with seabream presenting about twice the values of sole. Future ocean conditions elicited a decrease in CS and HOAD activities, and a counterbalanced increased of LDH activity. While CS activity of sole and seabream and LDH activity of sole were only significantly affected by $p\text{CO}_2$, LDH activity of seabream was significantly affected by both factors. These observed trends reflect a shift from aerobic to anaerobic pathways of substrate oxidation under future conditions, especially in seabream. The increase in glycolytic potential is a common tactic to enhance organism's tolerance to climate-related changes however is not sustainable on longer time-scales.

Keywords: Warm-hypercapnia acclimation, metabolic and heart rates, aerobic and anaerobic energy metabolism, early life stages, *Sparus aurata*, *Solea senegalensis*.

Introduction

In the last decades, the average temperature of the world's oceans has increased, and additional warming (up to 3 °C) is expected by the end of the century (IPCC 2013). Concurrently, atmospheric CO₂ concentrations are rising, and the continuous absorption of atmospheric CO₂ by oceans is causing a decline in ocean's pH, a process known as ocean acidification (Caldeira and Wickett 2005). If the rate of anthropogenic CO₂ emissions continues to rise, forecasts estimate an increase up to 1000 µatm by the year 2100 (Pörtner et al. 2014) and above 2000 µatm in some coastal areas (IPCC 2013). Exposure to ocean acidification may narrow the thermal tolerance window and, consequently, intensify the effect of increasing sea surface temperature on biological processes of marine ectothermic animals, such as growth, calcification, behavior and metabolism (Fabry et al. 2008; Hofmann et al. 2010; Hofmann and Todgham 2010; Melzner et al. 2009; Pörtner and Farrell 2008; Pörtner 2008).

Until recently, fish were assumed to be quite resilient to rising CO₂ because they are equipped with a powerful capability to maintain their homeostasis and compensate for extra and intracellular pH disturbances. However, recent studies have already report fish early stages vulnerability to ocean acidification (Franke and Clemmesen 2011; Frommel et al. 2014; Frommel et al. 2012; Munday et al. 2009; Munday et al. 2012; Pimentel et al. 2014; Tseng et al. 2013). The increase of seawater pCO₂ may cause CO₂ to enter by diffusion across gill epithelia into fish tissues and fluids and, if not actively compensated by HCO₃⁻ accumulation and/or H⁺ secretion, it may result in extracellular acidosis (Melzner et al. 2009). Plasma pH changes may constrain the capacity of oxygen supply and delivery (Pörtner et al. 2004) and negatively affect marine fish performance. Tissues may thereby become hypoxic, and the oxygen availability to maintain cell functions and/or cover extra costs from activities beyond those required for basic maintenance is predicted to decrease (Pörtner and Farrell 2008). Variations on the aerobic scope and aerobic performance of organisms may be a reflect of adjustments on a specific metabolic pathway and the extent to which that pathway is utilized (Pörtner 2010; Pörtner 2012; Pörtner and Farrell 2008; Strobel et al. 2013a). Such modifications of the metabolic machinery may progressively lead to a shift on the energy production mode. The activity of key metabolic enzymes, e.g. citrate synthase (CS), β-hydroxyacyl CoA dehydrogenase (HOAD) and lactate dehydrogenase (LDH) are biochemical markers that can reflect these specific

pathways and shifts. CS and HOAD are common and valuable indicators of the overall aerobic metabolic potential, specifically CS is a citric acid cycle enzyme located in the mitochondrial matrix and an indicator of mitochondrial adjustments (Somero and Childress 1980), and HOAD is used as an index for fatty acid oxidation and amino acids catabolism (Hochachka et al. 1983). Tissues with high potential for anaerobic glycolysis have been demonstrated to have high activity of cytosolic enzyme LDH, a terminal enzyme in the glycolytic pathway during anaerobiosis that is responsible for the anaerobic conversion of NADH to NAD⁺ and pyruvate to lactate (Newsholme and Leech 1988; Powers et al. 1997).

In order to contribute to the body of knowledge on fish physiological responses and adaptations to environmental challenges, we investigated some physiological mechanisms and specific metabolic pathways that species with different life strategies and locomotory abilities uses for adaptation to the new climate-related conditions. The present study was therefore designed to investigate the combined effects of warming (+4°C; 22°C) and acidification and ($\Delta\text{pH}=0.5$ units) on the oxygen consumption and heart rates, and on the metabolic enzymatic machinery during the early ontogeny of two fish species with different life strategies and locomotory abilities, specifically the seabream *Sparus aurata* (a continuous pelagic swimmers) and the flatfish *Solea senegalensis* (a benthonic poor-swimmer). More specifically, we quantified the activities of key enzymes, namely aerobic enzymes CS and HOAD and anaerobic enzyme LDH. These analyses were preformed in fifteen days post hatching larvae of *S. aurata* and flatfish *S. senegalensis*. Their ontogeny is a good example of interspecific variability in the larval fish development among sympatric species. Around fifteen days post hatch *S. senegalensis* larvae loses their bilateral symmetry, settle to the bottom and complete metamorphosis (changing from a pelagic to benthic mode of life) contrasting with *S. aurata* which is known to metamorphoses only in the second month of life (Parra and Yúfera 2001).

Material and Methods

Larval rearing

S. senegalensis and *S. aurata* larvae were acclimated to four different treatments, a cross-factor design of two temperatures and two $p\text{CO}_2$ levels: **(1)** control temperature [18 °C - the average sea temperature during the spawning season (Arias 1980; Kissil

et al. 2001)] and normocapnia ($p\text{CO}_2$ =370 μatm); **(2)** control temperature and hypercapnia ($p\text{CO}_2$ =1500 μatm); **(3)** warming (22 °C, +4 °C - warming scenario) and normocapnia; and **(4)** warming and hypercapnia. Newly-hatched larvae of each species were reared in 12 independent recirculating systems (three per treatment), each one composed by a 19 L cylindrical rearing tank connected to a 100 L sump. To ensure an accurate water temperature in each experimental treatment, the rearing tanks were placed inside 400 L water bath tanks. Temperature conditions were kept stable via seawater chiller systems, and pH levels were automatically adjusted by solenoid valves controlled by a Profilux system connected to individual pH probes (SCHOTT Instruments, Germany). pH adjustments were guaranteed by the injection of a certified CO₂ gas mixture via air stones or by aerating the water with CO₂ filtered air. Salinity, temperature and pH levels were monitored daily. Total alkalinity was measured according to Sarazin et al. (1999). The seawater carbonate chemistry was calculated for both species (see Table 1), using the CO2SYS software (Lewis and Wallace 1998). Senegal sole and seabream larvae were randomly distributed at a density of 70 larvae L⁻¹. *S. senegalensis* larvae were reared and collected in the same experiment published in Pimentel et al. (2014). Sole larvae started to feed on rotifers (*Brachionus plicatilis*) from 2 to 8 dph. *Artemia metanauplii* were introduced at 5 dph and their proportion in the diet was gradually increased, becoming the only prey offered at 8 dph. The feeding schedule of *S. aurata* larvae was adapted from (Fernández et al. 2008). Seabream larvae were fed on rotifers (*B. plicatilis*) between 2 and 15 dph, and *Artemia nauplii* from 10 to 15 dph.

Larvae of both species were collected at 15 dph, immediately placed in liquid nitrogen and then stored at -80°C for posterior enzymatic analyses.

Table 1. Seawater carbonate chemistry data for *Solea senegalensis* and *Sparus aurata* larvae under different climate change scenarios. Total carbon (C_T), carbon dioxide partial pressure (pCO_2), bicarbonate concentration (HCO_3^-) and aragonite saturation state of seawater (Ω_{arag}) were calculated with CO2SYS using salinity, temperature, pH and total alkalinity (A_T). Values are given in mean \pm SD.

Temperature (°C)	pH (Total scale)	A_T [$\mu\text{mol kg}^{-1}\text{SW}$]	C_T [$\mu\text{mol/kg}^{-1}\text{ SW}$]	pCO_2 [μatm]	HCO_3^- [$\mu\text{mol kg}^{-1}$]	Ω_{arag}
<i>Solea senegalensis</i>						
22.0 \pm 0.4	8.03 \pm 0.05	2335.7 \pm 89.1	2148.2 \pm 81.4	424.5 \pm 20.0	1985.3 \pm 75.3	2.24 \pm 0.08
22.1 \pm 1.0	7.51 \pm 0.05	2317.4 \pm 36.4	2314.7 \pm 36.7	1654.2 \pm 49.1	2194.9 \pm 34.8	0.78 \pm 0.01
18.2 \pm 0.4	8.02 \pm 0.04	2305.7 \pm 80.5	2141.8 \pm 76.8	400.0 \pm 66.7	1993.4 \pm 72.2	1.95 \pm 0.07
18.2 \pm 0.3	7.50 \pm 0.03	2281.1 \pm 61.9	2290.9 \pm 62.7	1607.9 \pm 24.8	2173.6 \pm 59.5	0.67 \pm 0.02
<i>Sparus aurata</i>						
18.4 \pm 0.3	8.09 \pm 0.07	2331.7 \pm 67.0	2055.2 \pm 60.5	342.3 \pm 73.7	1836.2 \pm 70.2	3.19 \pm 0.56
18.2 \pm 0.3	7.53 \pm 0.04	2318.6 \pm 50.7	2284.0 \pm 57.0	1484.7 \pm 91.0	2169.1 \pm 54.2	0.97 \pm 0.08
22.3 \pm 0.3	8.09 \pm 0.07	2308.3 \pm 78.8	1970.5 \pm 80.2	337.0 \pm 73.4	1736.9 \pm 98.7	3.49 \pm 0.49
22.1 \pm 0.5	7.53 \pm 0.04	2321.0 \pm 93.3	2292.2 \pm 83.2	1473.0 \pm 92.0	2137.0 \pm 99.5	1.16 \pm 0.10

Metabolism

Oxygen consumption measurements were determined according to Pimentel et al. (2014). For each species, six 15 dph larvae were incubated in sealed water-jacketed respirometry chambers (RC300 Respiration Cell, Strathkelvin Instruments Limited, UK), at each of the four experimental conditions. Water volumes were adjusted to larval size in order to allow routine activity. Chambers were immersed in Lauda water baths (Lauda-Königshofen, Germany) to control temperature. Oxygen concentrations were recorded with Clark-type O_2 electrodes connected to a multi-channel oxygen interface (Model 928, Strathkelvin Instruments Limited, UK). Blanks were run to correct for possible bacterial respiratory activity.

Routine heart rates

For each species, routine heart rates were accounted for 10 larvae (within the same size range) under a stereoscopic microscope (Leica S6D, Leica Microsystems). Larvae were allowed to acclimatize to chambers for some minutes, and measurements were taken after assuring that they were not under stress. Routine heart rates were defined as the number of heart beats per unit of time when larvae were motionless.

Enzyme activity

The enzyme activity of sole and seabream larvae was evaluated based on the activity levels of enzymes that reflect the capacity of oxidative pathways in catabolism, namely citrate synthase (CS), β -hydroxyacyl CoA dehydrogenase (HOAD) and lactate dehydrogenase (LDH). Homogenates were prepared using 100 mg wet tissue of whole larvae pooled from each of three replicates per treatment. Frozen samples of both species were homogenized in a buffer containing 150 mM imidazole and 1 mM EDTA at pH 7.4 in a glass/PTFE potter Elvehjem tissue grinder (Kartell, Italy) kept on ice. Homogenates were then centrifuged at 10000g for 10 min at 4 °C. Maximum activity levels of CS, LDH and HOAD were determined according to Driedzic and deAlmeidaVal (1996) and measured using a Shimadzu UV-1800 spectrophotometer (Shimadzu Scientific Instruments, Japan). CS activity was determined based on the reaction of acetyl CoA with DTNB [5,5' V dithio-bis (2-nitrobenzoic acid); extinction coefficient of 13600 M⁻¹ cm⁻¹] at 412 nm. CS activity was assayed in a buffer containing 0.25 mM DTNB, 75 mM Trisbase and 0.4 mM acetyl CoA at pH 8.0. The reactions were initiated by adding 0.5 mM oxaloacetate. Changes in absorbance were measured at 20 °C during 1 min. HOAD and LDH enzyme activities were measured following the oxidation of NADH (extinction coefficient of 6220 M⁻¹ cm⁻¹) at 340 nm. HOAD activity was assayed using 0.1 mM acetoacetyl CoA as substrate in a buffer containing 0.15 mM NADH, 1 mM EDTA and 50 mM imidazole at pH 7.5. LDH activity was assayed using 1 mM pyruvate as substrate in a buffer containing 0.15 mM NADH, 50 mM imidazole and 1 mM EDTA at pH 7.4.

All enzymatic activities were expressed as units (μ mol of substrate converted to product per minute) per total protein (mg). Furthermore, enzyme activity ratios (namely CS/HOAD and LDH/CS) were calculated in order to understand which type of metabolism is prevalent.

Statistical analysis

The effect of $p\text{CO}_2$ and temperature on metabolic and heart rates, CS, LDH, HOAD activities and enzyme activity ratios (CS/HOAD and LDH/CS) was evaluated for each species using a two-way ANOVA, followed by the Tukey post-hoc test. A three-way ANOVA and the Tukey post-hoc test were also used to evaluate differences between species. Normality and homogeneity of variances were verified by Kolmogorov-Smirnov and Levene's tests, respectively. All statistical analyses were performed for a significance level of $\alpha=0.05$, using STATISTICA 12.0 software (StatSoft Inc., USA).

Results

Metabolism and Routine heart rates

Metabolic rates of both species (Fig. 1) showed to be positively affected by temperature ($F_{1,20}=51.737$, $p=0.000$ and $F_{1,20}=54.652$, $p=0.000$, for sole and seabream, respectively), but negatively affected by hypercapnia ($F_{1,20}=13.442$, $p=0.002$ and $F_{1,20}=72.222$, $p=0.000$ for sole and seabream, respectively).

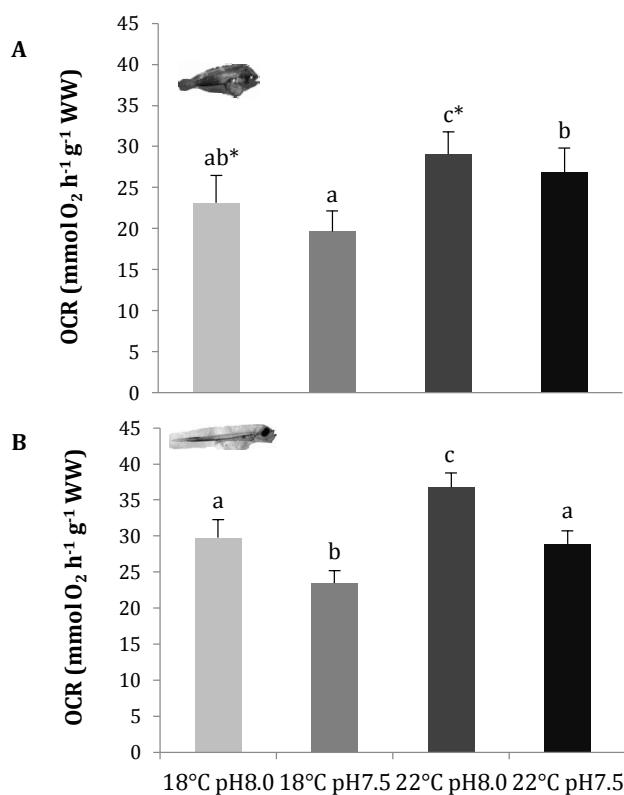


Figure 1. Impact of ocean warming and acidification on the oxygen consumption rates (OCR) of early stages of A) *Solea senegalensis* and B) *Sparus aurata*. Values are given in mean \pm SD ($n=6$). Different letters represent significant differences between the different climate scenarios, and asterisks represent significant differences between the two species within the same treatment ($p<0.05$).

Oxygen consumption rates of sole larvae increased significantly with warming from 23.1 ± 3.3 to $29.1 \pm 2.7 \text{ } \mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$ under normocapnia, and from 19.6 ± 2.5 to $26.8 \pm 3.0 \text{ } \mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$ under hypercapnia. Regarding seabream larvae, the lowest rates ($23.5 \pm 1. \text{ } \mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$) were observed under control temperature and hypercapnia, and the highest ($36.8 \pm 2.0 \text{ } \mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$) under warming and normocapnia. Oxygen consumption rates were also significantly different between both species ($F_{1,40}=44.976$, $p=0.000$), but no significant interaction between temperature, $p\text{CO}_2$ and species was found ($F_{1,40}=0.069$, $p=0.794$). Compared to sole larvae, the metabolism of seabream was higher, especially at normocapnic conditions. Heart rates of *S. aurata* and *S. senegalensis* (Fig. 2) were significantly affected by temperature and $p\text{CO}_2$, increasing with temperature ($F_{1,36}=930.43$, $p=0.000$ and $F_{1,36}=91.902$, $p=0.000$ for sole and seabream, respectively) and decreasing with $p\text{CO}_2$ ($F_{1,36}=11.85$, $p=0.001$ and $F_{1,36}=36.988$, $p=0.000$ for sole and seabream, respectively). Heart rates of sole larvae increased significantly with warming from 90.2 ± 2.4 to 120.3 ± 3.1 beats per minute under normocapnia, and from 85.3 ± 2.9 to 118.1 ± 4.3 beats per minute under hypercapnia. For seabream larvae, the lowest heart rate (74.3 ± 6.9 beats per minute) was observed under control temperature and normocapnia, while the highest value (127.5 ± 9.9 beats per minute) was reached under warming and normocapnia. No significant differences were found between both species ($F_{1,72}=0.26$, $p=0.614$), nor in the interaction between temperature, $p\text{CO}_2$ and species ($F_{1,72}=0.32$, $p=0.575$).

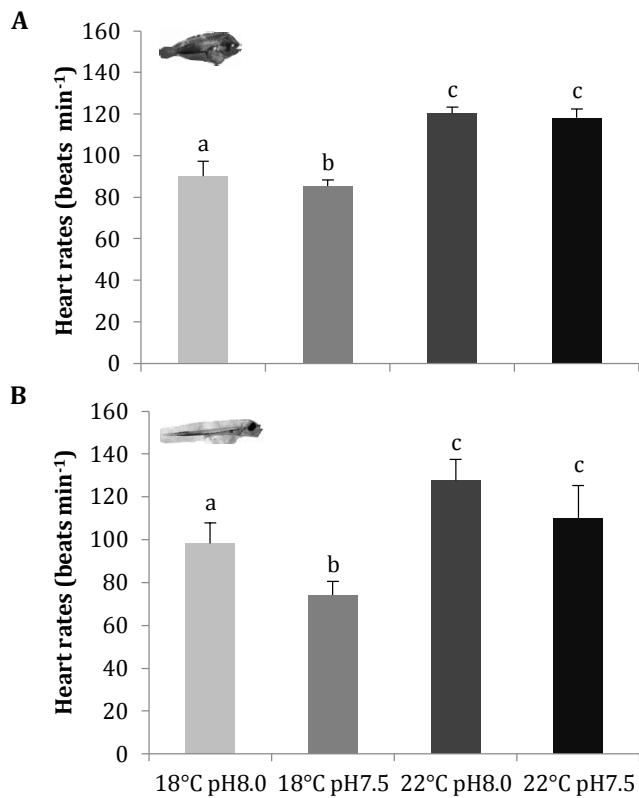


Figure 2. Impact of ocean warming and acidification on the routine heart rates of early stages of A) *Solea senegalensis* and B) *Sparus aurata*. Values are given in mean \pm SD (n=10). Different letters represent significant differences between the different climate scenarios, and asterisks represent significant differences between the two species within the same treatment ($p<0.05$).

Enzyme activity

The CS activity tended to increase with warming and to decrease with hypercapnia (Fig. 3). Although no significant effect of temperature was observed ($F_{1,8}=1.108$, $p=0.323$ and $F_{1,8}=1.101$, $p=0.325$ for sole and seabream, respectively), a significant effect was detected for $p\text{CO}_2$ in both species ($F_{1,8}=5.585$, $p=0.046$ and $F_{1,8}=10.939$, $p=0.011$ for sole and seabream, respectively). The CS activity of seabream larvae decreased with hypercapnia, from 1.9 ± 0.4 to $1.4 \pm 0.4 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ under control temperature, and from 2.1 ± 0.2 to $1.5 \pm 0.3 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ under warming (Fig. 3b). When comparing both species, the activity levels were significantly higher in *S. aurata* than in *S. senegalensis* ($F_{1,16}=20.329$, $p=0.000$).

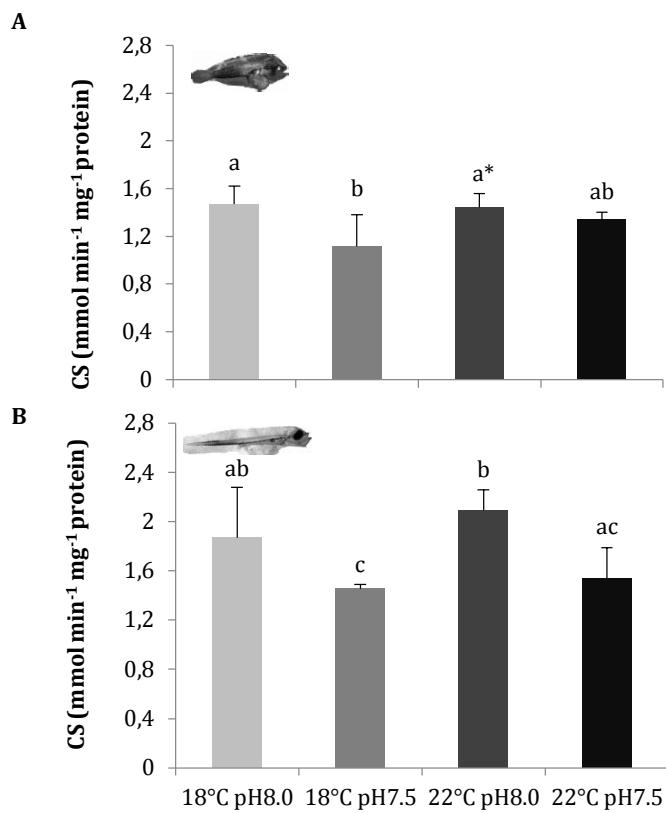


Figure 3. Impact of ocean warming and acidification on the enzyme citrate synthase (CS) activity of early stages of A) *Solea senegalensis* and B) *Sparus aurata*. Values are given in mean \pm SD ($n=3$). Different letters represent significant differences between the different climate scenarios, and asterisks represent significant differences between the two species within the same treatment ($p<0.05$).

The HOAD activity tended to increase with warming and decrease with hypercapnia (Fig. 4). HOAD levels of *S. senegalensis* were only significantly affected by $p\text{CO}_2$ ($F_{1,8}=7.108$, $p=0.029$), decreasing from 4.7 ± 1.5 to $3.5 \pm 1.0 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ under control temperature, and from 6.3 ± 1.3 to $4.0 \pm 0.5 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ under warming (Fig. 4a). On the other hand, HOAD levels of *S. aurata* were significantly affected by both environmental factors ($F_{1,8}=9.210$, $p=0.016$ and $F_{1,8}=14.652$, $p=0.005$ for temperature and $p\text{CO}_2$, respectively), decreasing with hypercapnia from 8.6 ± 1.3 to $7.0 \pm 1.3 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ under control temperature, and from 11.0 ± 0.5 to $8.2 \pm 0.4 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ under warming (Fig. 4b).

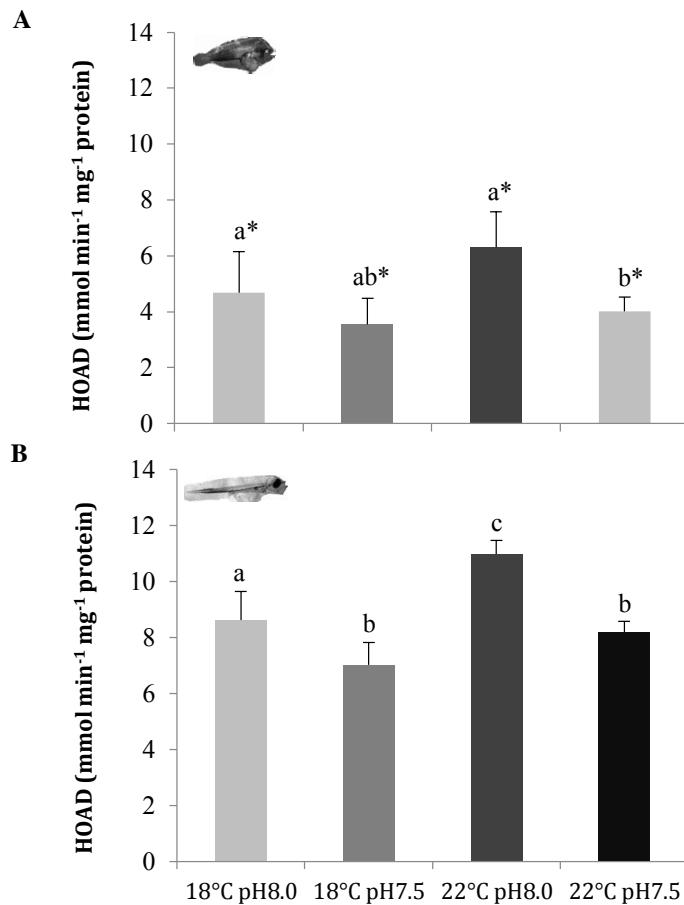


Figure 4. Impact of ocean warming and acidification on the enzyme β -hydroxyacyl CoA dehydrogenase (HOAD) activity of early stages of A) *Solea senegalensis* and B) *Sparus aurata*. Values are given in mean \pm SD ($n=3$). Different letters represent significant differences between the different climate scenarios, and asterisks represent significant differences between the two species within the same treatment ($p<0.05$).

Both species differed significantly in HOAD activity ($F_{1,16}=88.142$, $p=0.000$), with *S. aurata* presenting about twice the values of *S. senegalensis*. The LDH activity increased in both species with warming and hypercapnia (Fig. 5). Although, in *S. senegalensis* there was only a significant effect of $p\text{CO}_2$ ($F_{1,8}=6.006$, $p=0.039$), in seabream larvae a significant effect of temperature and $p\text{CO}_2$ was observed ($F_{1,8}=14.838$, $p=0.005$ and $F_{1,8}=97.058$, $p=0.000$, for temperature and $p\text{CO}_2$, respectively). The LDH activity of seabream larvae increased with hypercapnia, from 3.04 ± 0.14 to $4.75 \pm 0.23 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ under control temperatures (18°C), and from 3.81 ± 0.19 to $5.18 \pm 0.43 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ under warming conditions. No significant interaction

between both factors was observed ($F_{1,8}=0.115$ $p=0.743$ and $F_{1,8}=1.250$, $p=0.296$ for sole and seabream, respectively). Compared to *S. aurata*, the LDH activity of *S. senegalensis* was always lower, under normocapnia increase with warming from 1.11 ± 0.20 to 1.47 ± 0.30 $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$ and from 1.56 ± 0.40 to 1.48 ± 0.117 $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$ under hypercapnia. While significant differences were found between both species ($F_{1,16}=581.124$, $p=0.000$), and between $p\text{CO}_2$ and species ($F_{1,16}=25.932$, $p=0.000$), no significant interaction between temperature, $p\text{CO}_2$ and species was detected ($F_{1,16}=0.285$, $p=0.600$).

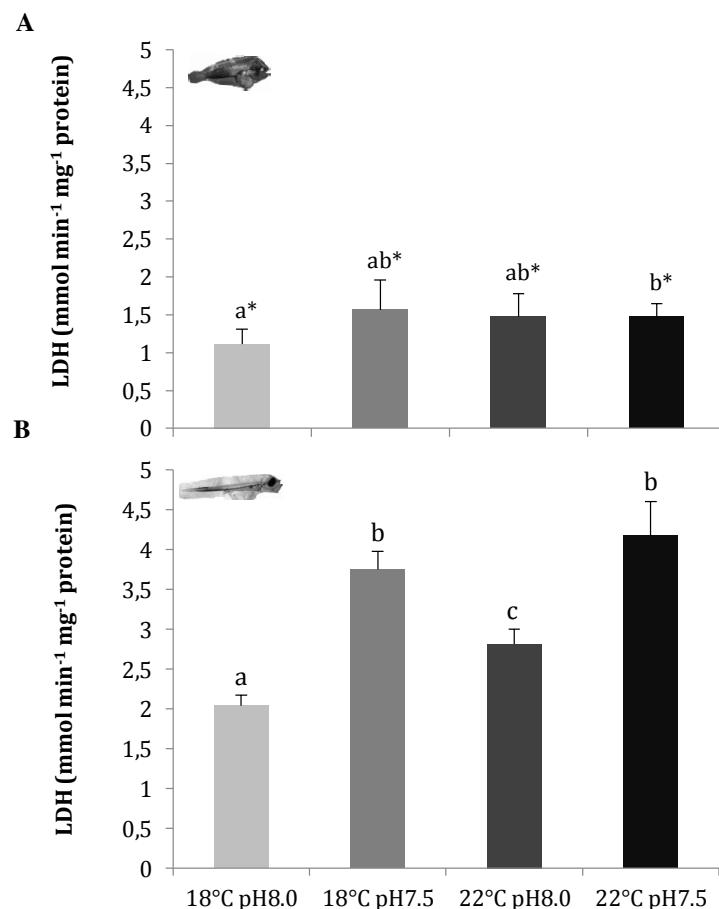


Figure 5. Impact of ocean warming and acidification on the enzyme lactate dehydrogenase (LDH) activity of early stages of A) *Solea senegalensis* and B) *Sparus aurata*. Values are given in mean \pm SD ($n=3$). Different letters represent significant differences between the different climate scenarios, and asterisks represent significant differences between the two species within the same treatment ($p<0.05$).

Enzyme activity ratios, namely CS/HOAD and, LDH/CS were calculated to assess the relatively contribution of oxidative and glycolytic enzymes of sole and seabream larvae (Table 2). In general, the CS/HOAD ratio was always lower than 1 and showed little variation among treatments, neither temperature ($F_{1,8}=1.014$, $p=0.343$ and $F_{1,8}=1.254$, $p=0.295$, for sole and seabream respectively) nor pCO_2 ($F_{1,8}=0.979$, $p=0.351$ and $F_{1,8}=0.646$, $p=0.445$, for sole and seabream respectively) had a significant effect on this activity ratio. For both species, the LDH/CS ratio tended to increase with both warming and hypercapnia however only hypercapnia had a significant effect ($F_{1,8}=17.942$, $p=0.003$ and $F_{1,8}=31.048$, $p=0.001$, for sole and seabream respectively). The LDH/CS ratio was always equal or lower than 1 in *S. senegalensis*, but higher than 1 in *S. aurata*. Moreover, a significant effect between species was detected for both ratios ($F_{1,16}=10.378$, $p=0.005$ and $F_{1,16}=83.888$, $p=0.000$ for CS/HOAD and LDH/CS respectively). While for the LDH/CS ratio a significant interaction was found only between pCO_2 and species ($F_{1,16}=13.308$, $p=0.002$), no significant interactions were detected for CS/HOAD ratio ($p>0.005$).

Table 2 – Ratios of enzyme activity (CS/HOAD and LDH/CS) of *Solea senegalensis* and *Sparus aurata* larvae under different climate change scenarios. Values are given in mean \pm SD ($n=3$). Different letters represent significant differences between the different climate scenarios, and for each ratio asterisks represent significant differences between the two species within the same treatment ($p<0.05$).

	<i>S. senegalensis</i>		<i>S. aurata</i>	
Treatments	CS/HOAD	LDH/CS	CS/HOAD	LDH/CS
18°C pH8.0	0.34 \pm 0.12	0.76 \pm 0.19 ^a	0.22 \pm 0.08	1.67 \pm 0.22 ^a
18°C pH7.5	0.33 \pm 0.09	1.40 \pm 0.23 ^{b*}	0.21 \pm 0.04	3.28 \pm 0.24 ^b
22°C pH8.0	0.23 \pm 0.03	1.03 \pm 0.23 ^{ab}	0.19 \pm 0.02	1.84 \pm 0.24 ^a
22°C pH7.5	0.34 \pm 0.05 *	1.09 \pm 0.10 ^{b*}	0.19 \pm 0.03	3.46 \pm 0.88 ^b

Discussion

The present findings support that enzymatic activity in different fish species reflects adaptations to different life strategies and locomotory abilities. The interspecific variability among these sympatric species besides being reflected on larval fish

development (Parra and Yúfera 2001) it is also reflected in their metabolic and enzymatic profiles. As expected the faster swimming fish species with higher metabolic demands (*S. aurata*) revealed a stronger aerobic capacity than the benthonic one (*S. senegalensis*), as suggested by the higher citrate synthase (CS) and lactate dehydrogenase (LDH) activities. Both species also differed significantly in β -hydroxyacyl CoA dehydrogenase (HOAD) activity, with *S. aurata* presenting about twice the values of *S. senegalensis*. Similar findings were observed by Johnston and Moon (1980), who showed higher HOAD levels in fish with greater swimming capacities.

Regarding the future ocean climate conditions, our results revealed that warming scenario increased the whole-organism metabolic rate (oxygen consumption) and heart rates of *Sparus aurata* and *Solea senegalensis*. This indicate that oxygen transport capacity of fish larvae did not decline with warming as reported for other fish species (e.g. Eliason et al. 2011). Contrarily, ocean acidification caused negative effects on larval fish oxygen consumption rates and cardiac functionality. Larvae of both species when exposed to high $p\text{CO}_2$ enter into a hypometabolic state. Although metabolic depression is commonly used by organisms to enhance tolerance to environmental stressful conditions (Hochachka and Somero 2002) it is also characterized by shutting down expensive processes, such as protein synthesis, specific ATP-consuming ion transporters, Na^+/K^+ ATPase and growth. This strategy is likewise known to limit aerobic scope and reduce animal fitness (Hochachka and Somero 2002; Pörtner et al. 2010; Storey and Storey 2004). Alongside with the above-mentioned metabolic depression, hypercapnia also caused cardiac failure (bradycardia) on seabream larvae, as previously reported for other species (Ishimatsu et al. 2004; Lee et al. 2003). Cardiac failure may cause blood pH disturbances and lower oxygen transport (Pörtner et al. 2005; Pörtner et al. 2004), limiting oxygen supply to tissues to sustain cellular aerobic capacity.

Ocean warming and acidification conditions also induced specific adjustments on enzymatic pathways. Specifically, warming elicited an increase in mitochondrial (HOAD) and glycolytic (LDH) enzymes activities, while hypercapnia induced the inhibition of CS and HOAD activities and the contra-balanced over-expression of LDH. These modifications indicate that a transition from aerobic to anaerobic metabolism occurred to sustain larval energetic demands under hypercapnic conditions. In fact,

the increase in glycolytic potential is a common tactic used by organisms to improve tolerance and survival under hypercapnia conditions (Hochachka and Somero 2002) however it is likely to be a tradeoff not bearable on longer time-scales. However, fish aerobic capacities not always decrease with hypercapnia exposure. Such responses have been demonstrated to be life-stage dependent, to vary among tissues and acclimation temperatures (Strobel et al. 2013a; Strobel et al. 2013b; Tseng et al. 2013). Here we argue that the decreased aerobic potential and whole-organism metabolic rates of both species might be a consequence of acid-base regulatory imbalance and hypercapnia-induced extracellular acidosis. Extracellular acidosis regulation is considered to be crucial to protect organisms from hypercapnia-induced disorders (Heisler 1989; Pörtner 2008; Seibel and Walsh 2003). Plasma pH decrease may cause blood pH disturbances and lower hemoglobin-O₂ affinity as well as lower oxygen transport (Pörtner et al. 2005; Pörtner et al. 2004), thus oxygen supply and availability on tissues may become insufficient or inadequate for maintenance of cell functions aerobically. Moreover, the energy available to essential processes may consequently become limited, which may progressively lead to a loss in fish larvae physiological functions.

The susceptibility to future ocean conditions revealed to differ among fish species, i.e. *S. aurata* showed much higher anaerobic enzyme activity (LDH) and LDH/CS ratio under hypercapnia. This may suggest an enhanced ability of *S. senegalensis* larvae to protect body fluids against such hypercapnia disorders, and subsequently may have a stronger acid-base regulation that better prepares them to endure future environmental changes. In fact, at 15 dph, the flatfish larvae already settled on the bottom and almost complete its metamorphosis – i.e. it is in a pre-juvenile stage. On the other hand, *S. aurata* larvae is much less developed, and its metamorphosis only happens 60 days after hatching (Parra and Yúfera 2001). Overall, our findings indicate that the metabolic capacity of fish larvae respond to variations in pCO₂ in ways that alter the net energy generation of fish larvae, especially for *S. aurata* larvae where anaerobic pathways played a significant role in energy production. This anaerobic metabolic transition might indicate that fish larvae were already above their critical thermal range. Furthermore, our results clearly suggest that hypercapnia narrowed thermal acclimation capacity of fish larvae, particularly of *S. aurata*. The planktonic existence of *S. aurata* larvae and the related higher metabolic rates and

cardiorespiratory demands may have contributed to the lower tolerance to future ocean conditions. In conclusion, it is of great importance to comprehend how acclimation to future ocean conditions affect the physiology of fish species with different life strategies, habitat and throughout different ontogenetic phases in a way to predict how long-term exposures will influence fish species at population-level.

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Author contributions

R.R., F.F. and M.S.P. designed the experiment; M.S.P., F.F. performed the experiment; M.S.P., J.M., F.F. and R.R. analyzed the data; M.S.P., F.F. and R.R. wrote the main paper. All authors discussed the results and their implications, and commented on the manuscript at all stages.

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CHAPTER 6

Oxidative stress and digestive enzyme activity of flatfish larvae in a changing ocean

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RESEARCH ARTICLE

Oxidative Stress and Digestive Enzyme Activity of Flatfish Larvae in a Changing Ocean

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Abstract

Until now, it is not known how the antioxidant and digestive enzymatic machinery of fish early life stages will change with the combined effects of future ocean acidification and warming. Here we show that high $p\text{CO}_2$ ($\sim 1600 \mu\text{atm}$) significantly decreased metabolic rates (up to 27.4 %) of flatfish larvae, *Solea senegalensis*, at both present (18 °C) and warmer temperatures (+4 °C). Moreover, both warming and hypercapnia increased the heat shock response and the activity of antioxidant enzymes, namely catalase (CAT) and glutathione S-transferase (GST), mainly in post-metamorphic larvae (30 dph). The lack of changes in the activity of CAT and GST of pre-metamorphic larvae (10 dph) seems to indicate that earlier stages lack a fully-developed antioxidant defense system. Nevertheless, the heat shock and antioxidant responses of post-metamorphic larvae were not enough to avoid the peroxidative damage, which was greatly increased under future environmental conditions. Digestive enzymatic activity of *S. senegalensis* larvae was also affected by future predictions. Hypercapnic conditions led to a decrease in the activity of digestive enzymes, both pancreatic (up to 26.1 % for trypsin and 74.5 % for amylase) and intestinal enzymes (up to 36.1 % for alkaline phosphatase) in post-metamorphic larvae. Moreover, the impact of ocean acidification and warming on some of these physiological and biochemical variables (namely, lower OCR and higher HSP and MDA levels) were translated into larvae performance, being significantly correlated with decreased larval growth and survival or increased incidence of skeletal deformities. The increased vulnerability of flatfish early life stages under future ocean conditions is expected to potentially determine recruitment and population dynamics in marine ecosystems.

Introduction

Ocean acidification and warming are among the most relevant environmental challenges that marine organisms will face in tomorrow's oceans [1–4]. The continuous absorption of atmospheric CO₂ by the oceans is expected to change seawater chemistry, with forecasts estimating a drop of 0.3–0.4 units in ocean pH by the year 2100. At the same time, the oceans are becoming warmer, and will continue as global surface temperature is expected to increase 1.1–6.4°C by the end of the century [5]. These environmental stressors may drive organisms outside their tolerance boundaries, compromising the overall fitness and survival of local populations.

Many organisms may cope with such climate-related changes, within limits, by adjusting mechanisms across levels of biological organization [4], including physiological protective mechanisms such as integrated heat shock and oxidative stress responses. When exposed to environmental fluctuations, organisms may be induced to produce heat shock proteins (HSP) to repair, refold, and eliminate damaged or denatured proteins [6]. Additionally, environmental stress may also induce the production of reactive oxygen species (ROS) [7]. The increase in ROS production may affect cellular integrity [8], and can injure cellular mechanisms by lipid peroxidation, one of the most frequent cellular injury processes where ROS react with membrane-associated lipids [7]. ROS production in marine organisms is controlled by efficient antioxidant capacity, characterized by a set of antioxidant enzymes which can together detoxify ROS [9].

When the above-mentioned protective mechanisms fail after exposure to environmental stress, organisms might limit the energy available, and growth, motility, ingestion, and digestion may suffer several functional disturbances [10]. In what concerns digestion, a correct maturation of the digestive system is essential to transform macronutrients from food into a form that can be easily digested, absorbed and assimilated, in order to supply dietary nutrients required for normal growth and development [11]. The digestive enzymes (pancreatic and brush border intestinal enzymes) are part of the metabolic regulatory mechanisms [10] and are thus widely used in studies as markers of fish larval development and as indicators of fish condition and physiological state [11–14]. The normal maturation of the enterocytes in developing fish larvae is characterized by a decrease of pancreatic enzyme activity (namely, trypsin and amylase), and by a marked increase in intestinal brush border membrane enzyme activity (such as alkaline phosphatase—ALP). This efficient brush border membrane digestion is representative of an adult mode of digestion [15]. A correlation between the major landmark events in digestive tract differentiation and the ontogenetic development of the digestive enzyme activities has been described in several fish species [16–19].

The activity of digestive enzymes is expected to be affected by external factors that modify metabolic functions, such as temperature and pH [10]. So far, the influence of ocean acidification on the digestive efficiency and enzymatic activity of marine organisms has been studied on marine invertebrate organisms [20–22]. The susceptibility of fish species to ocean acidification has received far less attention, since fish have developed an effective acid-base regulatory mechanism [23–25]. Nevertheless, the early life stages are expected to be more susceptible to changes in seawater pCO₂ and more prone to extracellular changes than juvenile and adult fish [24,26]. Indeed, several morphological, physiological and behavioral disturbances have been observed in fish early stages [26–34], including the target species of this study, the flatfish *Solea senegalensis*. In a previous study, the survival, growth and development of sole larvae showed to be negatively impacted by ocean warming and acidification [see 29], but the underlying mechanisms remain unknown.

Here we provide a comprehensive set of physiological and biochemical responses of *S. senegalensis* early life stages to ocean warming (+4°C) and acidification ($\Delta\text{pH} = 0.5$), which includes: i) oxygen consumption rates (OCR), ii) heat shock response (HSR; namely HSP70), iii) antioxidant enzyme activities (GST—glutathione S-transferase, and CAT—catalase), iv)

lipid peroxidation (MDA—malondialdehyde concentration), and v) digestive enzymatic activities (trypsin, amylase and ALP). Additionally, a correlation analysis was performed to link these parameters with the morphological data from our previous work [29].

Materials and Methods

Ethics statement

This study was authorized by the Portuguese National Authority for Animal Health (Direcção-Geral de Alimentação e Veterinária), and it was performed in strict accordance with the recommendations of the Animal Care and Use Committee of the Faculty of Sciences of the University of Lisbon.

Egg collection and larval rearing

Eggs of Senegal sole were collected from broodstock fish at IPMA—Estação Piloto de Piscicultura de Olhão (CRIP Sul, Olhão, Portugal) in June 2012, and transferred to the aquaculture facilities in Laboratório Marítimo da Guia (Cascais, Portugal). Senegal sole larvae were reared and collected in the same experiment published by Pimentel *et al.* [29].

After a short (2 h) acclimation period, eggs and larvae were exposed for one month to: i) 18°C—the mean sea surface temperature in summer (sSST) and normocapnia ($p\text{CO}_2 = \sim 400 \mu\text{atm}$), ii) 18°C and hypercapnia ($p\text{CO}_2 = \sim 1600 \mu\text{atm}$; $\Delta\text{pH} = 0.5$), iii) 22°C—the future sSST warming scenario for the western coast of Portugal in 2100 (+ 4°C) and normocapnia, and iv) 22°C and hypercapnia. This species inhabits the Western Iberian Upwelling Ecosystem, part of the Canary Current Upwelling System, one of the four major eastern boundary currents of the world. In these regions, actual $p\text{CO}_2$ levels may reach up to 500 μatm [35–37] and are thus expected to exceed the level of 1000 μatm projected for 2100 [5].

Larvae were reared in twelve recirculating seawater systems (three per treatment). Newly-hatched larvae were distributed randomly into three 19-L rearing tanks at a density of 70 larvae L⁻¹. Feeding was adjusted according to larval development at each experimental condition. Larvae opened the mouth around 2 dph and started to feed on rotifers, *Brachionus plicatilis*.

Enriched (AlgaMac-3050) *Artemia* metanauplii were introduced at 5 dph and their proportion in the diet was gradually increased, becoming the only prey offered at 8 dph. After larval settlement, frozen metanauplii were also introduced in the tank. Rotifer and *Artemia* density were adjusted twice a day to assure optimal prey density.

Temperatures (18.0 ± 0.2 and $22.0 \pm 0.2^\circ\text{C}$) were controlled via Heilea chillers (Guangdong, China). The pH was automatically adjusted in each tank via a Profilux (Kaiserslautern, Germany) connected to a pH probe (WaterTech pH 201S) and operating a solenoid valve connected to a CO₂ tank. The pH of each tank was also measured daily using a portable pH meter (SevenGo pro SG8, Mettler Toledo), in order to cross-calibrate the pH probes and to adjust the set points of the systems as required. Average pH values of the control and low pH treatments were 8.02 ± 0.05 and 7.51 ± 0.05 , respectively. The salinity was kept at 35.4 ± 0.4 . Ammonia and nitrite were monitored regularly and maintained within recommended levels.

Seawater carbonate system speciation (see S1 Table) was calculated weekly from total alkalinity (determined according to Sarazin *et al.* [38]) and pH measurements. Total dissolved inorganic carbon (C_T), $p\text{CO}_2$, bicarbonate concentration and aragonite saturation were calculated using the CO2SYS software [39], with dissociation constants from Mehrbach *et al.* [40] as refitted by Dickson & Millero [41].

Fish larvae were collected at 10 dph (pre-metamorphic stage), 20 dph (intermediate stage—undergoing metamorphosis) and 30 dph (post-metamorphic stage). Larvae were immediately placed in liquid nitrogen and then stored at -80°C for posterior biochemical analyses.

Oxygen consumption rates

Oxygen consumption measurements were determined according to previously established methods [42,43]. Nine pre-metamorphic (10 dph) and nine post-metamorphic (30 dph) larvae from each treatment (three per replicate) were individually placed in sealed water-jacketed respirometry chambers (RC300 Respiration cell, Strathkelvin, North Lanarkshire, Scotland) containing 1-µm filtered and UV-irradiated seawater from each treatment condition mixed with antibiotics (50 mg L⁻¹ streptomycin) to avoid bacterial respiration. Water volumes were adjusted in relation to animal mass (up to 10 mL) to minimize larval stress. Respiration chambers were immersed in water baths (Lauda, Lauda-Königshofen, Germany) to control temperature. The respiratory runs occurred after an acclimation period of about 2 h and lasted between 3 to 6 h. Oxygen consumption was also measured in chambers containing just water (blanks) for correction of possible bacterial respiratory activity. Oxygen concentrations were recorded with Clark-type O₂ electrodes connected to a multi-channel oxygen interface (Model 928, Strathkelvin, North Lanarkshire, Scotland). At the end of the respirometry trials, the mean minimum level of oxygen achieved was of 86.8 ± 6.6%.

Heat shock response, antioxidant enzymes and lipid peroxidation

Preparation of tissue extracts. After 10 and 30 days of acclimation to the different climate change scenarios, whole larvae were pooled from each replicate tank, comprising a total of three replicates per treatment. Homogenates were prepared using 150 mg wet tissue from each replicate tank. All samples were homogenized in 250 µL of phosphate buffered saline solution (PBS, pH 7.3, composed by 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na₂HP0₄ and 1.47 mM KH₂P0₄), by using a glass/PTFE Potter Elvehjem tissue grinder (Kartell, Italy). All homogenates were then centrifuged during 20 min at 14000 g at 4°C. HSP, antioxidant enzyme activities, lipid peroxidation and total protein expression were measured in the supernatant fraction. All enzyme assays were tested with commercial enzymes obtained from Sigma (Missouri, USA), and each sample was run in triplicate. The enzyme results were normalized by measuring the total protein content of the samples according to the Bradford method [44].

Heat shock response. HSP70 content (HSC70/HSP70) was assessed by ELISA (Enzyme-Linked Immunoabsorbent Assay) as previously described by Rosa *et al.* [43]. Briefly, a total of 5 µL of homogenate supernatant was diluted in 250 µL of PBS, and 50 µL of the diluted sample was added to 96-well microplates MICROLON600 (Greiner Bio-One GmbH, Germany) and incubated overnight at 4°C. Microplates were washed on the next day in 0.05% PBS-Tween-20 and 100 µL of blocking solution (1% Bovine Serum Albumin, BSA) was added to each well. For 2 hours, the microplates were incubated at room temperature in darkness. Then, 50 µL of a solution of 5 µg mL⁻¹ primary antibody anti-HSP70/HSC70 (that detects both 72 and 73 kDa proteins, which corresponds to the molecular mass of inducible HSP70 and constitutive HSC70, respectively) was added to each well. Wells were then incubated at 37°C for 90 min. The non-linked antibodies were removed by washing the microplates, which were then incubated for 90 min at 37°C with 50 µL of the secondary antibody [anti-mouse IgG Fab specific, ALP conjugate (1 µg mL⁻¹) from Sigma-Aldrich (Germany)]. After another wash, 100 µL of substrate p-nitrophenyl phosphate tablets (Sigma-Aldrich, Germany) was added to each well and incubated at room temperature (10 to 30 min). Subsequently, 50 µL of stop solution (3 M NaOH) was added to each well, and the absorbance was read at 405 nm in a 96-well microplate reader (BIO-RAD, Benchmark, USA). The amount of HSP70/HSC70 in the samples was then calculated from a standard curve of absorbance based on serial dilutions (from 0 to 2000 ng mL⁻¹) of purified HSP70 active protein (Acris, USA). The results were expressed in relation to the protein content of the samples (ng HSP70/HSC70 mg protein⁻¹).

Antioxidant enzymes. Glutathione S-transferase: GST activity was determined according to the procedure described by Rosa *et al.* [45] and Lopes *et al.* [46], optimized for a 96-well microplate. This assay uses 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, which conjugates with the thiol group of the glutathione (GSH), causing an increase in absorbance. 180 µL of substrate solution (composed by 200 mM L-glutathione reduced, Dulbecco's PBS and 100 mM CDNB solution) was added to each well of a 96-well Nunclon microplate (Thermo Scientific Nunc, USA), along with 20 µL of GST standard or sample. Equine liver GST was used as a positive control to validate the assay. The enzyme activity was determined spectrophotometrically at 340 nm by measuring the formation of the conjugate of GSH and CDNB. The absorbance was recorded every minute for 6 min, using a plate reader (BioRad, California, USA). The increase in absorbance per minute was estimated and the reaction rate at 340 nm was determined using the CDNB extinction coefficient of 0.0053 εµM (µM⁻¹ cm⁻¹) as follows:

$$\text{GST activity} = \frac{\Delta A_{340}/\text{min}}{0.0053} \times \frac{\text{Total volume}}{\text{Sample volume}} \times \text{dilution factor.}$$

The results were expressed in relation to the protein content of the samples (nmol min⁻¹ mg⁻¹ protein).

Catalase: The assay for the determination of CAT activity was based on Aebi [47]. In this assay, CAT activity is assessed by measuring the rate of removal of hydrogen peroxide (H₂O₂). The reaction can be followed by a decrease in absorbance as the H₂O₂ is converted into oxygen and water. At the end of the assay, H₂O₂ is consumed and CAT is inactivated. The total reaction volume of 3 mL was composed of 50 mM potassium phosphate buffer (pH 7.0) and 12.1 mM H₂O₂ as substrate. The reaction started by the addition of the samples into quartz cuvettes with an optical path length of 10 mm. The consumption of H₂O₂ [extinction coefficient of 0.04 εmM (mM⁻¹ cm⁻¹)] was monitored at 240 nm and 25°C, once every 15 s for a 180 s incubation period, using a Helios spectrophotometer (Unicam, UK). Standard CAT activity was measured using a bovine CAT solution (1523.6 U mL⁻¹) as a positive control for the validation of the assay. CAT activity was calculated using the following equation:

$$\text{CAT activity} = \frac{\Delta A_{240}/\text{min}}{0.04} \times \frac{\text{Total volume}}{\text{Sample volume}}.$$

The results were expressed in relation to the protein content of the samples (nmol min⁻¹ mg⁻¹ protein).

Lipid peroxidation. Lipid peroxidation was determined by the quantification of malondialdehyde (MDA), a specific end-product of the oxidative degradation process of lipids. The thiobarbituric acid reactive substances (TBARS) assay was used to quantify MDA as described by Rosa *et al.* [45]. Homogenates were treated with 8.1% sodium dodecyl sulfate, 20% trichloroacetic acid (pH 3.5), thiobarbituric acid and a 15:1 (v/v) mixture of n-butanol and pyridine. In the TBARS assay, the thiobarbituric acid reacts with the MDA to yield a fluorescent product, which was detected spectrophotometrically at 532 nm. MDA concentrations were calculated with the Microplate Manager 4.0 software (BIO-RAD, USA), based on an eight-point calibration curve (from 0 to 0.3 µM TBARS) using MDA bis (dimethyl acetal; Merck, Switzerland). The results were expressed in relation to the protein content of the samples (nmol mg⁻¹ protein).

Digestive enzymes

Preparation of tissue extracts. Two different groups of digestive enzymes were assayed: a) extracellular enzymes (more specifically, the pancreatic enzymes trypsin and amylase), and b) brush border enzymes linked to cell membranes (more specifically, the intestinal enzyme ALP).

Enzyme activities were measured in triplicates (using pooled larvae from each replicate tank) for each development stage (10, 20 and 30 dph larvae) under the different experimental conditions. Before homogenization, larvae were dissected in order to separate pancreatic and intestinal segments, as described by Cahu and Zambonino-Infante [48]. Samples were homogenized using a glass/PTFE Potter Elvehjem tissue grinder (Kartell, Italy) in 30 volumes (v/w) of ice-cold Tris-HCl (50 mM) and mannitol (2 mM) buffer at pH 7.0. The homogenates were then divided into two different aliquots of 1.5 mL and processed differently. Aliquots for assessing pancreatic enzymes were centrifuge at 3300 g (for 3 min) at 4°C, and the supernatants were removed for enzyme quantification. Intestinal brush border membranes for the determination of intestinal enzymes were purified according to Crane *et al.* [49]. Enzyme activities were expressed as specific enzyme activity, in units per milligram of protein (U mg^{-1} protein), and the soluble protein of crude enzyme extracts was quantified by the Bradford's method [44] using bovine serum albumin as standard.

Trypsin. Trypsin activity was assayed according to Holm *et al.* [50] using 0.1 MN α -benzoyl-DL-arginine p-nitroanilide (BAPNA) as substrate in 50 mM Tris-HCl buffer containing 20 mM CaCl₂ at pH 8.2. The changes in absorbance were measured at 25°C during 2 min at 407 nm, using a UV-1800 Shimadzu UV spectrophotometer (Japan). One unit of trypsin activity corresponded to 1 μmol of 4-nitroaniline liberated in 1 min per mL of extracellular enzymatic extract, based on the extinction coefficient of the substrate [8200 ϵM ($\text{M}^{-1} \text{cm}^{-1}$)].

Amylase. Amylase activity was quantified according to Metais [51] at 37°C and measured using soluble starch-iodine (0.3%) dissolved in Na₂HPO₄ buffer at pH 7.4 as substrate. Briefly, 50 μL of enzymatic extract was mixed with the substrate (3 g L⁻¹ starch in Na₂PO₄, pH 7.4) and incubated for 30 min at 37°C. The reaction was stopped with 20 μL of 1 N HCl. After the addition of 2 mL of N/3000 iodine solution, the absorbance was read at 580 nm, using a UV-1800 Shimadzu UV spectrophotometer (Japan). One unit of α -amylase activity was defined as 1 mg of starch hydrolyzed per min and per mL of extracellular enzymatic extract at 37°C.

Alkaline phosphatase. ALP was quantified according to the procedure described by Bessey [52] and Hausamen [53] using 5 mM p-nitrophenyl phosphate (PNPP) as substrate in 30 mM Na₂CO₃·H₂O and 1 mM MgCl₂·6H₂O buffer at pH 9.8. The enzymatic extract was mixed with the substrate solution and the change in absorbance was measured at 37°C during 2 min at 407 nm, using a UV-1800 Shimadzu UV spectrophotometer (Japan). One unit of ALP activity corresponded to 1 μmol of the substrate hydrolyzed in 1 min per mL of the brush border enzymatic extract (extinction coefficient of 18300 ϵM , $\text{M}^{-1} \text{cm}^{-1}$).

Statistical analyses

ANOVA was used to test whether significant differences existed between replicates of each experimental treatment. As no differences were found between replicates, all the samples from the same treatment were pooled and analyzed together. Three-way ANOVAs and Tukey HSD tests were then used to evaluate the effect of temperature, $p\text{CO}_2$ and developmental stage on the metabolism (OCR), HSR (HSP70), antioxidant (GST and CAT), lipid peroxidation (MDA) and digestive enzyme (trypsin, amylase and ALP) activities.

Pearson's correlation coefficients were used to analyze potential relationships between the variables analyzed in this study (OCR, HSR, lipid peroxidation, antioxidant and digestive enzymatic activities), and also with those obtained in our previous study with this species (namely survival, specific growth rates and skeletal deformities; see [29]).

All statistical analyses were performed for a significance level of 0.05, using Statistica 12.0 software (StatSoft Inc., Tulsa, USA).

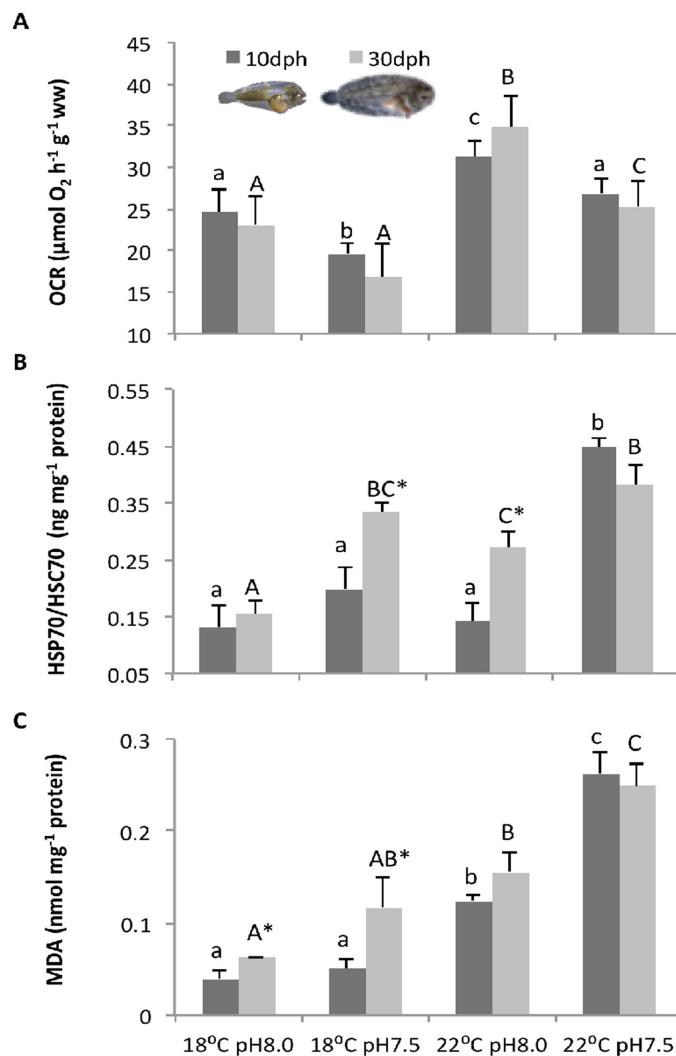


Fig 1. Impact of ocean acidification and warming on the metabolism, heat shock response and lipid peroxidation of *Solea senegalensis* larvae. A) Oxygen consumption rates (OCR), B) heat shock protein 70 (HSP70) concentrations, and C) malondialdehyde (MDA) levels in 10 and 30 dph larvae at different temperature and pH scenarios. Values are given as means + SD. Different letters (lower case for 10 dph larvae; capital letters for 30 dph) represent significant differences between the different climate scenarios ($p<0.05$). Asterisks represent significant differences between 10 and 30 dph larvae for the same treatment ($p<0.05$).

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Results

Oxygen consumption rates

The effect of warming and high $p\text{CO}_2$ on the metabolic rates of *S. senegalensis* larvae is presented in Fig 1A (see also S2 Table). OCR were significantly affected by temperature and $p\text{CO}_2$ ($p<0.05$), but not by developmental stage ($p>0.05$). Sole larvae displayed significantly higher OCR under normocapnia ($23.11 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$ at present-day temperature and $34.85 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$ at the future warming scenario). At higher $p\text{CO}_2$, OCR decreased significantly to 16.82 and $25.28 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$ (at present-day temperature and future warming scenario, respectively). No significant interaction was found between the three factors ($p>0.05$).

Heat shock response

The HSR of sole larvae was significantly ($p<0.05$) affected by temperature and $p\text{CO}_2$, and also by developmental stage (Fig 1B; see also S2 Table). Additionally, a significant interaction was observed between these three factors ($p<0.05$). The HSR (inducible HSP70) increased under hypercapnia in both pre- and post-metamorphic larval stages, especially under the warming treatment. In general, post-metamorphic larvae presented a stronger HSR than pre-metamorphic larvae (16.7 to 92.9 percentage points higher), except under the warming and high $p\text{CO}_2$ scenario, where HSR decreased 17.9 percentage points and the differences between stages were not statistically significant.

Antioxidant enzymes

The impact of high $p\text{CO}_2$ and environmental warming on antioxidant enzymes (CAT and GST) of *S. senegalensis* larvae is shown in Fig 2 (see also S2 Table).

CAT activity (Fig 2A) was significantly affected by developmental stage ($p<0.05$), but not by temperature and $p\text{CO}_2$ or by the interaction between factors ($p>0.05$). The highest value of CAT activity ($6.10 \pm 0.95 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein) was observed in the post-metamorphic larvae exposed to warming and high $p\text{CO}_2$. Pre-metamorphic larvae showed always lower values than post-metamorphic larvae, and no significant variation ($p>0.05$) was observed among treatments (between 2.42 ± 0.67 and $2.81 \pm 1.43 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein).

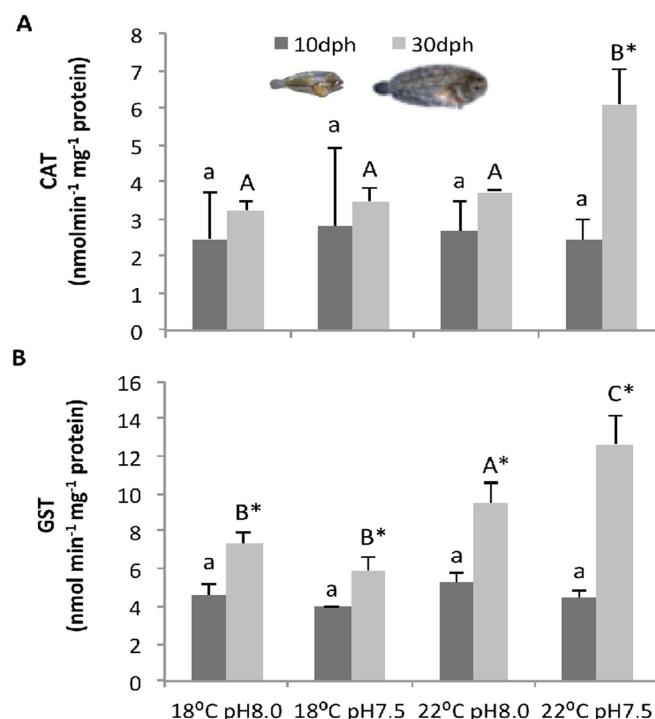


Fig 2. Impact of ocean acidification and warming on the antioxidant response of *Solea senegalensis* larvae. A) catalase (CAT), and B) glutathione S-transferase (GST) activities of 10 and 30 dph larvae at different temperature and pH scenarios. Values are given as means + SD. Different letters (lower case for 10 dph larvae; capital letters for 30 dph) represent significant differences between the different climate scenarios ($p<0.05$). Asterisks represent significant differences between 10 and 30 dph larvae for the same treatment ($p<0.05$).

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GST activity ([Fig 2B](#)) was significantly affected by temperature and developmental stage, as well as by the interactions between factors ($p<0.05$). GST activity in pre-metamorphic larvae was also lower than in post-metamorphic larvae ($p<0.05$), and similar in all treatments ($p>0.05$). In contrast, the GST activity of post-metamorphic larvae increased significantly with temperature ($p<0.05$). The highest value ($12.64 \pm 1.51 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$) was observed under the combined effect of warming and high $p\text{CO}_2$.

Lipid peroxidation

Lipid peroxidation (based on MDA levels) was also significantly affected by temperature, $p\text{CO}_2$, developmental stage, and the interaction between these three factors ($p<0.05$) ([Fig 1C](#), see also [S2 Table](#)). Lipid peroxidation increased significantly with warming in both developmental stages. The lowest value ($0.039 \pm 0.007 \text{ nmol mg}^{-1} \text{ protein}$) was found in pre-metamorphic larvae exposed to the present-day conditions. The effect of ocean acidification on MDA levels was only significantly noted under the warming scenario. In fact, the highest MDA values (0.26 ± 0.02 and $0.25 \pm 0.03 \text{ nmol mg}^{-1} \text{ protein}$ in pre- and post-metamorphic larvae, respectively) were found when larvae were exposed to the combined effects of higher temperature and $p\text{CO}_2$. MDA buildup was generally more pronounced in post-metamorphic larvae, except under the future combined scenario.

Digestive enzymes

The effect of warming and high $p\text{CO}_2$ on digestive enzymes of sole larvae is presented in Figs [3–5](#) (see also [S2 Table](#)). Both extracellular enzymes (trypsin and amylase) increased throughout development, while the brush border enzyme ALP significantly increased.

Trypsin activity ([Fig 3](#)) was significantly affected by temperature, $p\text{CO}_2$ and developmental stage, as well as by the interactions between factors ($p<0.05$). Trypsin activity increased with temperature only in 10 dph larvae. Regardless of temperature, trypsin activity decreased significantly with hypercapnia in both 10 and 20 dph larvae ($p<0.05$), but not in 30 dph larvae ($p>0.05$). The highest trypsin activity ($0.57 \pm 0.02 \text{ U mg}^{-1} \text{ protein}$) was observed in 10 dph larvae under warming and normocapnia, and the lowest value ($0.08 \pm 0.01 \text{ U mg}^{-1} \text{ protein}$) was observed under present-day temperature and hypercapnic conditions.

Amylase activity ([Fig 4](#)) was also significantly affected by the three factors (temperature, $p\text{CO}_2$ and developmental stage), as well as by most interactions between them ($p<0.05$). Amylase activity was also highest ($0.07 \pm 0.01 \text{ U mg}^{-1} \text{ protein}$) in 10 dph larvae under warming and normocapnia. Before metamorphosis, amylase activity decreased significantly ($p<0.05$) with warming and hypercapnia (up to $0.036 \pm 0.011 \text{ U mg}^{-1} \text{ protein}$), but showed no significant variation ($p>0.05$) at 20 dph (values between 0.018 ± 0.002 and $0.026 \pm 0.007 \text{ U mg}^{-1} \text{ protein}$) and 30 dph (values between 0.003 ± 0.001 and $0.018 \pm 0.002 \text{ U mg}^{-1} \text{ protein}$).

ALP activity ([Fig 5](#)) was significantly affected by $p\text{CO}_2$ and development stage ($p<0.05$), but not by temperature neither by the interaction of the three factors ($p>0.05$). ALP activity decreased with hypercapnia, especially when combined with warming ($p<0.05$). The lowest activity level of ALP ($0.007 \text{ U mg}^{-1} \text{ protein}$) was detected at 10 dph under warming and hypercapnic exposure, while the highest value ($0.019 \text{ U mg}^{-1} \text{ protein}$) was detected at 30 dph under warming and normocapnic conditions.

Correlation between variables

The correlations between the variables analyzed in the present study for 10 and 30 dph larvae are presented in Tables [1](#) and [2](#), respectively. [Table 2](#) also includes the correlations between the variables analyzed in the present study with those obtained in our previous study [[29](#)].

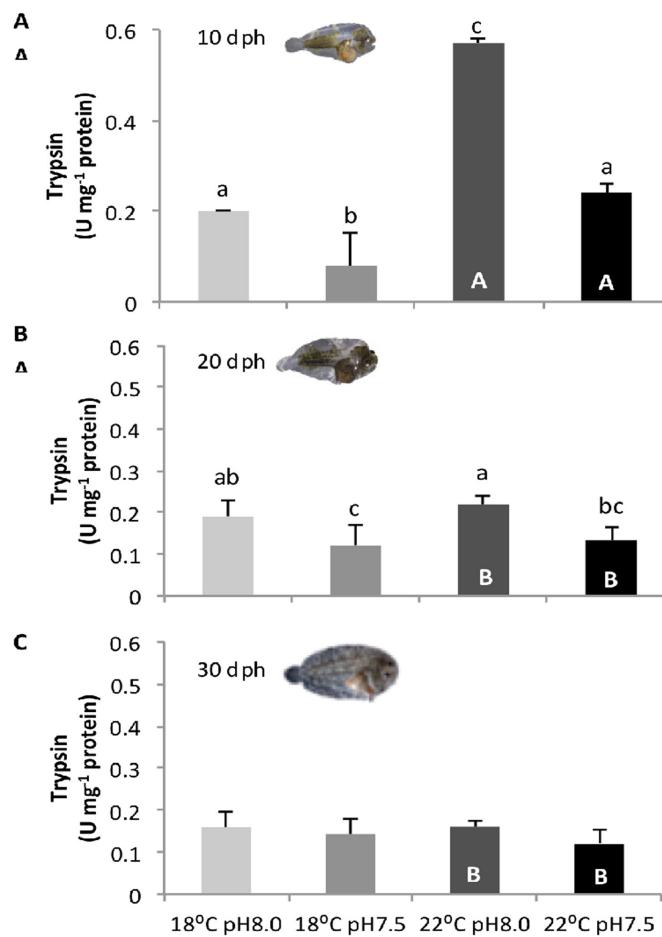


Fig 3. Impact of ocean acidification and warming on the trypsin activity of *Solea senegalensis* larvae. Enzyme activity in **A**) 10 dph, **B**) 20 dph, and **C**) 30 dph larvae at different temperature and pH conditions. Values are given as means + SD. Different letters represent significant differences between the different climate scenarios ($p < 0.05$). Lower-case letters indicate differences between treatments at the same development stage; capital letters represent differences between 10, 20 and 30 dph larvae for the same treatment.

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The metabolism of 10 dph larvae was positively correlated with GST ($r = 0.95$; $p = 0.041$) and trypsin ($r = 0.94$; $p = 0.044$), while the metabolism of 30 dph larvae was found to be positively correlated with amylase ($r = 0.92$; $p = 0.040$). Moreover, based on our previous findings [29], we found that the incidence of skeletal deformities in 30 dph larvae was positively correlated with HSP levels ($r = 0.99$; $p = 0.005$), while specific growth rates (SGR) were positively correlated with OCR ($r = 0.99$; $p = 0.014$) and amylase levels ($r = 0.97$; $p = 0.030$). On the other hand, survival was negatively correlated with HSP ($r = -0.93$; $p = 0.049$) and MDA levels ($r = -0.98$; $p = 0.025$). No other significant relationship was found ($p > 0.05$).

Discussion

Early life stages of marine fish are expected to be particularly sensitive to environmental stressors, due to the lack or low functional capacity of some organ systems (e.g., gill epithelium) and to the high rates of metabolism needed to fuel growth and development. In our previous study with *S. senegalensis* eggs and larvae [29], the exposure to future conditions caused a decline in

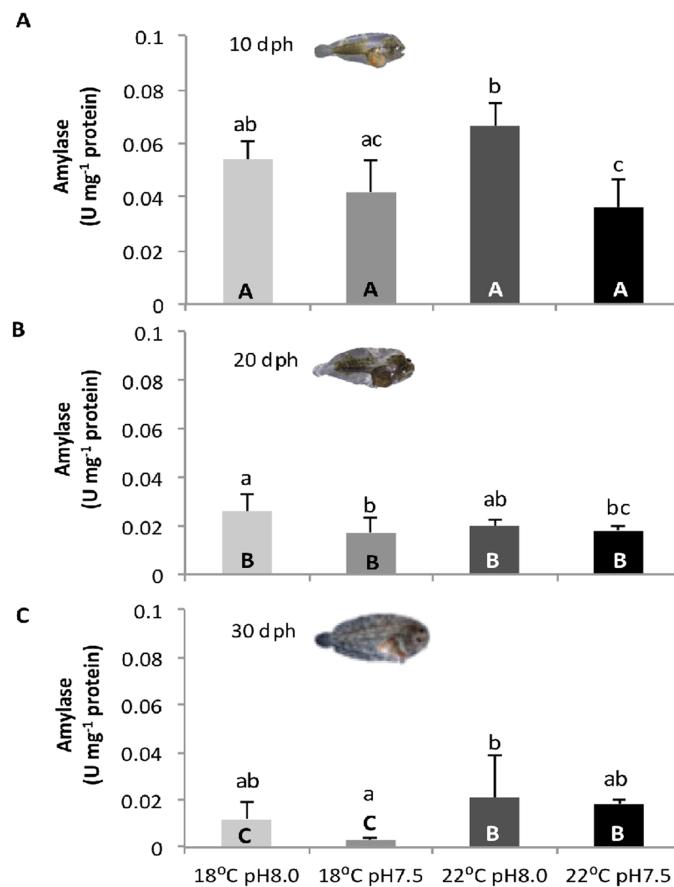


Fig 4. Impact of ocean acidification and warming on the amylase activity of *Solea senegalensis* larvae. Enzyme activity in A) 10 dph, B) 20 dph, and C) 30 dph larvae at different temperature and pH conditions. Values are given in mean + SD. Different letters represent significant differences between the different climate scenarios ($p < 0.05$). Lower-case letters indicate differences between treatments at the same development stage; capital letters represent differences between 10, 20 and 30 dph larvae for the same treatment.

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the hatching success, larval survival and growth of this flatfish species. Moreover, hypercapnia and warming amplified the incidence of skeletal deformities (by 32%), including severe deformities such as lordosis, scoliosis and kyphosis. Here we show that these climate change-related variables also affected the metabolism, HSR, lipid peroxidation, as well as the activity of antioxidant and digestive enzymes.

The metabolic rate of *S. senegalensis* larvae increased with temperature as expected (following normal Q_{10} values), but exposure to hypercapnic conditions triggered a 25% reduction in OCR. Metabolic depression, and the consequent reduction of total energy expenditure, is an important strategy to survive under acute environmental stress [54,55], because it allows organisms to put some biological processes in stand-by as a strategy for saving energy, prioritizing the survival of the individual [2,56]. Protein synthesis is an ATP-consuming process, and a reduced ATP demand of most cells might lead to a reduction in protein synthesis, which would by definition restrict growth [57,58]. Indeed, the lower OCR in sole larvae was strongly and positively correlated with lower SGR.

Most organisms display an integrated stress response (heat shock response and antioxidant enzyme activity) to prevent the increase in ROS formation [59] and the protein damage and

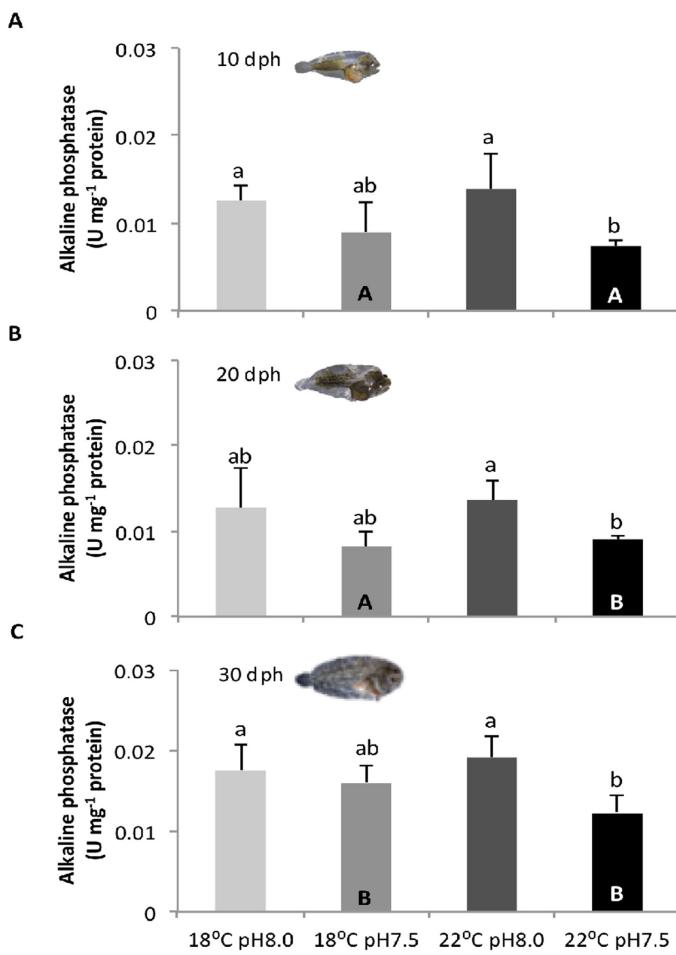


Fig 5. Impact of ocean acidification and warming on the alkaline phosphatase activity of *Solea senegalensis* larvae. Enzyme activity in A) 10 dph, B) 20 dph, and C) 30 dph larvae at different temperature and pH conditions. Values are given in mean + SD. Different letters represent significant differences between the different climate scenarios ($p < 0.05$). Lower-case letters indicate differences between treatments at the same development stage; capital letters represent differences between 10, 20 and 30 dph larvae for the same treatment.

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Table 1. Correlation analysis between physiological and biochemical variables of 10 dph *Solea senegalensis* larvae.

	OCR	HSP	MDA	CAT	GST	Trypsin	Amylase
HSP	0.03						
MDA	0.47	0.89					
CAT	-0.37	-0.46	-0.51				
GST	0.95	-0.29	0.17	-0.18			
Trypsin	0.94	-0.22	0.25	-0.05	0.98*		
Amylase	0.61	-0.77	-0.40	0.16	0.83	0.78	
ALP	0.50	-0.84	-0.52	0.09	0.74	0.65	0.98*

Pearson's coefficients between the variables analyzed in the present study, namely oxygen consumption rates (OCR), heat shock protein (HSP) concentrations, malondialdehyde (MDA) levels, antioxidant enzyme activities (catalase—CAT and glutathione S-transferase—GST) and digestive enzyme activities (trypsin, amylase and alkaline phosphatase—ALP). Asterisks represent significant correlations ($p < 0.05$).

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Table 2. Correlation analysis between physiological, biochemical and morphological variables of 30 dph *Solea senegalensis* larvae.

	Survival	SGR	Malformations	OCR	HSP	MDA	CAT	GST	Trypsin	Amylase
SGR	-0.27									
Malformations	-0.96*	0.05								
OCR	-0.16	0.99*	-0.03							
HSP	-0.93*	-0.03	0.99*	-0.12						
MDA	-0.98*	0.42	0.88	0.30	0.83					
CAT	-0.88	0.28	0.76	0.13	0.71	0.94*				
GST	-0.76	0.65	0.55	0.52	0.47	0.88	0.90*			
Trypsin	0.83	0.17	-0.81	0.31	-0.81	-0.80	-0.90	-0.62		
Amylase	-0.37	0.97*	0.13	0.92*	0.03	0.54	0.47	0.80	-0.03	
ALP	0.70	0.27	-0.69	0.41	-0.69	-0.69	-0.85	-0.55	0.98*	0.05

Pearson's coefficients between the variables analyzed in the present study, namely oxygen consumption rates (OCR), heat shock protein (HSP) concentrations, malondialdehyde (MDA) levels, antioxidant enzyme activities (catalase—CAT and glutathione S-transferase—GST) and digestive enzyme activities (trypsin, amylase and alkaline phosphatase—ALP), and those obtained in our previous study with this species [29], namely survival, specific growth rates (SGR) and the incidence of skeletal malformations. Asterisks represent significant correlations ($p<0.05$).

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unfolding [60] caused by environmental stressful conditions. The ability of elevated cellular HSP levels to strengthen thermal and chemical tolerance in animals is well documented [61–63]. In the present study, the exposure of sole larvae to warmer temperatures and higher $p\text{CO}_2$ levels triggered an increase in HSP70 levels in both developmental stages, thus indicating a stress response. Marine organisms possess also a powerful set of antioxidant enzymes that helps to detoxify ROS and reduce the negative effects on fitness [64,65]. Indeed, CAT and GST concentrations of post-metamorphic larvae increased by 88 and 72%, respectively, from present-day to forthcoming conditions. However, pre-metamorphic larvae may lack a fully developed antioxidant defense system and may be more exposed to tissue damage, as there were no differences in CAT and GST concentrations between treatments. Altogether, inducible HSP70, CAT and GST responses seem to constitute an integrated response of post-metamorphic larvae during exposure to warmer temperatures and hypercapnic conditions.

Despite the increment of HSR and antioxidant enzyme activities, this significant up-regulation was not effective against cellular injuries. Lipid peroxidation still increased under high temperature and $p\text{CO}_2$ conditions, as suggested by the higher MDA levels, a specific end-product of the oxidative degradation process of lipids. Environmental factors are known to be responsible for significant changes in MDA levels indicating that organisms are facing some adjustments due to oxidative stress conditions. In addition to the effect of temperature, high $p\text{CO}_2$ was further responsible for exacerbating the heat-induced cellular injuries. This matches findings in crustaceans that show an earlier onset of thermal limitation under elevated $p\text{CO}_2$ as a general principle [1,66,67].

Besides affecting the stress response (HSR and oxidative stress tolerance) of sole larvae, future ocean conditions also affected the activity of digestive enzymes. The ontogenetic development of the digestive system of sole larvae occurred as expected [16], characterized by a decrease in the activity of pancreatic enzymes followed by an increase in intestinal (brush border) enzyme activity. These opposing trends of ontogenetic variation may suggest the maturation of enterocytes, but further histological analysis would be necessary to confirm it. Regardless of this, elevated CO_2 conditions led to a general decrease in the activity of the digestive enzymes, both pancreatic and intestinal enzymes, especially in pre-metamorphic sole larvae. Morphological and physiological impairments in the digestive system (namely gut and

pancreas) of fish early life stages under ocean acidification have already been observed [27,28], but no connection has been established between altered functional development and digestive enzymatic activities.

All together, the results from the present study indicate that ocean warming and acidification pose significant stress to *S. senegalensis* larvae, especially to pre-metamorphic stages. Besides affecting the metabolism, HSP and antioxidant responses, lipid peroxidation and the activity of digestive enzymes, the impact of these climate change-related variables on some of these physiological and biochemical variables was further translated into fish performance. As mentioned above, lower oxygen consumption rates under hypercapnia were correlated with reduced larval growth. Moreover, the increase in HSP and MDA levels under high temperature and $p\text{CO}_2$ conditions, which are indicators of stress and tissue damage, was negatively correlated with larval survival. HSP levels were also positively correlated with the incidence of skeletal deformities. Other studies have shown that conditions that induce the heat shock response may also induce abnormal development [68–70]. In fact, environmental stress factors are among the most important factors that can induce skeletal deformities during fish development [71]. More studies should establish links between biochemical markers, physiological and morphological parameters in an attempt to demonstrate the effects from cellular processes up to the whole-animal level, in order to provide a more conclusive evidence of the sensitivity of marine fish early life stages to ocean climate change.

Supporting Information

S1 Table. Seawater carbonate chemistry data for the different climate change scenarios.

Total carbon (C_T), carbon dioxide partial pressure ($p\text{CO}_2$), bicarbonate concentration (HCO_3^-) and aragonite saturation state of seawater (Ω_{arag}) were calculated with CO2SYS using salinity, temperature, pH and total alkalinity (A_T). Values are means \pm SD.
(PDF)

S2 Table. ANOVA results. Results of three-way ANOVA evaluating the effect of temperature, $p\text{CO}_2$ and development stage on the oxygen consumption rate (OCR), heat shock proteins (HSP), lipid peroxidation (MDA—malondialdehyde), antioxidant enzymes (GST—Glutathione S-transferase, and CAT—catalase) and digestive enzymes (trypsin, amylase, and ALP—alkaline phosphatase) of *Solea senegalensis* larvae under the effect of ocean warming and acidification.
(PDF)

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Author Contributions

Conceived and designed the experiments: MSP FF RR. Performed the experiments: MSP FF MD. Analyzed the data: MSP FF MD JM RR. Contributed reagents/materials/analysis tools: JM MD PPF RR. Wrote the paper: MSP FF MD JM PPF MP HP RR.

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CHAPTER 7

7. GENERAL DISCUSSION AND FINAL CONCLUSIONS

7.1 Embryonic and larval survival

7.2 Biological responses of fish larvae

7.2.1 Growth impairments

7.2.2 Metabolic strategies and pathways

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7.2.4 Behavioural changes

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GENERAL DISCUSSION AND FINAL CONCLUSIONS

The present thesis contributes to the body of knowledge on larval fish acclimation to future ocean's warming and acidification, by characterizing their vulnerabilities and capacities to face climate change-related conditions in the ocean of tomorrow. Before evolutionary adaptation is possible, it is essential to analyze species' phenotypic plasticity in response to future oceans conditions. Acclimation is one of the most important mechanisms that will allow organisms to undergo future ocean changes (Donelson and Munday 2012). Species tolerance to ocean climate change conditions has shown to be species-specific, thus it is of paramount importance to understand the underlying mechanisms that species with different life strategies uses for adaptation to the new climate-related conditions. By studying a variety of physiological responses of different fish species to ocean environmental changes, the results of this thesis increases the knowledge of species stress tolerance limits essential for future predictions on species distribution shifts on local and global scales.

The experiments performed during this dissertation demonstrated that the susceptibility to ocean warming and acidification differed among *Solea senegalensis*, *Sparus aurata*, *Argyrosomus regius* and *Coryphaena hippurus* early stages. Changes on the different biological and physiological functions here investigated had severe repercussions on larval survival rates of each fish species. In wild, the survival chances of the most vulnerable early stages are expected to have profound impacts on fish recruitment and may constitute the bottleneck for species persistence in a changing ocean.

7.1 Embryonic and larval survival

The results obtained in the present dissertation showed that warming and acidification affected the resilience of fish early ontogenetic stages. The survival of fish embryos (hatching success) and larval survival of *S. aurata*, *A. regius* and *S. senegalensis* (Chapter 2 and 3) showed that embryos were more tolerant to ocean warming and acidification than larvae (Fig. 1). Contrarily to these findings, some studies have report that egg stage is significantly more vulnerable to acidification than larval stage (Jordaan and Kling 2003; Baumann et al. 2012). It can be argued

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that the morphological characteristics of fish eggs provide embryos a valuable physical protection during exposure to environmental stressful conditions, which is lost after hatching. The lack of such protection alongside with the planktonic existence of fish larvae and the related higher metabolic rates may contribute to the lower tolerance of larvae. Ocean warming elicited a more pronounced and negative impact on both embryonic and larval survival of *S. aurata*, *A. regius* and *S. senegalensis* than acidification, however, the negative effects of ocean acidification were intensified in the presence of increased temperature.

Failure of eggs to hatch successfully suggests that the predicted levels of ocean warming and acidification tested for each fish species was already outside their tolerance boundaries. The significant effect of ocean warming on hatching success was very similar within species, the percentage of decrease ranged between 10.71-11.54%. The success of embryos to hatch also decrease with ocean acidification, however on a smaller scale than warming (decrease percentages ranged between 3.85 and 7.14%). This decrease showed to be significant for *S. aurata* and *A. regius*, but not for *S. senegalensis*. These findings are coherent with several other studies, which indicates the species-specificity of fish embryo's responses to climate change. While some showed no significant changes in embryonic survival during exposure to predicted ocean acidification conditions (Munday et al. 2009; Franke and Clemmesen 2011; Frommel et al. 2012; Flynn et al. 2015), others reported that ocean acidification reduces significantly fish embryonic survival (Forsgren et al. 2013; Chambers et al. 2014). Despite non-significant, the synergistic effect of ocean warming and acidification caused the higher decrease in hatching success observed, with *S. aurata* exhibiting the higher decrease followed by *S. senegalensis*, and *A. regius* (26.42, 19.23 and 14.29% reduction, respectively). The underlying causes of changes in the hatching success process may be probably linked to abnormal cleavage patterns (van der Kraak and Pankhurst 1996), caused in this case by environmental changes. Moreover, warming and acidification may induce a drop in oxygen partial pressure (pO_2) (Walsh et al. 1991), increase carbon dioxide partial pressure (pCO_2), decrease pH in the perivitelline fluid or/and may inhibit the production of enzymes involved in hatching (Reddy and Lam 1991). Embryonic period represents a fundamental stage of fish life cycle, and modifications in the normal development of this ontogenetic phase can promote cascading effects on the performance and fitness of later stages of their life cycle

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(Chambers 1997), e.g. induce development deformities on on-growing stages and influence the larvae size at hatching.

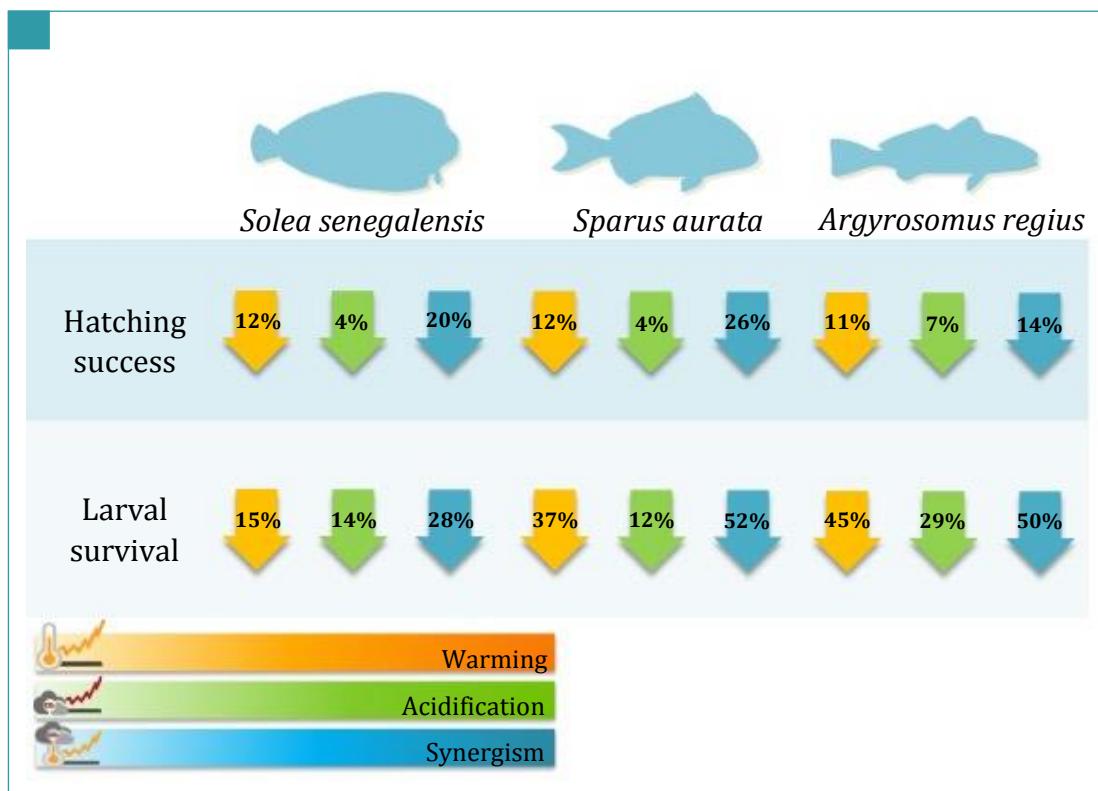


Figure 1. Summary diagram of the impacts of ocean warming and acidification on hatching success and larval survival of *Solea senegalensis*, *Sparus aurata* and *Argyrosomus regius*

Impacts on larval survival revealed to be more deleterious for the most active species with a planktonic existence, *S. aurata* and *A. regius* than for *S. senegalensis*. Exposure to the combined effect of higher $p\text{CO}_2$ and warmer temperature led to a synergistic effect of both factors, causing an additional increase in mortality. It was a consensus among the results obtained that the combination between ocean warming and acidification lowered the performance of fish larvae and elicited a higher reduction of survival rates rather than when analyzed separately. When compared to present-day conditions, *S. aurata* and *A. regius* displayed a decrease of 51.92 and of 50.00 % in survival rates, respectively, and *S. senegalensis* a decrease of 28.44%. These results are in line with the “oxygen- and capacity-limited thermal tolerance” model (OCLTT) which predicts that higher metabolic and the associated high demands on the cardiorespiratory systems makes animals more sensitive to increased temperatures (Pörtner and Knust 2007; Pörtner

2010), as is the case of the most active species *S. aurata* and *A. regius*. Notwithstanding, larval fish sensitivity to ocean climate-related changes have been also indicated to be highly variable within species. While some studies revealed larval relatively robustness in terms of survival (Munday et al. 2009; Munday et al. 2011a; Frommel et al. 2012; Hurst et al. 2012; Bignami et al. 2013; Frommel et al. 2013), Baumann et al. (2012) reported fish increasing vulnerability to future CO₂ levels. These reports alongside with the present findings provide strong evidence that it is not sufficient to predict how will fish early life stages undergo future climate changes based only in few species and on the effect of a single stressor alone. Fish larval stages represent a critical life phase for species' ecological success, therefore climate change-related impairments in survival may lead to substantial declines in adult populations, putting in jeopardy the species' persistence under a climate change scenario.

7.2 Biological responses of fish larvae under ocean climate changes

Based on the experimental results shown in the Chapters 2, 3 and 4, it can be argued that near-future ocean conditions have the potential to change significantly larval fish development, morphology, physiology and behavior. Generally, acclimation to the synergism between ocean warming and acidification showed to intensify the effects on many biological processes of the fish early-life stages analyzed in this thesis.

7.2.1 Growth impairments

Future ocean warming and acidification elicited a significant effect on larval growth. However, once again there were significant differences among fish species. Larval growth of *S. senegalensis* increased 23.69% with warmer temperatures and *S. aurata* and *A. regius* only increased 10.36 and 13.44%, respectively. However, while temperature had a positive and significant effect on larval growth of *S. senegalensis*, unexpectedly no differences were detected for *S. aurata* and *A. regius*. Contrarily to the effect of warming, ocean acidification elicited a decrease in larval growth of *S. senegalensis* and *S. aurata* (15.08 and 38.90%, respectively). However, *A. regius* growth was slightly higher (3.17 to 12.91% under warming and control

temperature) but not significantly different under such condition. It can be argued that as a consequence of a weak internal acid-base regulation (Langenbuch and Pörtner 2003), larval energy budget might be allocated away from growth towards maintenance (Pörtner 2010; Pörtner and Peck 2010). The differences between species reinforce the absence of consensus among studies, while some studies have reported decreased size and growth under high $p\text{CO}_2$ levels (Baumann et al. 2012; Frommel et al. 2014), others indicate that larvae may grow equally well or even faster under high $p\text{CO}_2$ conditions (Hurst et al. 2012; Hurst et al. 2013). If impacts on growth are truly species-specific, then ocean acidification and warming may have complex impacts on the dynamics of marine food webs, since larval growth and body size may mediate susceptibility to predation mortality (Anderson 1988).

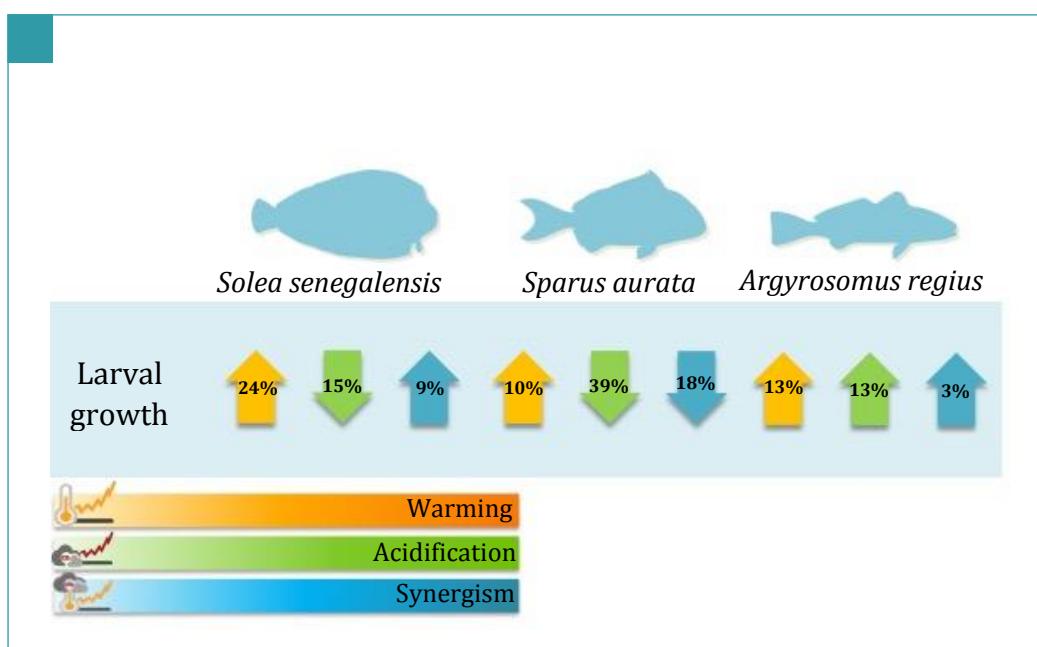


Figure 2. Summary diagram of the effects of ocean warming and acidification on larval growth of *Solea senegalensis*, *Sparus aurata* and *Argyrosomus regius*.

7.2.2 Metabolic strategies and pathways

During warming, metabolic rates of fish larvae increased with a $\text{Q}_{10} \approx 3$, which is consistent with previous studies on thermal responses of different fish species (Somero and DeVries 1967; Vanella and Calvo 2005; Beers and Sidell 2011; Bilyk and DeVries 2011). Exposure to future ocean warming did not induce negative impacts on metabolism and when combined with higher $p\text{CO}_2$ tended to mitigate

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some of the negative effects of higher $p\text{CO}_2$, except for the metabolism of *S. aurata*. The metabolic rates increment typically observed during warming conditions no longer occurred under ocean acidification. Contrarily, ocean acidification prompted considerably deleterious effects, leading to metabolic depression. A similar magnitude of metabolic depression was observed among species, with oxygen consumption rates of *S. senegalensis*, *C. hippurus* and *S. aurata* larvae decreased 27.18, 21.43 and 21.28%, respectively. Metabolic depression has been shown to induce a decrease in protein synthesis (Hochachka and Somero 2002; Storey and Storey 2004), which consequently leads to a reduction in growth, as previously mentioned for *S. senegalensis* and *S. aurata*. This metabolic adjustment likely indicates a possible ineffective control of the ion-regulation mechanisms of fish larvae under high $p\text{CO}_2$. Considered as one of the first line of defense against ambient hypercapnia, fish ion-regulatory machinery is known to be essential to maintain the internal ionic environment, and to preserve the cellular functions under ocean acidification. Maintenance of homeostasis is essential to avoid metabolic depression and energy allocation away from processes such as growth (Somero, 1985; Pörtner, 2010). Yet, we can only speculate about changes in extracellular acid-base status because neither extracellular pH_e nor intracellular pH_i , or $[\text{HCO}_3^-]_e$ were not assayed during this dissertation. Alongside with metabolic depression, results presented in Chapter 5 indicate that hypercapnia as also shown for other species (Perry and Reid 2002; Ishimatsu et al. 2004) caused cardiac failure (bradycardia) on fish larvae, which is considered a physiological perturbation that limits oxygen delivery to tissues (Lee et al. 2003). This cardiovascular adjustment limited larval tolerance to high CO_2 levels, especially to *S. aurata* that presented a steeper decrease (24.43%) than *S. senegalensis* (5.43%). Cardiac failure together with plasma pH decrease may cause blood pH disturbances and lower hemoglobin- O_2 affinity as well as lower oxygen transport (Pörtner 2004; Pörtner et al. 2005) therefore oxygen supply and availability on tissues may become insufficient or inadequate for maintenance of cell functions aerobically. Accordingly, results obtained in Chapter 5 also revealed changes in metabolic pathways and functional modifications of mitochondrial capabilities. These adjustments lead to inhibition of mitochondrial enzymes, citrate synthase and β -hydroxyacyl CoA dehydrogenase that are key enzymes for the overall aerobic metabolic potential. Consequently, anaerobic respiration pathways (given

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by the increase in lactate dehydrogenase activity) settled in together with protein denaturation and growth cessation, particularly for *S. aurata*, as mention above.

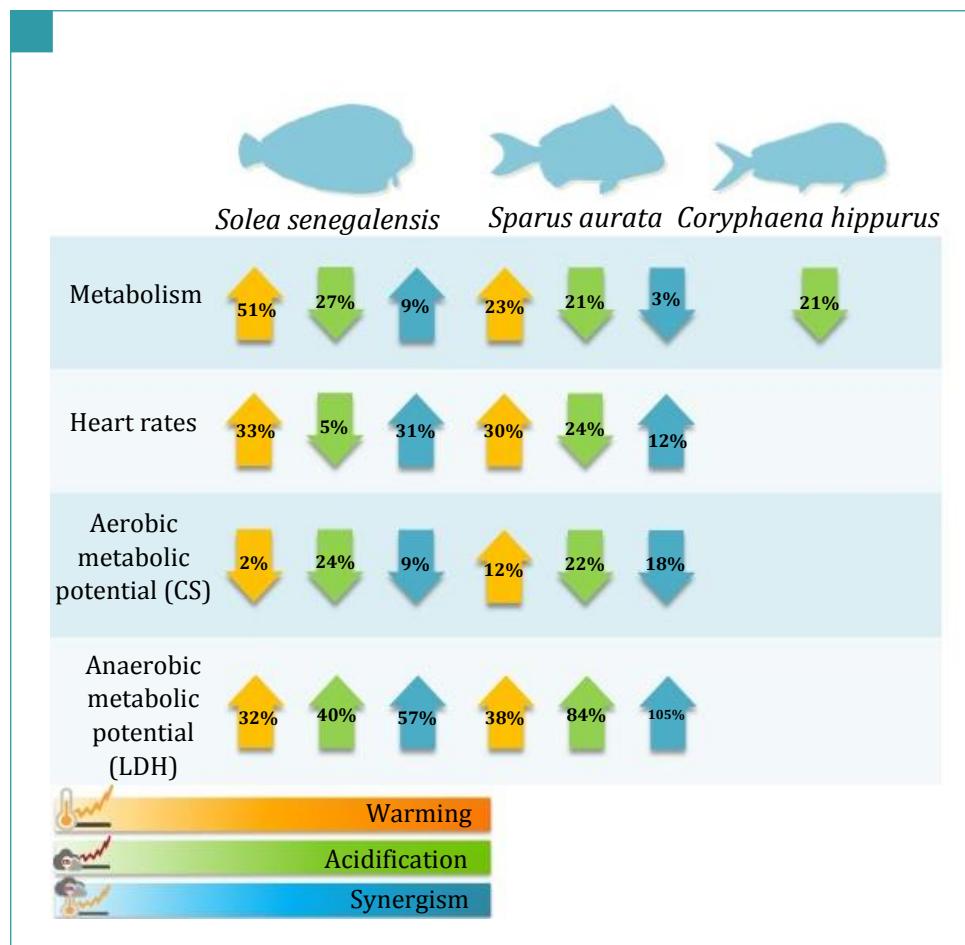


Figure 3. Summary diagram of the impacts of ocean warming and acidification on metabolism, heart rates and metabolic pathways of *Solea senegalensis*, *Sparus aurata* and *Coryphaena hippurus* larvae.

These modifications in the metabolic pathways occurred to maintain energy supply to life-sustaining functions under ocean acidification. However, this energy allocation might become constrained once aerobic scope is limited (Pörtner 2010). Concurrently, changes in cellular aerobic metabolism have also been recently published for *S. aurata* juveniles and for other fish species (Michaelidis et al. 2007; Strobel et al. 2013a; Strobel et al. 2013b; Tseng et al. 2013; Flynn et al. 2015). Although the decrease in aerobic capacity was contra-balanced with an increase in the glycolytic potential, such strategy it will not be viable in a long-term perspective. It can be argued that the decrease in aerobic potential might be the result of increased intracellular levels of bicarbonate, due to both increased $p\text{CO}_2$

and active pH buffering by bicarbonate uptake (Brauner and Baker 2009). All the above-mentioned adaptive strategies are considered crucial to protect organisms under short periods of hypercapnia, however it cannot be exclude potential negative impacts of long-term acidification. Therefore, such strategies will not be beneficial for organisms living under chronic conditions of high CO₂ in tomorrow's oceans.

7.2.3 Skeletal and otoliths developmental changes

Exposure to future ocean warming and acidification provide evidence that high pCO₂ will probably be also the main driver of morphological abnormalities, however warmer temperatures will also alter larvae morphology, specially within the synergistic scenario (Chapter 2 and 3). For both species, the synergistic scenario induced always a higher increase in malformations than when compared to both warming (84.05 and 44.46% for *S. senegalensis* and *S. aurata*, respectively) and acidification (38.42 and 8.18% for *S. senegalensis* and *S. aurata*, respectively) scenarios. These findings are in agreement with Baumann et al. (2012), however until nowadays there is no consistency of how acidification may affect skeletal development, with Munday et al. (2011a) finding no effects of CO₂ on the skeletal development of a reef fish. Skeletal abnormalities induced environmentally can be either caused by neuromuscular effects or by necessary changes for maintaining the biochemical integrity of bone (Divanach et al. 1996). The possible additional buffering of tissue pH with bicarbonate and non-bicarbonate ions in an attempt to maintain internal ion-regulation might have indeed also interfered with normal skeletal development, increasing the percentage of *S. aurata* and *S. senegalensis* larvae deformities. The greater vulnerability of *S. aurata* larval to warming was also indicated here by the greatest percentage of increase in deformations. Total malformations of *S. aurata* revealed to be always higher than for *S. senegalensis*, 68.12, 68.2, 59.4% higher in warming, acidification and synergistic scenarios, respectively. The malformations observed during fish early stages are expected to be in part responsible for the formation of spinal deformities (lordosis, scoliosis and kyphosis) in ongoing stages (Andrades et al. 1996; Koumoundouros et al. 2001). Furthermore, exposure to future ocean conditions provide also evidence that pCO₂ will change the mineralization of aragonite sensory structures (otoliths)

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of fishes, with responses dependent on increasing temperature (i.e. synergistic interaction of temperature and $p\text{CO}_2$).

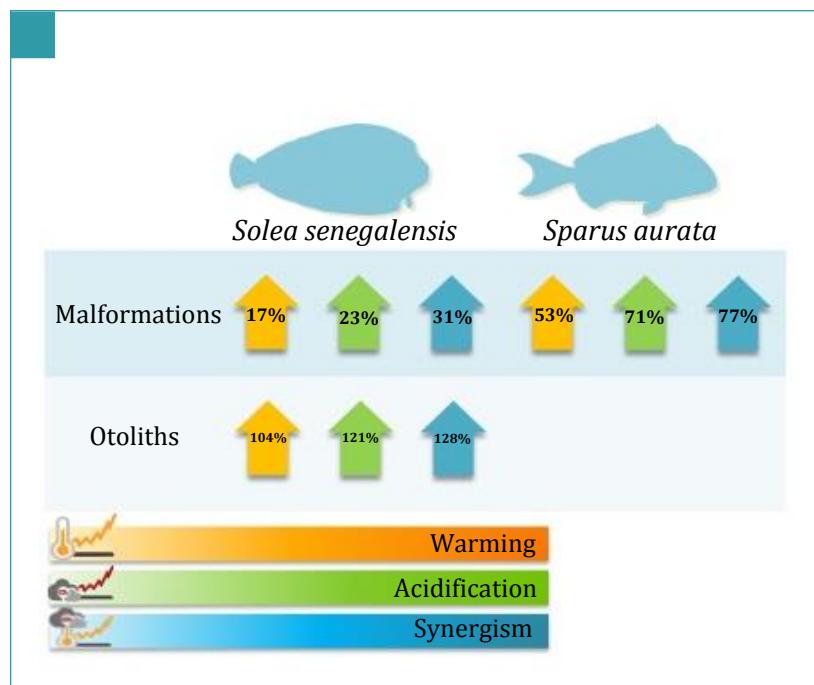


Figure 4. Summary diagram of the effects of ocean warming and acidification on malformations and otoliths of *Solea senegalensis* and *Sparus aurata* larvae.

Although there is limited information available on aragonite saturation and on the acid–base status of the endolymph of surrounding otoliths, it can be argued that extra-pH regulation in otolith endolymph may lead to an increase in calcium carbonate precipitation, accelerating $\Omega_{\text{aragonite}}$ formation. The overgrowth of these structures may thus cause major impacts on fish behavior, namely on larval orientation, acceleration and perception, which may reduce larval performance and increase mortality (Gagliano and McCormick 2004; Gagliano et al. 2008). Another example of results inconsistency is related to the effects of ocean acidification on these sensory structures. While Munday et al. (2011a) detected no effect on *Amphiprion percula*, the same authors (Munday et al. 2011b) and others (Checkley et al. 2009; Bignami et al. 2013) found significant effects on *Acanthochromis polyacanthus*, *Rachycentron canadum* and *Atractoscion nobilis*, respectively.

7.2.4 Behavioural changes

Concurrently with the above-mentioned results, behavioural patterns observed on Chapters 3 and 4 were also affected by warming and acidification. Swimming and foraging behaviours of *S. aurata* and capture rate of *A. regius* did not greatly increased with warming. Foraging behaviours did not follow the great increase in metabolic rates under warming, as expected. This fact may have further cause a reduction in the associated energy to satisfy the greater energetic demands at warming conditions, especially for *A. regius* that presented a lower increment in capture rate than *S. aurata* (approximately 70.00% lower).

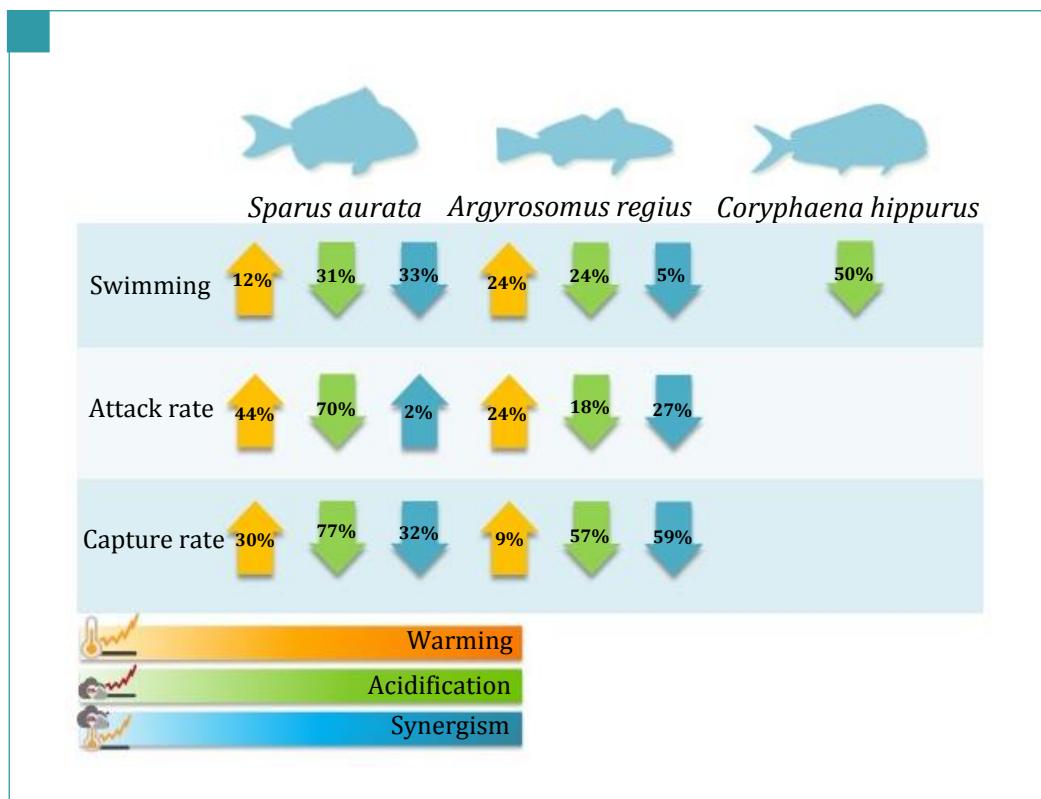


Figure 5. Summary diagram of the effects of ocean warming and acidification on behaviour of *Sparus aurata*, *Argyrosomus regius* and *Coryphaena hippurus* larvae.

Under such conditions larvae would rely on stored fuels to replenish the consumed ATP, which may cause depletion of stored energy and contribute to cell death, explaining in part the observed increase in larval mortality. Furthermore, within the present results it was detected that ocean acidification lead to more negative effects on larval fish behaviour than warming. Swimming and foraging behaviours decreased drastically, indicating that larvae entered into a lethargic state.

Coryphaena hippurus showed to be the most affected species with 38 and 52% increase of exhaustion (given by the decrease in swimming behaviour) when compared to *S. aurata* and *A. regius*, respectively. This indicated that early life stages of this circumtropical oceanic top predator will be more lethargic a signal that they will be struggling against this stress element. When comparing the foraging behaviors of *S. aurata* with *A. regius*, *S. aurata* larvae showed to be more affected by ocean acidification than *A. regius*. The decrease in foraging behaviors of *S. aurata* was 26 and 74% (capture and attack rate, respectively) higher than *A. regius* behaviour. The possible causes of the increased larval lethargic state may be either a consequence of the skeletal and otoliths changes mentioned-above, which may also interfere with fish larval behaviour (Powell et al. 2009), and/or as seen for other fish species the accumulation of HCO₃⁻ and Cl⁻ reduction resultant from acid-base balance regulation (Gagliano and McCormick 2004; Gagliano et al. 2008; Brauner and Baker 2009) under ocean acidification may also interfere with the normal function of brain neurotransmitters, GABA-A receptors, causing behavioural deviations (Nilsson et al. 2012). The reverse of GABA-A receptor function in the brain has been shown to drive a wide variety of behaviour and sensory impairments in marine organisms, namely loss of olfactory and lateralization (Domenici et al. 2012), auditory preferences (Simpson et al. 2011), sensory preferences, visual threat perception (Ferrari et al. 2012a), learning abilities (Ferrari et al. 2012b) and decision-making (Domenici et al. 2012; Ferrari et al. 2012a). The consequences of increasing lethargic state during hypercapnia in a long-term to fish welfare remain unclear, but may ultimately affect growth rates, and as a consequence of the decrease in food uptake, larval survival may thus become depending on individual resistance to starvation.

7.2.5 Antioxidant defense mechanisms

The antioxidant defense mechanisms, presented in Chapter 6, comprised by heat shock and antioxidant responses, have been described as one of the first lines of protections against thermal and oxidative stress in many marine organisms (e.g. Abele et al. 2011). During exposure to the synergism between warming and acidification, fish larvae showed to display an integrated heat shock and antioxidant responses. HSP70/HSC70 and antioxidant enzymes overexpression

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under warming and acidification is essential so that oxidative stress is either not detected or only occur momentarily (Abele et al. 2011).

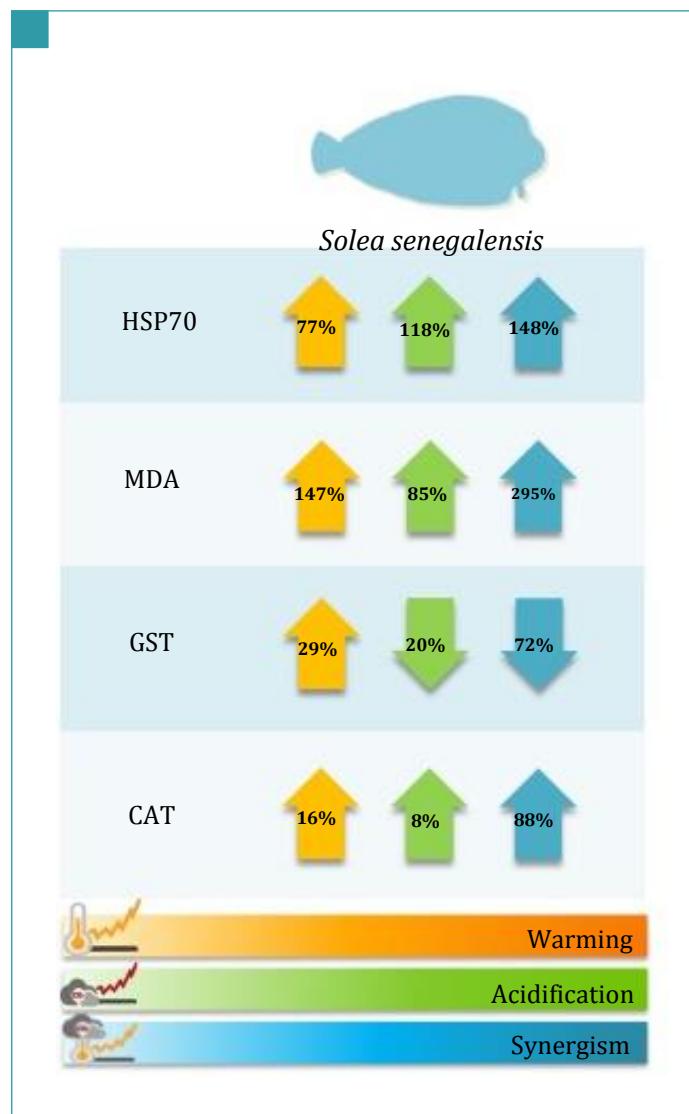


Figure 6. Summary diagram of the effects of ocean warming and acidification on antioxidant defense mechanisms of *Solea senegalensis* larvae.

While the activation of heat shock response partially reduced negative stress effects and increased thermal tolerance limits exhibited by *S. senegalensis* under warming, the opposite trend was observed for ocean acidification. The greater larval sensitivity to environmental hypercapnia was reflected in the higher increment of lipid peroxidation and on lower antioxidant enzymes (GST and CAT) concentrations that did not follow the pronounced increase of heat shock proteins (HSP70/HSC70), and did not participate in ROS elimination. Besides being reported to increase the generation of ROS, ocean acidification also showed here to suppress larval antioxidant defense, accelerating oxidative stress and

possible reducing thermal tolerance limits. Under synergistic conditions stress HSP70/HSC70 and antioxidant enzymes (GST and CAT) were activated in an attempt to maintain the redox balance, protect cellular integrity and mechanisms of fish larvae. However, contrarily to what was expected the up-regulation of HSP, GST and CAT demonstrated not to be an effective defense against ROS formation and extreme cellular lipid peroxidation damage.

These protective mechanisms - HSP and antioxidant enzymes - are critical biological tools to improve organism's survival. However, protein synthesis is energetically costly, especially under metabolic depression (Tomanek and Somero 1999; Tomanek 2010). Thus, cellular protection and maintenance is achieved at the cost of significantly energetic investments (Somero 2002; Hofmann 2005; Dong et al. 2008). It is important note that one of the possible consequences of increased lipid peroxidation is bone cellular components modification (Lall and Lewis-McCrea 2007). This may be one of the pathways that affect the function and integrity of cells, causing a reduction in bone formation and stimulation in bone resorption, with further negative consequences for fish skeletal development (Lewis-McCrea and Lall 2007). Indeed, in Chapter 6 it was reported that lipid peroxidation increment was highly correlated with larval skeletal abnormalities ($R^2=0.88$).

7.2.6 Digestive enzymatic constraints

During larval development, warming as predicted elicited both positive effects on digestive enzymatic activities, as reported for other species (Gelman et al. 2008). Temperature influences the biochemical reactions involved in larval digestion process, by increasing the amino acid digestibility. Specifically, pancreatic (trypsin and amylase) and intestinal (alkaline phosphatase, ALP) enzymatic activity of *S. senegalensis* larvae increased with temperature (184.64, 10.07 and 22.89%, respectively). This increase alongside with the expected increase in the rate of reaction (Love 1970) accompanied the higher larval metabolic demands. Besides temperature, pH is also an important factor shaping the digestion process, as expected digestive enzymes showed a tendency to decrease with acidification. *S. senegalensis* larvae showed a decrease of trypsin, amylase and ALP (59.92, 29.15 and 22.83%, respectively).

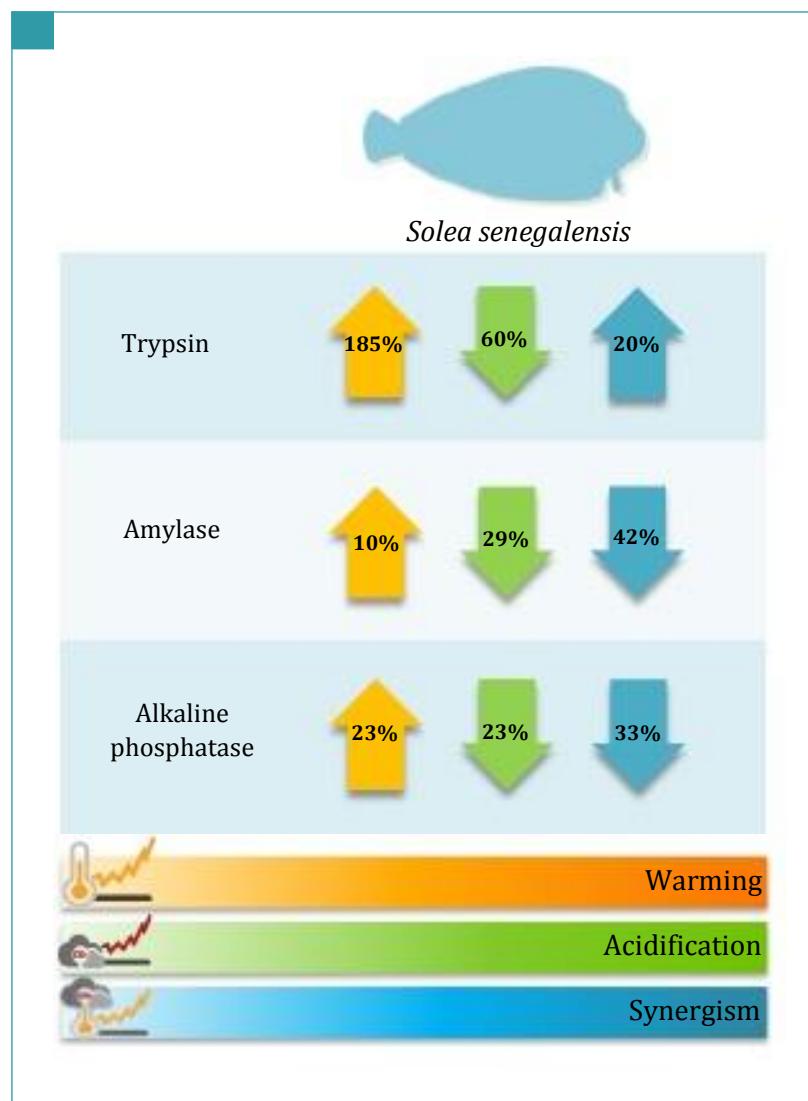


Figure 7. Summary diagram of the effects of ocean warming and acidification on digestive enzymes of *Solea senegalensis* larvae.

At the same time that digestion becomes a slower process, the gastric secretions and the direct absorption of nutrients may also decrease. The decreased synthesis of digestive enzymes might be either a consequence of limited amount of energy available for digestion and assimilation caused by energy changes to maintain homeostasis or/and can be either a consequence of direct intra and extracellular pH changes elicited by hypercapnia. Under ocean acidification, morphological and physiological abnormalities at the digestive system (namely gut and pancreas) of fish early life stages have been already shown (Frommel et al. 2012; Frommel et al. 2014), however no connection was established between altered functional development and digestive enzymes activities.

7.3 Final remarks and Future perspectives

In conclusion, the present description of fish larvae responses to climate change related-variables provides a valuable insight of how predicted warming and acidification may impact the development of wild fish larvae and their future performance in a changing ocean. As it has been shown for early stages of many other marine taxa, the susceptibility of fish early life stages to climate change was here reinforced. The planktonic existence of the most active fish species and the related higher metabolic rates and cardiorespiratory demands may have contributed to the lower tolerance to future ocean conditions. Within the past few years the body of literature concerning the effect of predicted ocean warming and acidification on fish larvae has increased, however, studies have been only focusing on non-commercial species and on the separately effects of warming or acidification. Yet, the increasing temperature is predicted to co-occur with other environmental stressors, such as accumulation of CO₂ in the oceans and increasing hypoxia events. The combination between environmental stressors may intensify the deleterious impacts (Pörtner et al. 2014), challenging the early stages of many fish species. In accordance with the OCLTT concept, carbon dioxide level here tested caused a narrowing of the thermal performance window of fish early life stages, and lead the organism earlier to the limits of its thermal acclimation capacity. Future research is thus required to study on a long-term basis the combined effect of ocean warming and acidification on larval fish performance to increase our understanding of potential synergistic effects. Based on the present findings, it is also crucial to evaluate such effects on species with different life styles, habitat or niche preferences, climate zones and throughout distinct phases of ontogeny in a way to distinguish tolerant from acutely intolerant species. Acclimation approaches, such as shown in the present thesis, are essential to determine which mechanisms will allow organisms to face future ocean changes and in this specific case to evaluate fish larvae susceptibilities especially those of commercially important fish species. Moreover, further research should establish links between biochemical markers, physiological and morphological parameters in an attempt to demonstrate the effects from cellular processes up to the whole-animal level, in order to provide a more conclusive evidence of the sensitivity of marine fish early life stages to ocean climate change.

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Potential for adaptation to new forthcoming conditions is also essential, as already stated by Darwin (1859) "*It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is the most adaptable to change*". Predicting the effects of climate change on fish populations depends not only on measuring the effects of climate stressors on performance, but also on the potential for adaptation through genetic changes. Some genotypes may be the comparative winners in tomorrow's ocean, strong inter-individual variation, and pre-adaptive genetic traits, may promote resilience in a changing ocean. Given the time frame in which ocean warming and acidification are expected to occur, it is important not to forget that species may have the opportunity to adapt. Although remains poorly known the mechanism of transgenerational acclimation, some studies have already shown that parental acclimation can modify the response of fish larvae to climate change conditions (e.g. see Donelson et al. 2012; Salinas and Munch 2012; Murray et al. 2014; Schade et al. 2014; Welch et al. 2014). It is therefore expected that transgenerational acclimation might moderate the negative impacts of future ocean conditions on fish larvae.

Fish larval stages represent a critical life phase for species' ecological success. Both the disruption and thrive of fish early stages have the potential to induce cascading effects over the entire ecosystems while triggering important economic consequences. Therefore, climate-related challenges may potentially influence fish larvae recruitment and dispersal success, affecting the distribution and dynamics of fish larvae under future ocean conditions. Further it can lead to substantial declines in adult populations, putting in jeopardy the species' persistence in a climate change scenario. Although challenging, it would be important to scale up the physiological impairments of early life stages to potential population-level consequences for species. Future efforts should thus focus on how these environmental factors may affect fish species at population-level and transgenerational contexts in a way to help ocean managers and policy-makers to take proactive measures and to improve and justify actions to help endangered and commercially important fish species to adapt to the threat of climate change. Yet, it is inevitable not to be aware and to think that further efforts to reduce global anthropogenic CO₂ emissions by nations could help to perpetuate and preserve species persistence in tomorrow's ocean.

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