Combined effects of ocean acidification and warming on a large pelagic fish, the European sea bass (*Dicentrarchus labrax*)

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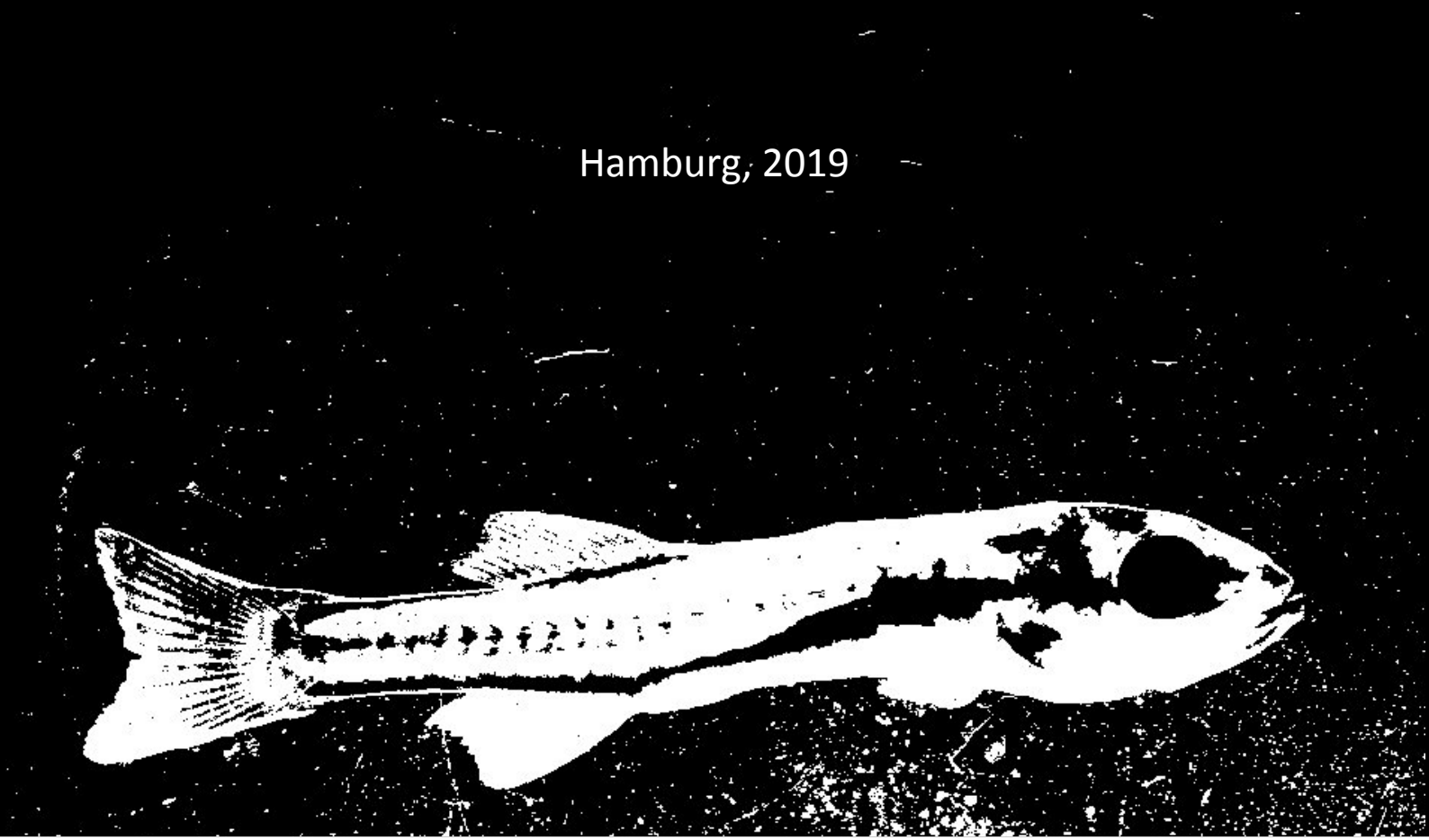

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Louise Cominassi

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"After all this time.

After all of these seasons.

After your own decision,

To go to the water for a reason.

It's only the ocean and you."

Jack Johnson

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My supervisor, Myron Peck, for giving me the opportunity to carry out the research I fancied, for your trust and your advices. I would like to thank you particularly for introducing me to all the amazing people from the Larval Fish community with whom I was able to share my love for science and travel.

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GENERAL ABSTRACT

During the last centuries the human population has made major technical advancements in a number of industries. The development of these anthropogenic activities has led to an increase in greenhouse gas emissions such as carbon dioxide, altering the properties of the atmosphere. As a result, atmosphere heat retention has increased, causing global warming and by transfer ocean warming. In addition, the excess in carbon dioxide ejected in the atmosphere is substantially taken up by the world's oceans, buffering the earth climate but negatively impacting marine life. Indeed, carbon dioxide reacts with the seawater and releases hydrogen ions, leading to the acidification of the ocean which can be measured by a rise in the partial pressure of CO_2 and a decrease in pH. This process, called ocean acidification, together with ocean warming, threatens marine organisms.

Over the last few decades, a number of experiments have examined the impact of either projected future levels of partial pressure of CO_2 (PCO_2) or future temperature on marine organisms and ecosystems. However, there are still important gaps in knowledge on how the two stressors might interact with each other and their combined effects, especially in more complex species. A number of commercial fish species are part of the top of the trophic food chain and thereby are of particular importance to investigate, not only to support food security but also as fundamental species of their ecosystems. It is thus crucial to obtain reliable knowledge and dependable quantitative data to assess the future of these species in the altered environment and help management decisions.

This thesis has examined the effect of three levels of PCO_2 (650, 1150, 1700 µatm; pH 8.0, 7.8, 7.6) at two temperature levels (15 and 20°C), on a variety of key fitness traits in European sea bass larvae (*Dicentrarchus labrax*). Temperature significantly affected the growth rates and the critical swimming speed of the individuals and likely induced energy trade-offs between the two underlying mechanisms. Ocean acidification and ocean warming acted synergistically on otoliths development and bone calcification.

In this thesis, we also address important caveats regarding 1) interaction of ocean acidification and warming with food availability and 2) potential for acclimation. Until now, the majority of the research work has incorporated significant levels of feeding when conducting experiments. The present work demonstrates that an interaction exists between acidification, warming and food supply and that the negative impact of ocean acidification observed in sea bass juveniles exposed to warm conditions can be mitigated by elevated food rations. When fed with restricted rations, changes in energy partitioning are likely to happen at the cost of digestive efficiency through a reduction in enzyme activities. Despite the ecologic relevance of feeding rations, it has also been noticed that previous studies have been mostly of short duration. They therefore may not accurately predict the long-term effects of future conditions on marine populations. In order to investigate the potential for sea bass to adapt or acclimate we conducted a transgenerational study. We assessed the performance in the progeny of

parents conditioned to the projected environment. Results highlighted the potential for transgenerational plasticity in the species since swimming capacity was first reduced at warm temperature but the negative impact of temperature disappeared in the offspring.

Overall, this dissertation demonstrates that ocean acidification may act synergistically with warming, threatening sea bass populations. The exact effects, however, are very difficult to predict given that they rely on other factors, such as food availability and parental conditioning.

ZUSAMMENFASSUNG

In den letzten Jahrhunderten hat die menschliche Bevölkerung in einer Reihe von Branchen große technische Fortschritte erzielt. Die Entwicklung dieser anthropogenen Aktivitäten hat zu einem Anstieg der Treibhausgasemissionen wie Kohlendioxid geführt und die Eigenschaften der Atmosphäre verändert. Infolgedessen hat die Wärmespeicherung in der Atmosphäre zugenommen, was zu einer globalen sowie Ozeanerwärmung führt. Darüber hinaus wird der Überschuss an Kohlendioxid, der in die Atmosphäre ausgeworfen wird, im Wesentlichen von den Weltmeeren, die das Erdklima puffern, aufgenommen. Dies kann jedoch marine Organismen negativ beeinträchtigen. In der Tat reagiert das Kohlendioxid mit dem Meerwasser und setzt Wasserstoffionen frei, die zur Versauerung des Ozeans führen. Das kann an einem Anstieg des Partialdrucks von CO_2 und einem Abfall des pH-Wertes gemessen werden. Dieser als Ozeanversauerung bezeichnete Prozess, bedroht zusammen mit der Erwärmung des Ozeans die Meeresorganismen.

In den letzten Jahrzehnten haben viele Experimente die Auswirkung zukünftiger PCO_2-Werte (CO_2 Partialdruck) oder zukünftiger Temperaturen auf marine Organismen und Ökosysteme untersucht. Es gibt jedoch immer noch wichtige Wissenslücken darüber, wie die beiden Stressoren miteinander und mit ihren Auswirkungen interagieren könnten, insbesondere bei den komplexeren Arten. Eine Reihe kommerzieller Fischarten gehört zur höchsten Stufe der trophischen Nahrungskette. Da sie wichtige Arten im Ökosystem sind, aber auch um die Ernährungssicherheit zu fördern ist es daher von entscheidender Bedeutung, verlässliche Kenntnisse und verlässliche quantitative Daten zu erlangen, um die Zukunft dieser Arten in dem veränderten Lebensraum zu beurteilen und Managemententscheidungen zu treffen.

In dieser Doktorarbeit wurden wichtige Fitnessmerkmale der Laven des europäischen Wolfbarschs (*Dicentrarchus labrax*) auf die Wirkung von drei PCO_2-Spiegeln (650, 1150, 1700 µatm; pH 8.0, 7.8, 7.6) auf zwei Temperaturniveaus (15 und 20°C) untersucht. Die Temperatur beeinflusste signifikant die Wachstumsraten und die kritische Schwimmgeschwindigkeit der Individuen und führte wahrscheinlich zu Energieverschiebungen zwischen den beiden zugrunde liegenden Mechanismen. Die Versauerung der Ozeane und die Erwärmung der Ozeane wirkten sich synergistisch auf die Entwicklung der Otolithen und das Kalzifizieren der Knochen aus.

In dieser Arbeit werden auch wichtige Wissenslücken in Bezug auf 1) die Wechselwirkung von Ozeanversauerung und Erwärmung mit der Verfügbarkeit von Nahrung und 2) das Potential für Akklimatisierung angesprochen. Bis jetzt wurden in dem Großteil der Forschungsarbeiten bei der Durchführung von Experimenten erhebliche Mengen an Futter gefüttert. Die vorliegende Arbeit zeigt, dass eine Wechselwirkung zwischen Versauerung, Erwärmung und Nahrungsverfügbarkeit besteht und, dass die negativen Auswirkungen der Ozeanversauerung, die bei Wolfsbarschlarven beobachtet wurden, die warmen Bedingungen ausgesetzt sind, durch erhöhte Nahrungsrationen gemildert werden konnten. Bei einer Fütterung mit eingeschränkten Rationen, gehen Änderungen der Energieaufteilung wahrscheinlich zu Lasten der Verdauungseffizienz aufgrund einer Verringerung der Enzymaktivitäten. Trotz der ökologischen Relevanz von der Fütterung von Rationen, wurde auch festgestellt, dass frühere

Studien größtenteils von kurzer Dauer waren. Daher können sie die langfristigen Auswirkungen zukünftiger Umweltbedingungen auf Populationen möglicherweise nicht genau vorhersagen.

Um zu untersuchen, ob sich Wolfsbarsch anpassen oder akklimatisieren kann, haben wir eine generationenübergreifende Studie durchgeführt. Wir bewerteten die Leistungsfähigkeit bei den Nachkommen von Eltern, die den projizierten Umweltbedingungen ausgesetzt waren. Die Ergebnisse zeigten das Potenzial für die Plastizität der Art über Generationen, da die Schwimmkapazität bei warmen Temperaturen zwar verringert wurde, die negativen Auswirkungen der Temperatur jedoch bei den Nachkommen verschwanden.

Insgesamt zeigt diese Dissertation, dass die Ozeanversauerung synergistisch mit der Erwärmung die Wolfsbarschpopulationen bedrohen kann. Die genauen Auswirkungen sind jedoch sehr schwer vorherzusagen, da sie von anderen Faktoren abhängig sind, wie etwa der Verfügbarkeit von Nahrung und der Konditionierung der Eltern.

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CHAPTER 1: General Introduction

1.1 Climate Change (CC)

Climate change (CC), particularly global warming and ocean acidification, is impacting the distribution and productivity of both terrestrial and marine organisms (Intergovernmental Panel on Climate Change IPCC 2014). Given that human well-being is largely supported by aquatic ecosystems and species (e.g. food security, cultural heritage, communities economies) (Hidalgo et al. 2018; FAO 2019), it appears essential to limit the impact of future conditions. The impact of projected changes in CC might be reduced thanks to international policy initiatives and sustainable management, yet good management relies on good scientific knowledge.

Over the history of the planet, climate has changed markedly due to natural process but, in the last 150 years, changes have occurred faster and in a greater way. Climate is the average weather condition occurring over a certain period of time, from months to millions of years (IPCC 2014). Variation in natural climate is independent from external factors and can happen randomly, in a chaotic manner, or following cycles such as season or multi-millennial scale patterns (IPCC 2014; Harley et al. 2006). Since the development of human activities over the last centuries, changes in earth climate system have been observed (Harley et al. 2006). The Industrialization and concomitant burning of fossil fuels and other anthropogenic processes, such as land clearing, has led to increases in emissions of greenhouse gases, and especially carbon dioxide (CO_2), into the atmosphere (Sabine et al. 2004; Orr et al. 2005; Raupach et al. 2007; IPCC 2014). The accumulation of anthropogenic gases in the atmosphere is causing significant changes in heat retention resulting in the greenhouse effect and ultimately global warming. Since the pre-industrial level, a drastic increase of 40% of the concentration of the atmospheric CO_2 has been recorded. Global atmospheric CO_2 concentrations today exceed levels of concentration reported for the past 800 000 years (IPCC 2014) (Figure 1.1), with measured values reaching up to 415 ppm in May 2019 at Mauna Loa Observatory (Keeling Curve, Scripps Institution of Oceanography). Oceans act as a buffer for global warming by absorbing close to one third of the excess CO_2 emitted (Quéré et al. 2015).

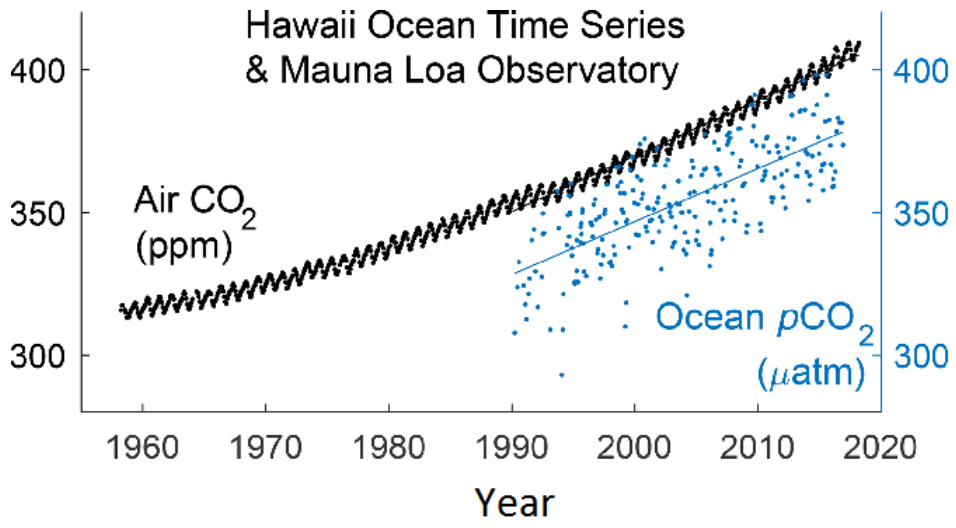

Figure 1.1 Atmospheric CO_2 (black dots) measured at the Mauna Loa Observatory in Hawaii beginning in 1958 and surface ocean pCO_2 data (blue dots) from the Hawaii Ocean Time Series (HOT) station. Black and blue lines indicate linear trends after 1990. Atmospheric CO_2 increased by 1.86±0.11 ppm year^{-1}. Surface ocean pCO_2 increased by 1.95± 0.017 µatm year^{-1}. pCO_2 is calculated using the Microsoft Excel macro CO2sys, with (Mehrbach et al. 1973) refit coefficients for the dissociation constants of H_2CO_3 and HCO_3^-, and (Dickson & Millero 1987) dissociation constant for HSO_4^- (taken from Fennel et al. 2018).

1.1.1 Ocean Warming (OW)

The majority of the planet experiences surface warming. An increase of 0.85°C in temperature over the land and the oceans has been measured between 1880 and 2012 (IPCC 2014). In seawater from the surface down to 75 m an average warming since 1971 of 0.11 °C per decade has been reported over all latitudes (IPCC 2014). Furthermore, deep waters (≤ 700 m) warmed 0.1°C from 1961 to 2003 (Bindoff et al. 2007) and traces of warming have been recorded at even greater depth (~2000 m) between 1957 and 2009 (IPCC 2014). Effects of OW are numerous (Harley et al. 2006), for instance the combination of thermal expansion and freshwater input from melting ice is expected to cause sea level rise (Rhein et al. 2013). Furthermore, phenomenon like El-Niño and large changes in ocean dynamic and circulation, are predicted to be more common (Timmermann et al. 1999). A gradient in pressure will form between continental regions experiencing stronger warming and coastal areas characterized by wind fields along oceans limits (IPCC 2014). A strong gradient will also lead to an increase in storm frequencies and an alteration in patterns of precipitation impacting coastal salinity, turbidity and terrestrial input of nutrients and pollutants (Harley et al. 2006). While the average temperature of the sea surface is predicted to rise by 2.2°C or 3.7°C (according to RCP6.0 and RCP8.5, respectively) (Figure 1.2) by 2100, global OW is predicted to escalate by 0.6 (RCP2.6) to 2.0°C (RCP8.5) in the upper 100 m and 0.3°C (RCP2.6) to 0.6°C (RCP8.5) at a depth of 1000 m (Collins et al. 2013).

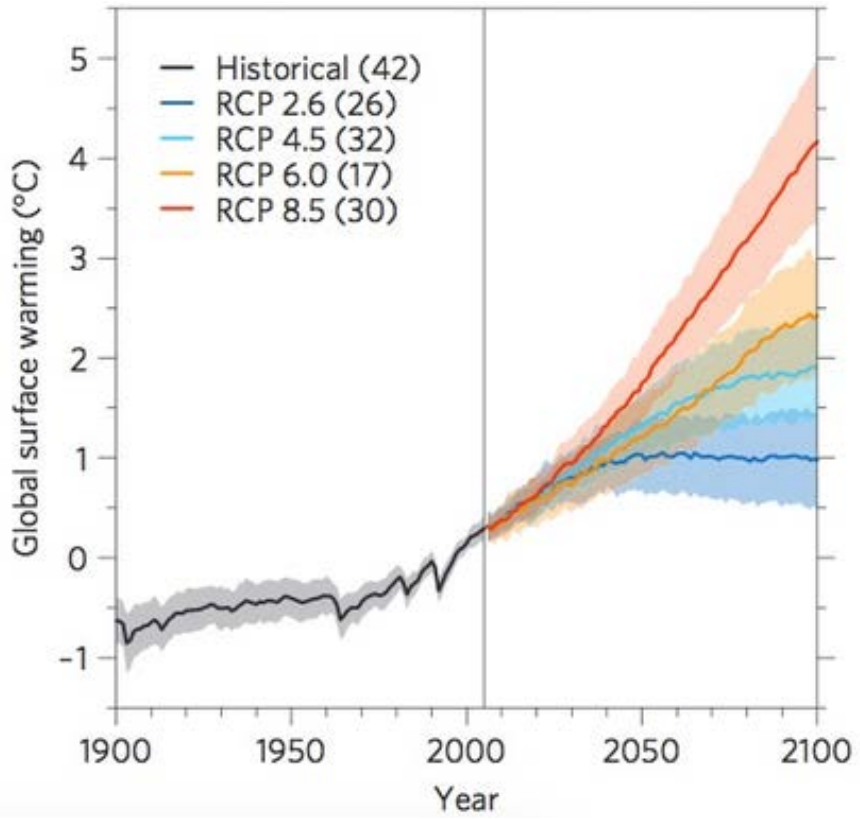

Figure 1.2 Global temperature change (mean and one standard deviation as shading) relative to 1986–2005 based on IPCC scenarios (Representative Concentration Pathways, RCP) run by CMIP5 (modified from Knutti & Sedláček, 2013). The number of models is given in brackets.

1.1.2 Ocean Acidification (OA)

The absorption of exceeding CO_2 by the oceans comes at a cost, which is ocean acidification (OA) (Doney et al. 2009). Atmospheric CO_2 dissolves in seawater and react with the water molecules (H_2O) to form carbonic acid (H_2CO_3). The carbonic acid dissociates itself further into bicarbonate ions (HCO_3^-) and carbonate ions, freeing hydrogen ions (H^+) (Figure 1.3). The release of hydrogen ions increase the acidity of the water which is measured as a decrease in pH (Caldeira & Wickett 2005; Doney et al. 2009; Feely et al. 2004). The decrease in oceanic pH levels causes a reduction in the availability of calcium carbonate ($CaCO_3$) polymorphs (aragonite, calcite and vaterite) (Feely et al. 2004; Doney et al. 2009).

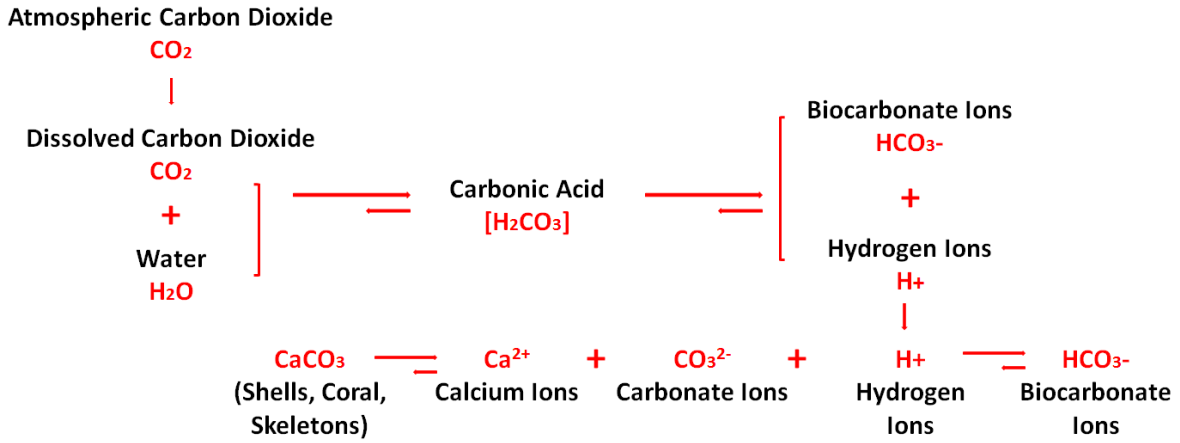

Figure 1.3 Schematic representation of carbonate chemistry. Carbon dioxide dissolves in the ocean and reacts with seawater to form carbonic acid and subsequently bicarbonate, carbonate and hydrogen ions.

Since the beginning of industrialization, pH of the global ocean has displayed a decrease in pH of 0.1, which consists in an increase of H^+ of 26%. The pH of water surface of oceans is projected to decrease even further in the next 100 years by 0.13 to 0.5 units depending on the considered Representative Concentration Pathway (RCP) scenarios developed by the IPCC (Caldeira & Wickett 2005; Meehl et al. 2007) (Figure 1.4). These expected changes in pH correspond to a rise in CO_2 partial pressures (PCO_2) levels to 730 and 1000 ppm by 2100 (Meehl et al. 2007).

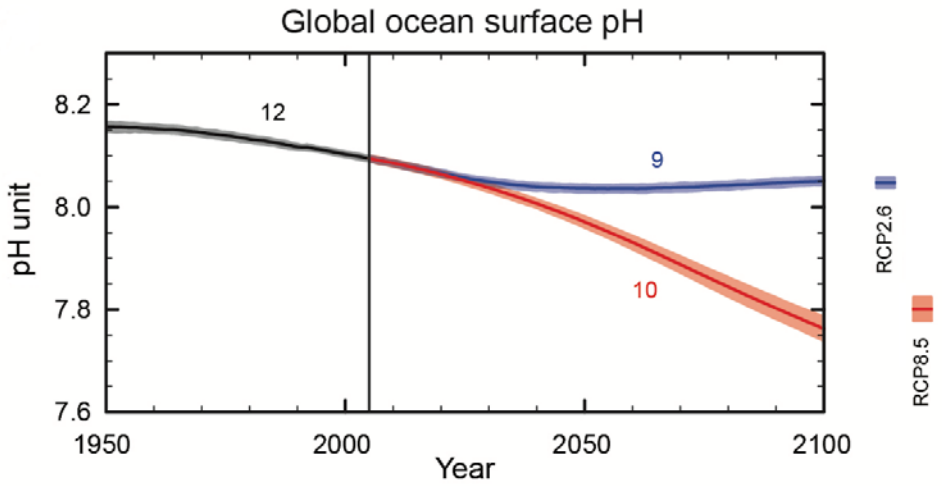

Figure 1.4 Global pH change (mean and one standard deviation as shading) relative to 1986–2005 based on IPCC scenarios (Representative Concentration Pathways, RCP) (taken from IPCC 2014). The number of models is given in color on the graphic.

The net effect of CO_2 uptake by the ocean is expressed by a decrease in pH and carbonate ion concentration, and by an increase in bicarbonate ion concentration and ocean PCO_2. The

change of biogeochemistry of the ocean is now recognized to be a threat to marine organisms and numerous research or conducted since the past decade to try to understand its potential impact (Hofmann & Todgham 2010; Kroeker et al. 2013; Harvey et al. 2013; Cattano et al. 2018). To investigate the impact of OA on species biology, it is important to comprehend its regional dynamics. At high latitudes, pH is expected to be even lower than the global average. Indeed, CO_2 solubility increases with lower temperatures resulting in higher gas exchange at the sea surface. In addition, this phenomenon is positively reinforced by the melting of sea ice induced by OW (Maneja et al. 2013). Coastal areas are also likely to face important increases in PCO_2 levels due to the inflow of terrestrial organic carbon. In the water, this input of terrestrial organic carbon is oxidized chemically or by microorganisms which further releases CO_2. It has been reported that due to the significant oxygen consumption, coastal region can experience drastic variation in pH and PCO_2 with levels reaching up to 3200 µatm (Melzner et al., 2012).

1.2 Effects of CC on marine organisms

Climate strongly influences the majority of oceans characteristics such as temperature, chemistry, circulation, stratification, nutrient supply and even solar radiation (IPCC 2014). When confronted to important climate-induced changes, like ocean warming (OW) or ocean acidification (OA), species can either migrate, acclimate, adapt or die (Barry et al. 2011). Ecological responses to climate effects can thus be observed at the individual-level with possible changes in the physiology, morphology or behavior of the organisms at different ontogenic stage, but also at the population- and community-level, characterized by changes in dispersal, recruitment or species abundance (Harley et al. 2006).

1.2.1 Effect of OW on marine organisms

All species are constrained to a range of temperatures, known as thermal window, outside of which they cannot survive. This window might me be more or less wide depending on the adaptation of the organism to its environments. For example, thermal window of stenotherm organisms is rather narrow compared to eurythermal species tolerant to a more extended range of temperatures (Hochachka & Somero 2002). Vulnerability of marine species, confronted with OW and extreme temperatures, depends on this thermal specialization (Pörtner & Peck 2010). Within this window of tolerable temperatures, an optimum exists at which individual performance and species fitness is at its maximum. Outside of this optimal range in temperatures, warming may have deleterious effects on the organism, eventually leading to death (Pörtner & Peck 2010) (Figure 1.5). Marine species have demonstrated different potentials responses when confronted to temperatures without their thermal window: species can migrate, acclimate or adapt. Species unable to respond to change in temperatures are likely to become locally or globally extinct.

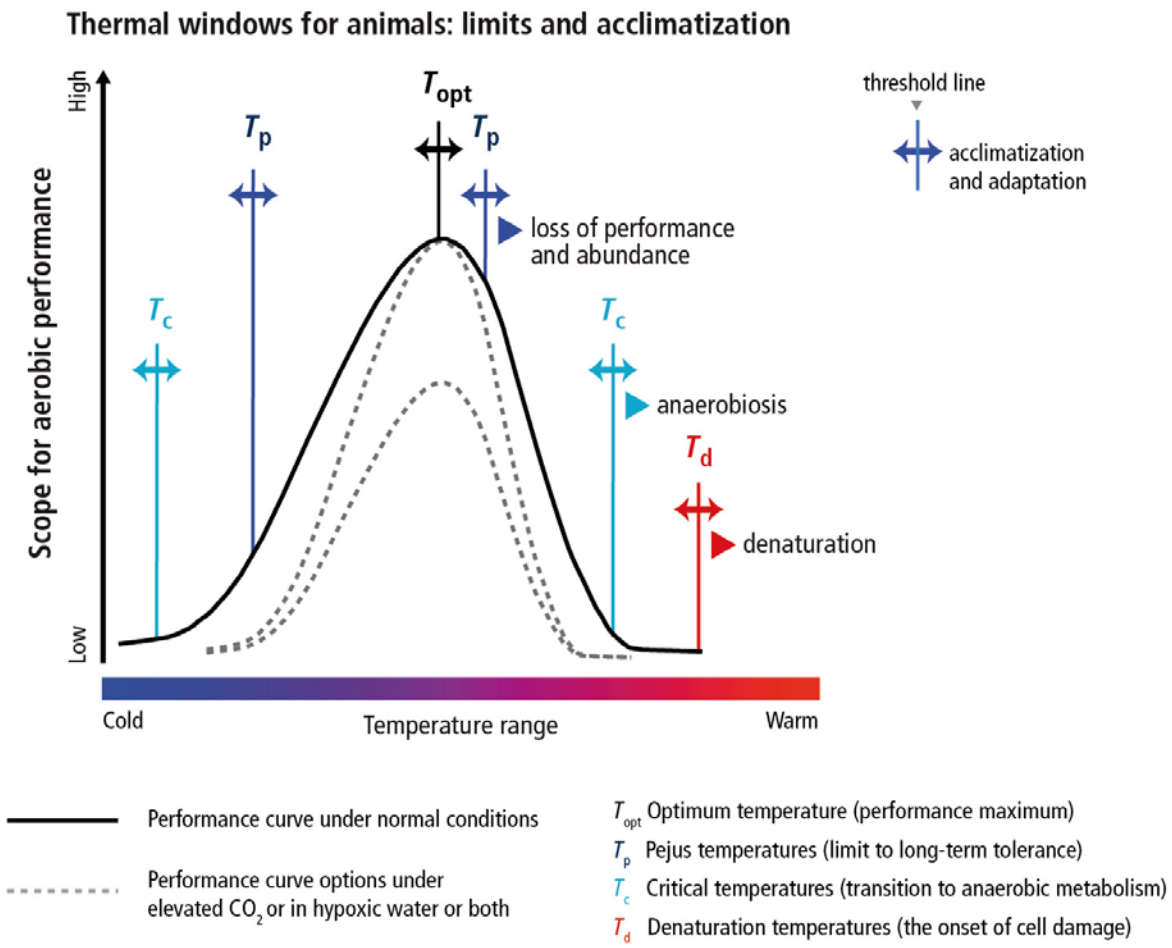

Figure 5. **The thermal windows of aerobic performance display optima and limitations by pejus, critical and denaturation temperatures** (modified from Pörtner & Farrell, 2008). The synergistic effects of multiple factors reduce the scope for aerobic performance and limit the tolerable range in temperatures.

To avoid sub-optimal temperatures, species can migrate to more suitable areas. The geographic distribution of marine biota is influenced by global patterns in temperature (Hochachka & Somero 2002). Therefore, OW is the most powerful driver responsible for changes in organisms distribution, abundance and also community and ecosystem structure (Perry et al. 2005; Pörtner 2008). OW is likely to induce poleward migrations in order for species to stay within their thermal window (Tissot et al. 1993). This hypothesis has been supported by Pörtner et al. (2014) who even predicted a modification in community patterns with a decrease in species richness in low latitude due to the poleward redistribution trend. According to Poloczanska et al. (2013), marine species have, on average, already shifted the limits of their distributions by 72.0 ± 13.5 km per decade (based on data sets > 19 years in length). Recent evidence suggests that the center of abundance of some fish species, such as the yellowfin whiting (*Sillago schomburgkii*), has been shifting poleward since 1950, but that this shift has dramatically accelerated over the past 10 years due to an increasing frequency of heatwaves (Smith et al. 2019). Escaping stressful environments however, depends on the

availability of suitable habitat but also on the locomotor ability of the fish which is normally much reduced or negligible for very early life stages.

Organisms can also acclimate to an environmental stressor. The physiological responses of marine species to global warming depend largely on their ability to maintain their energy homeostasis (Sokolova et al. 2012; Sokolova & Lannig 2008). Indeed, as water temperatures increase, so does the minimum energy required to maintain their key physiological functions (e.g. growth, reproduction, feeding) (Sandersfeld & 2015; Weber et al. 2016). Thermal acclimation mostly occurs through the role of phenotypic plasticity, and is often described as the ability to adjust a performance trait in response the warm environment (Munday et al. 2013). This mechanism is determined by the fish species, life stages, social status and life history (Figure 1.6) and can take place relatively fast. For example, Sparks et al. (2017) noticed a significant difference between the development rates of Pacific salmon (*Oncorhynchus nerka*) embryos. Embryos exposed to the cold environment take 2.5 times longer to develop than embryos maintained into the warm environment.

Marine species might cope with warm conditions through genetic adaptations. Adaptation depends on the species capacity to generate new genetic alternative and /or if the species possess ample variation upon which selection can operate (Munday et al. 2013). Genetic adaptation occurs generally through a longer time scale. This mechanism might not act fast enough to enhance species evolution compromising the ability of species with long generation times or overexploited species to cope with environmental stressor (Dulvy et al. 2003).

1.2.2 Effects of OA on marine organisms

Changes in properties of the ocean due to the uptake in anthropogenic CO_2 is likely to impact marine species. Effects of OA have been first investigated in calcifying organisms, since the capacity of these species to build their calcium carbonate shells is likely to be influenced by the reduction in carbonate saturation state (Byrne 2011; Kleypas & Langdon 2006; Kroeker et al. 2013). Rising levels of partial pressure of CO_2 (PCO_2), however, can also have direct effects on other marine organisms (Pörtner et al. 2004). Teleost fish as well are composed of calcified structures such as bones and otoliths. Its skeleton, however, is constituted principally of calcium phosphate and believed to be less sensitive to decrease in carbonate saturation (Toppe et al. 2007). Although European sea bass (*Dicentrarchus labrax*) displayed faster acidification with high level of PCO_2, fish skeleton also exhibit higher frequency of deformities in the column and in the jaw (Crespel et al. 2017). Moreover, fish do still own calcium carbonate structures, ear bones (otoliths) mostly composed of aragonite. Otoliths grow with the fish and their growth is marked by periodic increments commonly used to estimate the age of fish (Checkley et al. 2009). A number of studies have observed increasing otoliths size in fish conditioned to future levels of acidification (Checkley et al. 2009; Bignami et al. 2013). Changes in otolith shape have also been reported and those changes are worrisome since

otoliths are involved in auditory and swimming capacity (Bignami et al. 2013). This alterations, however, appeared to be highly species-specific with some fishes showing no change in otolith growth at near future CO_2 levels (Munday et al. 2011; Simpson et al. 2011).

In fish, the fitness response (e.g. survival, growth) to OA differs depending on the level of exposure, the species or the developmental stage. A number of studies have found an increase in mortality in a number of species due to exposition to high level of acidification (Munday et al. 2010; Baumann et al. 2012; Miller et al. 2012). But contrastingly, the survival of the eggs of a tropical fish (*Amphiprion percula*) was unaffected (Munday et al. 2009).

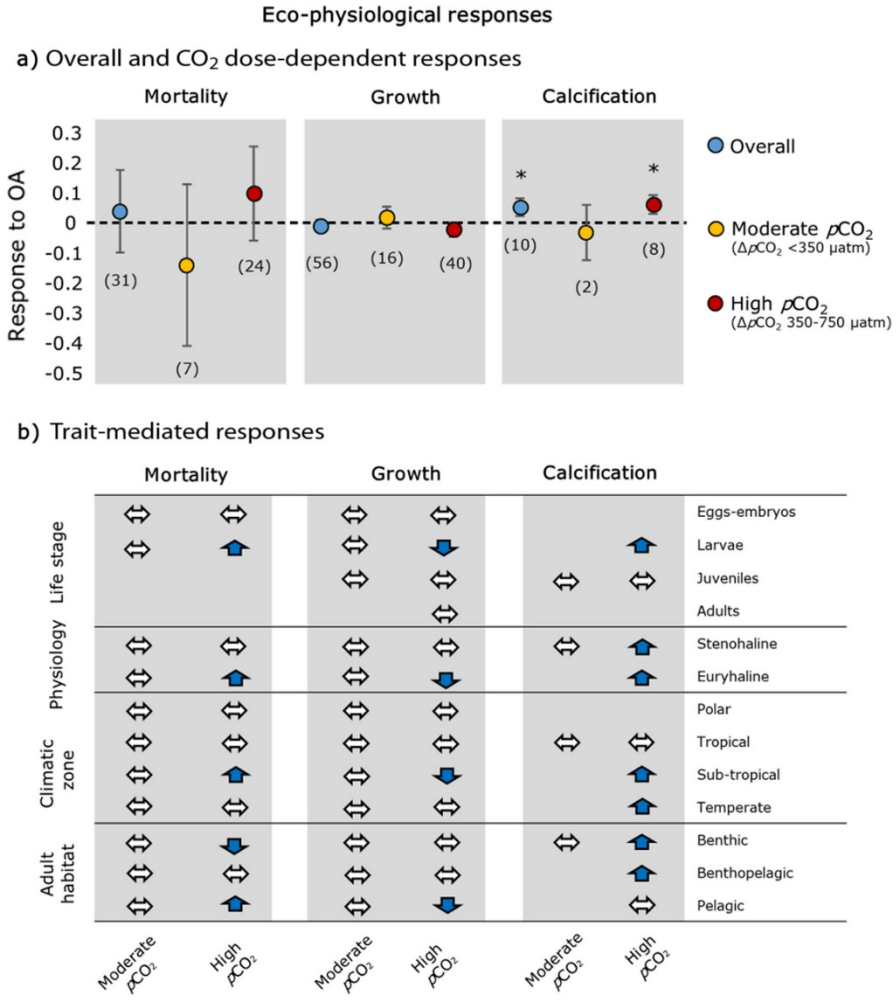

Figure 1.6 Ocean acidification (OA) effects on fish eco-physiological responses (mortality, resting metabolic rate and metabolic scope). (a) Overall and CO_2 dose-dependent responses. The number of contrasts considered per response is shown in parentheses. Asterisks indicate significant difference from zero ($P < 0.05$). (b) Summary of trait-mediated responses for each CO_2 projection relative to fish mortality, resting metabolic rate and metabolic scope. Arrows show the directionality of the responses: up arrow, significantly positive; down arrow, significantly negative; side to side arrow, null (taken from Cattano et al. 2018).

Similarly investigation of OA impact on fish growth has resulted in a number of contrasting findings. For example, divergences were observed among tropical fish species at similar life stages. Miller et al. (2012) found a decline in growth in juvenile anemonefish (*Amphiprion melanopus*). Whereas the spiny damselfish (*Acanthochromis polyacanthus*) was unaffected by projected conditions of acidification at juvenile stage (Munday et al. 2009). Similarly, in temperate species, fish growth has been found to be reduced, increased or unaffected by elevated levels of PCO_2. Atlantic herring (*Clupea harengus*) larvae, reared at 1800 to 4200 µatm PCO_2, displayed reduced growth rate (Maneja et al., 2015). While the work of Pimentel et al. (2016) showed increased growth in the early life stage of meagre (*Argyrosomus regius*) exposed to elevated PCO_2 levels, Despite the substantial research efforts over the last decade to assess the impact of OA (Cattano et al. 2018; Kroeker et al. 2013) (Figure 1.6), gaps in knowledge still remain, specially concerning the potential of adaptation in temperate fish.

1.2.3 Combined effects of OA and OW

Multiple climate drivers interact in nature to impact the vital rates of marine biota (Frost et al. 1999). The combined influence of this multitude of factors is challenging to predict since interactions can be either additive (the combined effect is roughly the sum of their individual effects), synergistic (the combined effect is larger than the sum of the effects of the independent stressors) or antagonistic (the combined effect is less than the effect of either stressor) (Vinebrooke et al. 2004). Understanding the cumulative effect of multiple stressors on marine organisms is currently one of the top priorities for ecologists (Hodgson & Halpern, 2018). Recently, an effort has thus been to assess the combined effects of OW and OA. The combination of acidification and warming was found to have an interacting effect on reef fishes behavior, with impact on species lateralization (Domenici et al. 2014) or on feeding rates (Nowicki et al. 2012). Additive impacts were also observed and have been reported to affect metabolic rates in two species of cardinalfishes (Munday et al. 2009). Overall, however, the majority of the studies highlighted synergistic effects between elevated temperatures and PCO_2 levels, resulting in greater negative effects on survival (Pimentel et al. 2016), swimming ability (Watson et al. 2018) and the outcome of predator-prey interactions (Allan et al. 2017). Therefore, warming cannot be dissociated of PCO_2 levels to accurately understand the effects of ocean acidification and evaluate the potential of fish species to cope with the future environment.

The combined effects of acidification and warming in marine fishes is still largely understudied, especially on large pelagic fish. It is critical to focus the research effort on these species which are fundamental both ecologically and economically. Indeed, in addition to be top predators and thereby contributing to the support of marine ecosystems functioning (Casini et al., 2009; Frank et al. 2005), large pelagic fish represent a major food source worldwide (FAO 2019). Furthermore, these species are supposedly less resilient to OAW since they encounter relatively stable conditions in the open sea (Munday et al. 2008; Pörtner 2008)

in comparison to the high fluctuations in temperatures and pH experiences in coastal habitats (Hofmann et al. 2011; Waldbusser & Salisbury 2014).

1.3 Sensitivity to environmental stressors with ontogenic stage

The larval stage of fish represent a transitional period during which individuals undergo major changes in structure, physiology, size and morphology (Peck & Moyano 2016), as such it is characterized by the highest potential for sensitivity to environmental stressors (Knutti & Sedláček 2013). Water-breather were first thought to be particularly sensitive to increase in environmental CO_2 concentration compared to terrestrial animals due to the lower level of partial pressure of CO_2 (PCO_2) of their body fluids (Ultsch & Jackson 1996). Indeed, rise in CO_2 concentration in the environment is likely to lower further the CO_2 excretion potential of the vertebrate, leading to respiratory acidosis and the necessity to actively discharge hydrogen ions. Nonetheless, they appeared protected against acidification thanks to an effective osmotic- and ionic-regulatory system capable of compensate for changes in intracellular and extracellular pH (Melzner et al. 2009). Organs such as kidney, intestine and mainly gills, are able to buffer blood pH by excreting hydrogen ions and absorbing bicarbonate ions, a reaction catalyzed by one enzyme, the carbonic anhydrase (CA) (Melzner et al. 2009; Perry & Gilmour 2006). For example, the Atlantic cod (*Gadus morhua*) is capable of fully compensate acidosis after experiencing 24h of hypercapnia in both intracellular and extracellular compartments (Larsen et al. 1997). On the other hand, the gulf toadfish (*Opsanus beta*) endures acidosis when conditioned to two levels of PCO_2 (<1000 µatm and >1900 µatm) but after 2 to 4 hours, this state was fully compensated and the fish blood showed elevated concentrations of HCO_3^- (Heuer et al. 2012).

Buffering the blood pH is a reaction costly in energy. The excretion of ions H^+ is linked with the inflow of Na+ while the HCO_3^- is coupled with the outflow of Cl^-, exchanges or facilitated by the Na+/H. Exchangers can operate thanks to the energy provided by the Na^+/K^+ ATPase. To preserve electroneutrality, Cl^- is transported to seawater via chloride channels while the bicarbonate is transferred to the extracellular fluid (Melzner et al. 2009; Perry & Gilmour 2006). Internal regulation of pH is thus associated with an increase in bicarbonate ions. PCO_2 level is defined by the integration of a concentration in bicarbonate ions and a level of pH. The extracellular pH might briefly decrease, the HCO_3^- concentrations, however, increase right away and after a certain period of time, the pH gets back to its initial value. Although pH is again close to its ideal level, HCO_3^- concentrations and PCO_2 have changed drastically (Figure 1.7). Eggs and early larval stages are thus developing stages, they do not own completely functional gills yet, therefore their potential for compensation and regulation of internal acidosis might not be effective making them likely less robust to hypercapnia (Frommel et al. 2012; Baumann et al. 2012). Falk-Petersen (2005) suggested that larvae might be able to involve chloride cells and possibly other channels across the whole body surface to regulate their internal pH, but its process is likely to be less efficient.

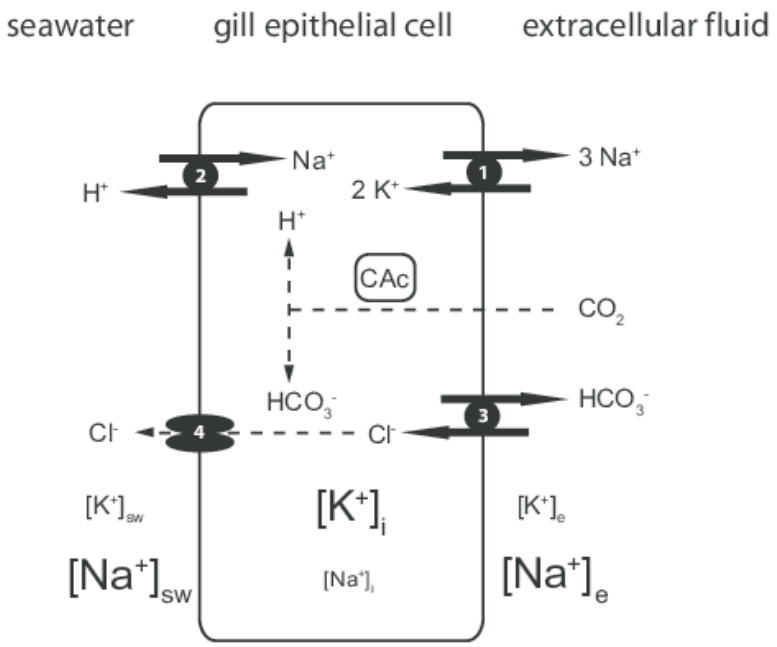

Figure 1.7 Simplified schematic depiction of an epithelial gill cell (ionocyte) of a teleost fish (taken from Melzner et al. 2009) (adapted from Perry & Gilmour, 2006)) (1) =Na^+/K^+ ATPase, (2)= Na^+/K^+ exchanger, (3)=Cl^-/HCO_3^- exchanger, (4)=Cl^- channel (e.g. CFTR), CAc = cytoplasmic carbonic anhydrase.

Eggs and early larval stages are especially susceptible to thermal changes. The thermal range of marine biota varies between and within species with the earliest as well as reproductive stages being particularly susceptible to thermal effects (Harley et al. 2006; Pörtner & Peck 2010). This is linked with the oxygen- and capacity-limited thermal tolerance (OCLTT) which depends on the body size, the development of organ functioning and the metabolism plasticity (Pörtner & Farrell 2008). The thermal tolerance window of embryonic and larval fish is much narrower than that of adults making them highly sensitive to temperature changes (Rombough 1997; Pörtner & Farrell 2008) (Figure 1.8). For example, Drost et al. (2016) observed that while maximum temperature for heart rate in adult Arctic cod (*Boreogadus saida*) was around 10°C, it was only 7.6°C in larvae. Thermal constraints for the Arctic cod is thus unlikely related to thermal tolerance in adults but most likely governed by temperatures encountered in the summer which are beyond the thermal window of the larvae. Since early life stages are constrained within such a narrow window, they are likely exposed to strong evolutionary pressures likely to induce adaptive changes and subsequently impacting the functioning of the next developmental stage (Pörtner & Farrell, 2008). Without a fully developed homeostasis, regulatory system and a rather narrow thermal window, early life stages are likely to be strongly impacted by the effects of ocean acidification and warming (OAW).

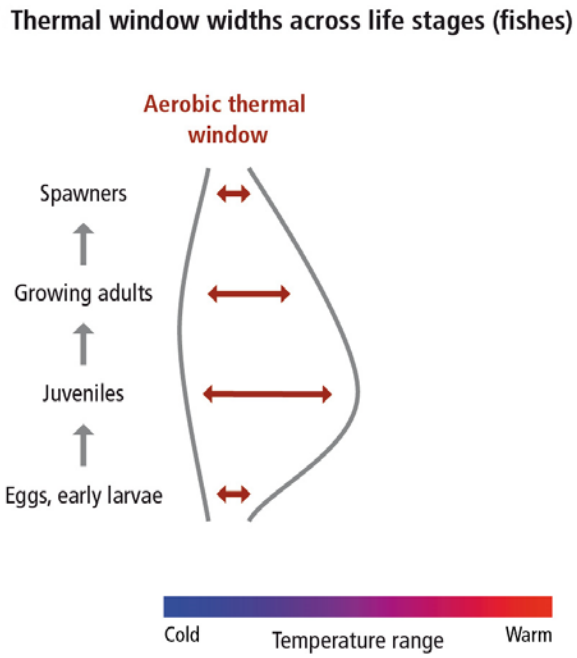

Figure 1.8 Positions and widths of windows on the temperature scale shift with life stage (taken from Pörtner & Farrell, 2008).

1.4 Transgenerational Acclimation

1.4.1 Potential for Adaptation and Acclimation

To date, effects of ocean acidification (OA) are investigated over short periods of time but OA will proceed over a much longer time frame. Therefore, changes in seawater chemistry will happen over several generations. Hence, organisms and populations can potentially adapt or acclimate with time, especially species with shorter lifespan, with a greater number of generation selecting for maximal adapted genotype to the stressor in a short time period (Melzner et al. 2009; Welch et al. 2014). Until now, only a few studies have looked into the potential for adaptation in marine species exposed to acidification. A number of studies have conducted experiments and exposed organisms to different levels of partial pressure of CO_2 (PCO_2) over a few days or weeks while marine species can experiment these conditions throughout all their life and over generations. By not considering adaptive potential occurring after long-term conditioning, effects of OA cannot be fully assessed and might be either over- or underestimated (Sunday et al. 2014; Gaylord et al. 2014; Foo and Byrne 2016).

Potential for species to develop tolerance to a new environment can be expressed through adaptation or acclimation. Adaptation to environmental constraints implicates selection on genetic variation within the population. According to the experienced environment, some features will enable individuals to survive or reproduce better (adaptive traits) in given conditions, and the genes that determine these traits will be selected from one generation to the next. This process is irreversible and occurs over the population through long time scale.

Acclimation is a form of phenotypic plasticity which is characterized by the capacity for one genotype to express on phenotype according to the environment (Whitman & Agrawal 2009). The expressed phenotype enables the organism to either maintain or increase its performance in the new environment (Munday 2014). Donelson et al. (2012), observed that the spiny damselfish kept at elevated levels of temperature displayed less change in metabolic rate compared to the individuals which undergo acute warmer conditions. This phenomenon is called phenotypic buffering (Reusch 2014). Acclimation can occur within a generation, it is then referred to as reversible or developmental acclimation. When it happens from one generation to the other or across a number of generations, it is mentioned as transgenerational acclimation (Munday 2014). Species confronted to highly variable environment, defined by daily or seasonal environmental changes, often demonstrate reversible acclimation. Developmental acclimation is characterized by a permanent change in the phenotype induced after exposition to a specific environment during early life (Angilletta 2009). According to Scott & Johnston (2012), zebrafish (*Danio rerio*) swam better in elevated temperature when they were first exposed to these warm conditions at embryonic stages. Similarly, exposure to hypoxia during embryonic or larval phases can have lasting effects on fish metabolism (Cadiz et al. 2017; Robertson et al. 2014).

1.4.2 Transgenerational Plasticity (TGP)

Transgenerational acclimation, also referred to as transgenerational plasticity (TGP), takes place when the conditioning of the parents to environmental conditions affects the response of the offspring in the new environment and this without genetic alterations (Mousseau & Fox 1998; Salinas & Munch 2012). Cases of TGP have been observed in an extended scope of traits in numerous taxa (Salinas et al. 2013). Acclimation through transgenerational plasticity occurs via diverse non-genetic mechanisms and does not require a selection of genotype from one generation to the other (Bonduriansky 2012). These mechanisms involve changes in nutritional provisioning and transfer of hormones or proteins, or transfer of epigenetic marks (Jablonka & Raz, 2009). TGP is beneficial if it leads to better performance in offspring after parental exposure in the new environment.

A range of studies have looked at the TGP for species response to climate change starting with OW. Performance such as the aerobic scope (Donelson et al. 2012) or growth (Salinas & Munch 2012) in offspring were improved once parents experimented similar warmer environment. Similarly, TGP OA-dependent were observed in a number of traits and parents conditioning to high CO_2 levels lead to positive response of their offspring in a high- CO_2 environment (Miller et al. 2012; Murray et al. 2014). For example, size impairment induced by high levels of PCO_2 in Sydney rock oysters disappeared once parents where maintained at the elevated PCO_2 levels during reproduction (Parker et al. 2012). In the same way, Miller et al. (2012) found that negative effects on the metabolic rate, growth and survival induced by high PCO_2 levels in juvenile cinnamon anemonefish (*Amphiprion melanopus*) were absent or reversed when

parents first experienced the same conditions. Alleviation with previous parental conditioning was also reported after OA-induced behavioral impairments in the species (Allan et al. 2014). Murray et al. (2014) highlighted that breeding season characterized by different levels of pH lead to more or less sensitive Atlantic silverside (*Menidia menidia*) offspring. The offspring spawned late in the season at low pH were more tolerant to elevated CO_2 compared to the individuals produced earlier in the season when environmental pH of the parents was much higher. Contrastingly, with high CO_2 conditions, the juvenile's tropical spiny damselfish showed damage chemical conspecific alarm cues but this was not either attenuated or reversed when parents were first exposed demonstrating no capacity for transgenerational acclimation of impaired predator avoidance (Welch et al. 2014). Welch et al. (2014) suggested that genetic adaptation would be necessary for the spiny damselfish to reduce the impact of OA. This body of evidence highlighted that effects of OA on marine species might be overestimated when assessed by short-term experiment. Transgenerational acclimation has the potential to offset negative impact of PCO_2 levels observed in some organisms in one generation. Assessing the potential for transgenerational acclimation is thus essential to have a holistic evaluation of the effects of OAW on any species.

1.5 A model species: the European sea bass (*Dicentrarchus labrax*)

1.5.1 European sea bass ecology and fisheries

The European sea bass is a coastal marine fish of key interest not only for its economic and cultural value in Europe but also for ecotoxicology and evolutionary studies. The European sea bass is a temperate species with an extended geographical distribution (Pickett & Pawson 1994). The Atlantic species can be found from Morocco to Norway, in the Black Sea and in the Mediterranean Sea (Pickett & Pawson 1994). More recently, it has even been reported spawning along the North of the Norwegian coast and in the Baltic sea, consequence of climate change according to the authors (Bagdonas et al. 2011). Sea bass can experience depth greater than 225 m but are more common in shallow waters (de Pontual et al. 2019). It inhabits coastal waters and can be encountered in estuarine areas and coastal lagoons, and occasionally rivers. Sea bass can thus tolerate a wide range of conditions in terms of temperature and salinity and as such is considered as an euryhaline (0-40 ppt salinity, Eroldoğan et al. 2004) and eurytherm (2-32°C, Pickett & Pawson 1994) fish. Shallow bays like estuaries and lagoons offer shelter and abundant food for juvenile stages (Jennings and Pawson 1992; Pickett & Pawson 1994; Perez-Ruzafa & Marcos 2014) (Figure 1.9). Fish undergo metamorphosis and subsequent period of growth (~4 years) in these nurseries (Jennings & Pawson 1992). These apparently ideal areas are, however, characterized by high environmental variability associated with high metabolic costs likely to affect reproduction success (Perez-Ruzafa & Marcos 2014). Therefore, during winter, mature sea bass migrates

from coastline to deeper offshore pre-spawning grounds where temperature is more stable (Pickett & Pawson 1994; Perez-Ruzafa and Marcos 2014). The European sea bass exhibits sexual growth dimorphism and maturity occurs at three to four years for males, and four to five years for females (Perez-Ruzafa & Marcos 2014). Spawning periods are regulated by temperatures with the reproduction taking place between December and March in the Mediterranean and between March and June in the Atlantic Ocean. In average 200 000 eggs kg-1 are spawned per female and eggs are fertilized externally. Sea bass eggs are pelagic and hatch after three to five days. Hatching size of larvae is about 4 mm. Larvae reach post-larval stage at about 22 mm after two to three months, time during which they slowly migrate to the inshore nursery areas due to decreasing temperatures in the coastal feeding grounds (Kennedy & Fitzmaurice 1968; Kennedy & Fitzmaurice 1972; Pickett & Pawson 1994). The European sea bass is characterized as a generalist top predator. Its diet is highly adaptable feeding mainly on Mysidacea, Amphipods at larval stage and opportunistically on a broad range of prey such as cephalopods, crustaceans and fish at the juvenile and adult stages (Pickett and Pawson 1994).

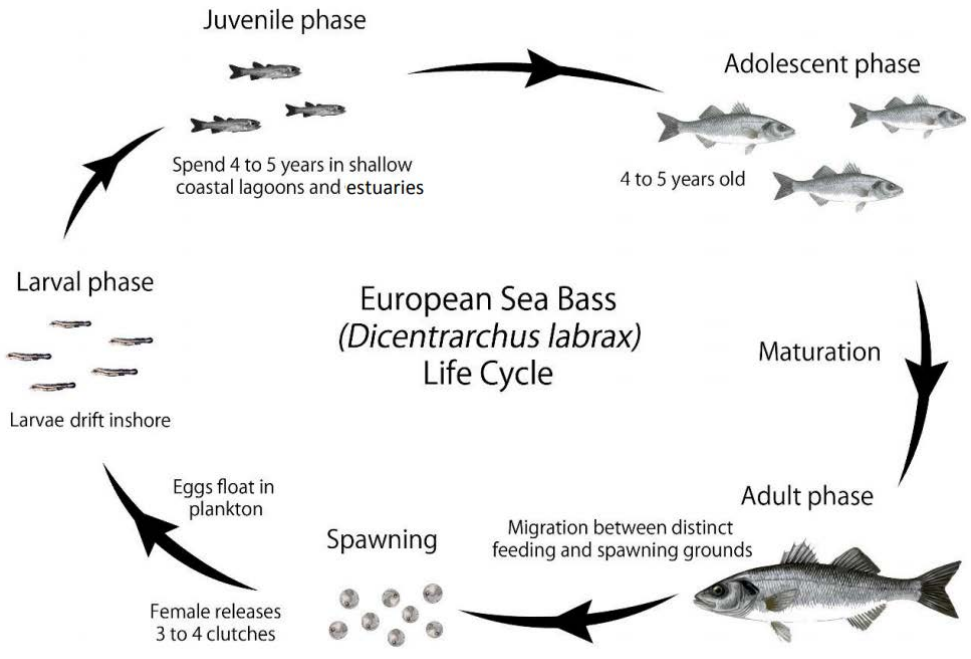

Figure 1.9 Life cycle of the European sea bass (*Dicentrarchus labrax*) (modified from Dando & Demir 1985; Pawson et al. 2007).

The European sea bass is one of the most abundant but concurrently the most exploited fish species found along the northeast Atlantic coast (Perez-Ruzafa & Marcos 2014). It is highly regarded for capture and recreational fisheries and is a highly productive species in aquaculture (Pickett & Pawson 1994; FAO 2010). Capture trend started in 1950 and increased exponentially to reach its maximum with 11 826 tones caught in 2003. Since 2005, sea bass stocks have declined, the total biomass reported between 2011 and 2012 being 32% lower than the total biomass recorded in the three previous years. Landings values counted 8 401

tones in 2014 and 5 751 tones in 2016 (FAO 2016). Recreational fisheries have a major share in capture fisheries, as it is estimated to account for 30% to 50% of the total catch in the Atlantic (ICES 2018). Sea bass is mostly caught in the North Sea and the English Channel by European fleet, with France reporting the highest catch, using pelagic trawls, seine and hooks lines. The fish is also caught in the Mediterranean Sea to a lesser extent, with largest catch originating from Italy and Egypt in recent years (FAO 2018). Despite its recent establishment, sea bass aquaculture, on the other hand, has shown an important upwards trend with an increase from 3 921 tones in 1990 to 156 449 tones in 2014. In 2016, sea bass production has accounted for 96% of the total fish production of the year (aquaculture 165 915 tons vs. fisheries 6919 tons in 2016; (FAO 2018). Sea bass was the first non-salmonid marine species domesticated and commercially cultured in Europe (Bagni 2005). The fish was historically farmed in coastal lagoons and tidal reservoirs before the start of intensive rearing trials in the early 1970s. In the beginning of the 1980s, mostly in France and Italy, the animal was fully domesticated with the control of reproduction and larval rearing (Bagni 2005; Chatain & Chavanne, 2009), and in 2000, more than 50 000 tons were produced. In 2010, annual production was about 120 000 tons and contrary to fisheries, production is established mainly in the Mediterranean area (FAO 2012).

Conditions for the domestication of sea bass are fully controlled enabling its rearing and maintenance in the laboratory. Sea bass has, thus, been the focus of several monographs (Barnabe et al. 1976; Pickett & Pawson 1994; Vázquez and Muñoz-Cueto 2014) providing thoughtful description of its biology. A significant amount of work has been done on its ecology (Handelsman et al. 2010; Pope et al. 2014; Bento et al. 2016), physiology (Claireaux and Lagardère 1999; Henderson et al. 2011), or nutrition (Kousoulaki et al. 2015; Parpoura & Alexis, 2001). Its facilitated rearing and extensive bibliography make the sea bass the suitable marine fish species model of the temperate latitudes. As such, sea bass has become, in the past decade, one of the first used specimen in academic ecotoxicology studies (Roméo et al. 2000; Claireaux et al. 2013).

1.5.2 European sea bass and climate change – the previous state-of-art

The European sea bass is an ectothermic species and as such its metabolism, physiology and behavior are largely influenced by the temperature of the environment. Temperatures influence sea bass metabolism, its metabolic scope and active metabolic rate increase distinctly from 10°C to 20°C, and reach an optimum between 20-24°C (Claireaux & Lagardère 1999). Thus, when confronted to temperatures below 10°C, a reduction in the metabolic scope in sea bass appeared likely to cause conflicts in energy budget and subsequently, increased mortality risks which may explain the northern distribution limit (Claireaux & Lagardère 1999; Henderson et al. 2011), but maintained between 20-24°C sea bass are likely to display maximum performance, at least in adults.

The impact of temperatures in sea bass has been investigated on a number of fitness traits starting with growth (Alliot et al. 1983; Koumoundouros et al. 2001; Person-Le Ruyet et al. 2004; Vagner et al. 2007). The Mediterranean sea bass fingerlings had a higher growth rate, food consumption and feeding conversion rate at elevated temperatures (22°C) than at lower ones (15°C) (Alliot et al. 1983), and maxima for growth rate, feed intake and feed efficiency in juveniles have been suggested to be between 24-27.5°C by (Person-Le Ruyet et al. 2004). Similarly, in the European sea bass, the development of eggs, and skeletal development during the larval phase, was significantly accelerated when fish were exposed to high temperatures (Koumoundouros et al. 2001; Vagner et al. 2007). A number of studies also reported effects of temperatures in sea bass development (Sfakianakis et al. 2006; Georgakopoulou et al. 2007). Indeed, in addition to increasing the rate of development, it influenced the apparition of malformations. Temperatures have reported to impact frequency of deformation of the branchiostegal rays (Georgakopoulou et al. 2007) and column anomalies such as lordosis (Sfakianakis et al. 2006).

Changes in temperature can alter the species performance and for example its ability to swim (Koumoundouros et al. 2002, 2009; Claireaux et al. 2006, 2007; Leis et al. 2012). Sea bass are strong swimmers (Pickett & Pawson 1994), maximum swimming speed is linked to active metabolic rate and as such strongly influenced by temperature. Swimming speed is reduced at low temperatures, maximum speed is observed at 22-24°C corresponding to the temperature optimum for metabolic scope in juveniles (Claireaux et al. 2006). Swimming speed has been measured in larvae acclimated to different temperatures (Leis et al. 2012) but the optimum for swimming capacity for this ontogenic stage is yet to be determined.

Until now, only a couple of studies have investigated the impact of ocean acidification (OA) on the European sea bass. Sea bass larvae were shown to be largely robust. Despite a small decrease in growth when exposed to 980 µatm PCO_2, larvae displayed an increase in survival and a faster bone mineralization with a reduction in macroscopic deformities at the highest OA condition (1520 µatm PCO_2). Whereas gene expression and the development of the digestive track appeared unaffected by low or high levels of partial pressure of CO_2 (PCO_2) (Crespel et al., 2017). Behavioral responses to OA have been explored on juveniles, reporting a high resilience of sea bass to future OA scenarios. Individuals reared at high level of acidification (1000 µatm) exhibited similar patterns of activity (movement or stillness), boldness (exploration of the arena) and level of interaction with neighbors than when reared at ambient condition (585 µatm) (Duteil et al. 2016). Both studies suggest that sea bass is relatively capable of coping with projected conditions of OA.

Combined effect of ocean acidification and warming (OAW) (RCP8.5) on sea bass survival, development morphology and metabolism have been investigated by Pope et al. (2014). Larval mortality decreased with elevated temperature, elevated level of PCO_2 and their interaction. Temperature affected morphology of the larvae, animals displayed a larger eye diameter and a decrease in carbon-nitrogen ratios suggesting higher developmental stage, but

PCO_2 did not (Pope et al. 2014). After metamorphosis, fish exposed to high levels of acidification and temperatures turned out to be heavier with a lower aerobic scope and juveniles maximum metabolic rate was under high temperature (Pope et al. 2014). The effects of OAW on sea bass, however, remained largely understudied.

AIMS AND THESIS OUTLINE

The aim of the present work was to **evaluate the potential for a warm temperate fish species, the European sea bass, to cope with the combined effect of temperature and acidification**. While there is some understanding of single stressor effects, whether it is elevated levels of partial pressure of CO_2 (PCO_2) or temperature, the comprehension of multi-stressors effects is far more complex and there is a necessity of addressing some gaps in knowledge and shortcomings. This dissertation thus tries to obtain reliable quantitative physiological data, under realistic acidification and warming scenarios, to assess the ecology of a commercial with a long-lived species. This has been realized first through a short term-experiment examining the responses of a number of physiological features and secondly via a long-term study considering the potential for acclimation and adaptation to ocean acidification and warming (OAW).

In Chapter 2, my co-author and I **explored the potential synergistic effects of temperature and acidification on** traits determinant of the Darwinian fitness, **growth and swimming performance**. Understanding how these fundamental parameters are affected gives an insight on the ecology of the fish in the new environment. We also compared our results to previous literature and briefly review data existing on the effects of ocean acidification (OA), ocean warming (OW) and OAW on the swimming performance in fish. Changes in swimming capacity were tied with potential trade-offs and changes in energy partitioning.

The fish larval stage represents a transitional sensitive period; we were thus surprised to found that while temperature influences the swimming capacity of the fish, OA has little to no effect on sea bass larvae fitness. **In Chapter 3**, the co-authors and I **assessed thus the effect of OAW on other aspects of the development at a sub-organismal levels.** Using a staining technique we first evaluated otoliths growth and formation. Subsequently we examined substantial changes in skeleton structure by following calcification rates and comparing the frequency of deformities among treatments.

During the first short experiment individuals were fed *ad libitum*. Since trade-offs were observed in Chapter 2, we wondered if **the significant food supply, and thus the supply in energy, was possibly minimizing the effect of OAW** and hiding significant energy trade-offs. Therefore **in Chapter 4**, a three-factorial experiment was conducted, integrating different feeding levels, to examine if food availability helps the individuals to cope with the combined stressors. The potential interaction between OAW and food ration was investigated at the organism level but also by exploring underlying mechanisms likely to explain differences in growth

After assessing physiological responses in a number of traits through short-term experiments we tried to realize a more holistic assessment of the future of the species by looking into the potential for acclimation and adaptation. Indeed, there is an obvious scarcity in knowledge regarding potential for transgenerational acclimation to OAW, especially in species with a long

life-span. In Chapter 5, the co-authors and I tested if parental conditioning to elevated PCO2 and a short exposure to warm temperature offsets negative effects of the new environment on the swimming performance.

List of Manuscripts and Contributions

The chapters of this doctoral thesis are based on the following manuscripts:

1. **Combined effects of ocean acidification and temperature on larval and juvenile growth, development and swimming performance of European sea bass (*Dicentrarchus labrax*).** Louise Cominassi, Marta Moyano, Guy Claireaux, Sarah Howald, Felix C. Mark, José-Luis Zambonino-Infante, Nicolas Le Bayon, Myron A. Peck (2019). Plos ONE 14, 9.

2. **Combined effects of ocean acidification and temperature alter the calcification of inner body structures in larval European sea bass (*Dicentrarchus labrax*).** Louise Cominassi, Marta Moyano, Guy Claireaux, Sarah Howald, Costantino Parisi, Felix C. Mark, Myron A. Peck. (Manuscript draft)

3. **Food availability modulates the combined effects of ocean acidification and warming on fish growth.** Louise Cominassi, Marta Moyano, Guy Claireaux, Sarah Howald, Felix C. Mark, José-Luis Zambonino-Infante, Myron A. Peck (2019; submitted to Scientific Reports).

4. **Transgenerational tolerance to the effects of ocean acidification and warming in larval European sea bass.** Louise Cominassi, Guy Claireaux, Marta Moyano, Sarah Howald, Felix C. José-Luis Zambonino-Infante, Mark, Myron A. Peck. (Manuscript draft)

Author	Contributions
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Marta Moyano	Conceptualization, Data Curation, Software, Supervision, Validation, Writing – Review & Editing
Guy Claireaux	Conceptualization, Funding Acquisition, Project Administration, Supervision, Validation, Writing - Review & Editing
Sarah Howald	Data Curation, Investigation, Writing - Review & Editing
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CHAPTER 2: Fish fitness in response to acidification and warming

Combined effects of ocean acidification and temperature on larval and juvenile growth, development and swimming performance of European sea bass (*Dicentrarchus labrax*).

Louise Cominassi[1], Marta Moyano[1], Guy Claireaux[2], Sarah Howald[1,3], Felix C. Mark[3], José-Luis Zambonino-Infante[4], Nicolas Le Bayon[4], Myron A. Peck[1]

Ocean acidification and ocean warming (OAW) are simultaneously occurring and could pose ecological challenges to marine life, particularly early life stages of fish that, although they are internal calcifiers, may have poorly developed acid-base regulation. This study assessed the effect of projected OAW on key fitness traits (growth, development and swimming ability) in European sea bass (*Dicentrarchus labrax*) larvae and juveniles. Starting at 2 days post-hatch (dph), larvae were exposed to one of three levels of partial pressure of CO_2 (PCO_2) (650, 1150, 1700 µatm; pH 8.0, 7.8, 7.6) at either a cold (15°C) or warm (20°C) temperature. Growth rate, development stage and critical swimming speed (U_{crit}) were repeatedly measured as sea bass grew from 0.6 to ~10.0 (cold) or ~14.0 (warm) cm body length. Exposure to different levels of PCO_2 had no significant effect on growth, development or U_{crit} of larvae and juveniles. At the warmer temperature, larvae displayed faster growth and deeper bodies. Notochord flexion occurred at 0.8 and 1.2 cm and metamorphosis was completed at an age of ~45 and ~60 days post-hatch for sea bass in the warm and cold treatments, respectively. Swimming performance increased rapidly with larval development but better swimmers were observed in the cold treatment, reflecting a potential trade-off between fast grow and swimming ability. A comparison of the results of this and other studies on marine fish indicates that the effects of OAW on the growth, development and swimming ability of early life stages are species-specific and that generalizing the impacts of climate-driven warming or ocean acidification is not warranted.

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2.1 Introduction

Over the last 150 years, the burning of fossil fuels has contributed to an increase in atmospheric CO_2 from approximately 280 to 410 ppm and a further increase (730 to 1020 ppm) is anticipated by the end of 2100 (Meehl et al. 2007; IPCC 2014). This increased concentration of atmospheric CO_2 has enhanced greenhouse warming around the globe at a rate of ~ 0.2°C per decade in the past 30 years (Hansen et al. 2006) and, after dissolving in the oceans, it is causing ocean acidification (OA). During the 20th century, the pH of ocean surface waters has decreased by 0.1 pH units and a further reduction of 0.3-0.5 pH units is expected to occur by the end of the present century (Caldeira & Wickett 2005). These changes in partial pressures of CO_2 (PCO_2), pH and temperature of the oceans have impacted the distribution, physiological performance, morphology and behavior of marine organisms (Kroeker et al. 2013). The effect of the interaction between OA and warming (OAW) on marine flora and fauna is difficult to predict, especially since impacts are often life stage- and species-specific, and ocean warming (OW) could either offset (Mcculloch et al. 2012) or aggravate impacts of OA.

Gaining a mechanistic, physiologically-based understanding of how ocean acidification and warming (OAW) affects marine flora and fauna is essential for reliable projections of future effects of climate change (Hollowed et al. 2013). Studies examining the consequences of OA on marine organisms have mainly focused on calcifying invertebrates (Byrne 2011; Mostofa et al. 2016) with far fewer studies conducted on fish (Catalán et al. 2019). In fish, accumulating bicarbonate is a classical response aimed at regulating acid-base balance when the internal milieu acidifies. The net increase of HCO_3^- in plasma occurs in exchange for Cl^-, predominantly at the gills (Brauner & Baker 2009), but this HCO_3^- / Cl^- exchange eventually reaches a species-specific threshold beyond which acid-base regulation may occur at the expense of internal ionic/osmotic balance (Cameron & Iwama 1987). Juvenile and adult fish have efficient acid-base and osmo-ionic regulatory systems and are particularly tolerant to environmental hypercapnia and acidification (Heuer & Grosell 2014). Young larvae, however, have not yet developed full regulatory capacity and, consequently, they are expected to be more sensitive to changes in internal PCO_2 and pH (Pörtner & Peck 2010; Hurst et al. Mathis, 2013). This appears to be the case as several studies have reported decreased survival and/or abnormal development in marine fish larva exposed to OA (Baumann et al. 2012; Flynn et al. 2015; Sswat et al. 2018) and behavior can be impacted via impaired sensory abilities such as olfaction (Munday et al. 2009). Within the thermal tolerance window, warming increases rates of biochemical reactions and, thus, overall energy requirements and oxygen demand. After a certain thermal threshold is exceeded, however, deterioration in cellular activities occurs, resulting in reduced tissue and organismal-level performance. Decrements in performance may be due to the limited capacity of the oxygen transport chain to sustain temperature-driven increases in ATP production by mitochondria (Pörtner & Farrell 2008; Hofmann & Todgham 2010; Leo et al. 2017). It might also be explained by the 'multiple performances – multiple optima' MPMO) hypothesis, which posits that each physiological activity has its own

thermal optimum which can shift with life stage and the nature of the thermal challenge (Clark et al. 2013).

In early life stages of marine fish, growth and development lead to improvements in locomotor performance, a fundamental trait that influences food acquisition, predator avoidance and habitat connectivity (e.g. between spawning sites and larval nursery areas) (Leis 2006). Hence, locomotor performance is a key determinant of Darwinian fitness. Critical swimming speed (U_{crit}; Brett 1964) is a popular measure of swimming performance, estimating the athleticism of fish. The U_{crit} is also a well-established index to monitor the ontogeny of swimming performance in marine fish larvae, especially in tropical species (Fisher et al. 2010). Moreover, U_{crit} has been frequently used to evaluate the effects of environmental factors (e.g. temperature, dissolved oxygen concentration, presence of toxins and pathogens) on the physiological performance of fish (Beamish 1978). Therefore, when examining the effects of OA and/or OW on organismal-level performance, examining U_{crit}, along with growth and development, provides an integrated measure of physiological impact with clear ecological relevance.

The European sea bass (*Dicentrarchus labrax*) is one of the most important commercial and recreational fish species in the Northeast (NE) Atlantic and Mediterranean Sea, and potential sensitivity to stressors can negatively impact the productivity of this species and its fisheries (Torrecillas et al. 2007). Due to its importance as an aquaculture target, standard rearing protocols exist for rearing sea bass early life stages, and swimming performance has been well measured, including inter-individual variability and repeatability (Guy Claireaux & Lefrançois 2007). We examined the effects of OAW on the somatic growth, development and swimming capacity throughout the larval and early juvenile phase of sea bass reared at two temperatures (15°C and 20°C) and three PCO_2 levels (650, 1150, 1700 µatm; pH 8.0, 7.8, 7.6). We compared our results with previous studies conducted on sea bass and critically reviewed the literature published on the effects of OA, OW, and OAW on the swimming ability of marine fish early life stage.

2.2 Materials and Methods

The present work was performed within Ifremer-Centre de Bretagne facilities (agreement number: B29-212-05). Experiments were conducted according to the ethics and guideline of the French law and approved by the governmental ethics committee of the Brittany region (Comité d'Ethique Finistérien en Experimentation Animal, CEFEA, registering code C2EA-74) (Authorization APAFIS 4341.03).

2.2.1 Animals and experimental conditions

2.2.1.1 Water parameters

The larvae and post-larval juveniles were incubated within 6 different ocean acidification and warming (OAW) treatments. The acidification conditions included three different CO_2 partial pressures (PCO_2). For the control treatment, the targeted level of CO_2 was set to approximately 650 µatm, today's ambient situation in coastal waters of Brittany (Pope et al. 2014; Duteil et al. 2016), with an annual mean PCO_2 level of 603 µatm (range 284-888 µatm) in the Bay of Brest, in 2014 (Salt et al. 2016). Climate projections indicate that the oceans will reach about 1000 µatm PCO_2 in the next 130 years (IPCC 2014; Caldeira & Wickett 2005; Doney et al. 2009). A second treatment was based on the IPCC Representative Concentration Pathway (RCP) 8.5 scenario projecting a ΔPCO_2 of ~500 µatm above current values (labelled Δ500, approx. 1150 µatm) (IPCC 2014). Sea bass juveniles and adults are usually found in coastal waters and estuaries where the impact of PCO_2 might be exacerbated (Pickett & Pawson 1994; Lonthair et al. 2017). Wallace et al. (2014) reported PCO_2 values >2000 µatm in northeast US estuaries, while values up to 3000 µatm were recorded in coastal areas of the SW Baltic Sea (Melzner et al. 2012). Based on those data, and PCO_2 values of European estuaries provided by Frankignoulle et al. (1998), a higher CO_2 treatment of Δ1000 µatm from ambient level (labelled Δ1000, approx. 1700 µatm) was applied representing CO_2 condition encountered occasionally by the adults and may become more common under future climate. Acidification conditions were crossed with two temperatures: a 'cold' (ambient condition) and a 'warm' (global warming) treatment. Under ambient condition, larvae were reared at 15°C and juveniles experienced 15 to 18°C (natural, seasonal differences reflecting ambient summer conditions in the Bay of Brest (see http://marc.ifremer.fr/en/results/temperatureandsalinity/mars3dchannelbayofbiscaymodel/(typevisu)/map/(zoneid)/sudbzh#appTop) (Chauvaud et al. 2001; de Pontual et al. 2019). In the warm treatment, larvae were reared at 20°C and juveniles experienced 20 to 23°C (5°C warmer than the cold treatment). The 5°C increase was based on the 'business-as-usual (RCP 8.5) scenario predicted by the general circulation models (GCMs) by the end of the century (IPCC 2007). We applied constant temperatures for larvae and seasonally changing temperatures for juveniles to better depict thermal conditions experienced by these life stages in the wild. Due to relatively fast rates of growth and development, sea bass larvae experience less differences in temperature compared to juveniles. Larvae hatch offshore, and then juveniles enter estuaries in the late spring and grow in these waters through the summer months (Vinagre et al. 2012; Anastasiadi et al. 2017).

The sea water was pumped from a depth of 20 m approximately 500 m from the coastline in the Bay of Brest, passed through a sand filter (~500 µm), heated (tungsten, Plate Heat Exchanger, Vicarb, Sweden), degassed using a column, filtered using a 2 µm membrane and finally UV sterilized (PZ50, 75W, Ocene, France) assuring high water quality. Replicate treatment tanks (n = 3 for larval rearing and n = 2 for juvenile rearing) were supplied with sea water via header tanks where water PCO_2 was controlled using an IKS Aquastar system (IKS

Computer Systeme GmbH, Germany). This design used interdependent treatment replicates which was corrected using tank as a random factor in the analysis (Cornwall & Hurd 2016). The system continuously measured water pH and controlled a solenoid valve connected to a CO_2 cylinder. This valve controlled the amount of CO_2 injected into the header tank water, which supplied the fish rearing tank. Temperature and pH were checked daily with a WTW 3110 pH meter (Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW Sentix 41, NBS scale) before feeding the fish. The pH meter as well as the IKS Aquastar system were calibrated daily with NBS certified WTW technical buffers pH 4.01 and pH 7.00 (Xylem Analytics Germany, Weilheim, Germany). Total alkalinity was measured once a week following the protocol of Anderson & Robinson (1946), and Strickland & Parsons 1972): a 50 ml sample of filtered tank water (200 µm nylon mesh) was mixed with 15 ml HCl (0.01 M) and pH was measured immediately. Total alkalinity was then calculated with the following formula:

$$TA = \frac{V_{HCl} \cdot c_{HCl}}{V_{sample}} - \frac{(V_{HCl} + V_{sample})}{V_{sample}} \cdot \frac{\{H^+\}}{\gamma_{H^+}} \quad \left[\frac{mol}{l}\right]$$

With: TA – total alkalinity [mol * l^{-1}], V_{HCl} – volume HCl [l], c_{HCl} – concentration HCl [mol * l^{-1}], V_{sample} – volume of sample [l], H^+ – hydrogen activity (10^{-pH}), γ^{H+} – hydrogen activity coefficient (here $\gamma^{H+} = 0.758$).

The Microsoft Excel macro CO2sys (Lewis et al. 1998) was used to calculate seawater carbonate chemistry, the constants after (Mehrbach et al., 1973) (as cited in CO2sys) refit by (Dickson & Millero 1987) (as cited in CO2sys), were employed. Values of pH are presented on the free proton concentration scale (pH_{free}) (Waters & Millero 2013). Oxygen saturation (WTW Oxi 340, Xylem Analytics Germany, Weilheim, Germany) and salinity (WTW LF325, Xylem Analytics Germany, Weilheim, Germany) were measured once a week together with total alkalinity, from juvenile stage onwards (Table 2.1).

2.2.1.2 Animals

Larvae were obtained from an aquaculture facility (Aquastream, Ploemeur-Lorient, France) at 2 days post-hatch (dph) (20.01.2016). Brood stock fish were caught in the sea off Morbihan, France. Four females (mean weight 4.5 kg) were crossed with ten males (mean weight 2.4 kg) which spawned naturally using photothermal manipulation. Conditions in the aquaculture facility during breeding were as followed: 8h45 light and 15h15 darkness, 13°C, 35 psu, pH 7.6. Spawning of eggs took place on 15.01.2016; larvae hatched on 18.01.2016 and were transported to our laboratory facilities on 20.01.2016.

Table 2.1 Water parameters during the larval (L) and juvenile (J) rearing done in this study. Larval period from 21.01.2016 (3 days post-hatch (dph)) until 04.03.2016 (46 dph) and 18.03.2016 (60 dph) for warm (W) and cold (C) life condition respectively; juvenile period until 24.10.2016 (280 dph) and 08.02.2017 (387 dph) for warm (W) and cold (C) life condition respectively. Values show mean ± SE overall replicate tanks per condition. Temperature (Temp.) and pH (free scale) were measured daily; salinity and total alkalinity (TA) and oxygen weekly; PCO_2 was calculated with CO2sys. Inflow sea water (SW) parameters were measured in 2017 and 2018 and annual average values are shown. A, Ambient PCO_2 (650 µatm); Δ500, ambient + 500 µatm CO_2; Δ1000, ambient + 1000 µatm CO_2.

Treatment	pH_{Free} (-)	Temp. (°C)	Salinity (psu)	O2 (% airsat.)	TA ()	PCO_2 (µatm)
L C A	7.95±0.01	15.3±0.0	33.0±0.1	-	2364±17	656±16
L C Δ500	7.77±0.01	15.3±0.0	33.0±0.1	-	2382±19	1041±26
L C Δ1000	7.58±0.00	15.3±0.0	33.0±0.1	-	2394±26	1682±26
L W A	7.88±0.01	20.0±0.1	33.1±0.1	-	2369±21	832±13
L W Δ500	7.79±0.01	20.0±0.1	33.1±0.1	-	2383±22	1057±30
L W Δ1000	7.60±0.01	20.0±0.1	33.1±0.1	-	2380±23	1672±33
J C A	7.97±0.01	16.0±0.2	34.2±0.1	90.9±0.5	2396±18	655±18
J C Δ500	7.75±0.01	16.0±0.2	34.2±0.1	92.2±0.6	2404±19	1107±21
J C Δ1000	7.55±0.01	16.1±0.2	34.2±0.1	90.9±0.6	2399±19	1841±40
J W A	7.92±0.01	21.9±0.2	35.0±0.2	90.2±0.9	2418±12	788±22
J W Δ500	7.78±0.01	21.8±0.2	35.0±0.2	90.5±0.7	2420±15	1133±43
J W Δ1000	7.59±0.01	21.9±0.2	35.0±0.2	91.3±0.6	2423±12	1808±65
SW cold	8.05±0.01	14.5±0.5	33.0±0.2	101.2±0.6	2434±21	522±18
SW warm	7.95±0.02	21.2±0.4	32.7±0.1	102.3±1.4	2433±28	723±33

Larval rearing was performed in a temperature-controlled room using black, 35-L tanks initially stocked with *ca*. 5000 larvae $tank^{-1}$ in order to accommodate our sampling design. Allocation among experimental tanks took place at 3 dph (21.01.2016). During the following three days, the temperature for the warm condition was increased 1°C during the first day and 2°C during each of the following days. The PCO_2 conditions were applied directly after fish allocation to the experimental treatments. Starting at 7 dph (mouth opening), larvae were fed with live brine shrimp (*Artemia salina)* nauplii, hatched from High HUFA Premium cysts (Catvis, AE's-Hertogenbosch, Netherlands). From 7 to 16 dph a concentration of ~ 120 nauplii per larva day^{-1} was delivered, after 16 dph concentration was ~800 nauplii per larva day^{-1}. Until 33 dph, larvae were fed newly hatched (24-h old) nauplii. Older larvae were fed with nauplii enriched with cod liver oil and dry yeast for 24 h. The nauplii were transferred from their storage tanks (one per temperature condition) to the larval rearing tanks using peristaltic pumps at ad libitum feeding concentrations continuously during 6 hours. Larvae experienced a 15-h photoperiod (7:00 to 22:00), the light intensity progressively increased with larval age, from total darkness to 96 lux according to S2.1 Table. Median larval mortality

(n = 18 tanks) was 30%, see S2.2 Table. Flow rate through the larval tanks was 0.18 L min^{-1}, corresponding to a water exchange of 30% per hour, and organic compounds were removed from the tanks using a protein skimmer.

Fish were moved from the larval to juvenile tanks at 50 dph and 65 dph for fish reared in the warm and cold life conditions, respectively. Fish were counted, and all individuals from replicate tanks at one condition (temperature x PCO_2) were pooled in the same tank for about three weeks and then randomly allocated to two 670-L treatment tanks (S2.1 Figure). Having only two replicates limited our ability to estimate variance but dividing the fish randomly removed any potential effect of larval rearing tank. Mortality of 24.8 to 43.4% occurred after loading to the juvenile tanks, likely due to handling stress (S2.3 Table). Juveniles were fed daily with commercial fish food (Neo Start) (Le Gouessant, Lamballe, France). Food was distributed ad libitum via automatic feeders. Size and amount was adjusted all through the juvenile rearing period, as recommended by the supplier. This amount was calculated according to the tank biomass, number of fish and temperature. Around 200 dph, for example, 90 g tank-1 day-1 and 160 g tank-1 day-1 of food pellets was distributed for the 15°C-reared fish (mean biomass ~ 6.9 kg) and the 20°C-reared fish (mean biomass ~ 4.2 kg), respectively. Photoperiod was adjusted each week to mimic natural conditions. Uneaten food and feces were siphoned from tanks each day (after pH measurements). Water flow rates maintained oxygen saturation levels above 90%.

2.2.2 Swimming tests

2.2.2.1 Larvae

Swimming tests and morphological measurements were performed from 15 dph until the end of the larval stage i.e., when the caudal fin was completely formed. Swimming experiments were conducted on larvae from the six treatments (PCO_2 x T) conditions. In each swimming trial, 3 randomly selected larvae from each replicate tank (9 larvae per treatment) were measured. Trials were conducted every 3 to 5 days, and 6 to 7 trials were conducted per treatment.

All measurements of U_{crit} were performed on an individual larva swimming in one lane (24 x 3 x 2.5 cm) of a custom-made Brett-type flume (Stobutzki & Bellwood 1997). Water was pumped (universal, EHEIM, Germany) from a header tank into the flume and velocity was adjusted using a valve calibrated to water flow rate. A laminar flow was made by passing the water through a honeycomb section (length = 10 cm) placed upstream and a mesh screen was located at the downstream end of the lane. Pilot trials with dye ensured that cross-sectional water velocity in the lane was homogenous. Treatment water conditions of the tested larva were maintained in the flume. Temperature was controlled using a cooling/heating system

(Tr10, TECO, Italy) and PCO_2 was maintained by injecting CO_2 directly in the water of the header tank via a gas diffuser.

A larva was introduced into the swimming lane and was acclimated to the lowest water velocity for 5 min (Table 2.2). The water velocity was then increased at a rate of 0.5 BL s^{-1} every 3 min until the larva was unable to swim against the flow and drifted to the downstream mesh screen. At the beginning of each trial, the average length of the fish in each replicate tank was determined so that a standard velocity increment could be established (among all trials). Larvae swam in the middle of the chamber suggesting minimal or no wall effects. Once the test was completed, the larva was euthanized with an overdose of anesthetic (Tricaine methane-sulfonate MS222, PharmaQ Limited, Hampshire, United Kingdom, as prescribe by the European legislation to minimize fish stress), digitally photographed under a stereomicroscope (Leica MZ 16, Wetzlar, Germany) and stored in 4% formalin. Body length (BL), body height (BH) and tail flexion angle, were measured using ImageJ (Rasband 2014). For preflexion larvae, BL was equal to the notochord length, which corresponded to the length from the tip of the snout to the end of the notochord. For flexion and postflexion larvae, BL was determined by measuring standard length, corresponding to the distance from the tip of the snout to the posterior end of the hypural plate. The notochord angle was also measured to estimate the size at which larvae reached the postflexion stage. Unfortunately, due to the small sample size of larvae < 10 mm in length (n=45 for 15°C and n=54 for 20°C), it was not possible to run a logistic regression to calculate the mean larval size at which 50% of the larvae completed flexion. Instead, we determined the size at which postflexion was first observed.

2.2.2.2 Juveniles

Juveniles from four treatments were tested: cold and warm temperatures at both ambient PCO_2 (650 µatm) and RCP8.5 (Δ1000; 1700 µatm). When tested, juveniles in the cold and warm treatments were 242 and 233 dph and had a mean (± s.e.m) BL of 83.2(0.8) and 119.9(1.2) mm, respectively. For each treatment, thirty individuals (~ 15 per replicate tank) were tested. For each run, fish were tested in groups of 8 or 5 individuals, for 15°C and 20°C- reared fish, swimming in a 46 x 14 x 14 cm chamber of the Loligo® Systems swim tunnel (Denmark). A streamline and homogenous flow was maintained using honeycomb section (confirmed by dye tests). Fish were tested at their treatment water conditions (see section 2.2.1.1). Water velocity was calibrated with a vane-wheelflow meter (HFA, Höntzsch GmbH, Germany), and controlled by an AC motor (Santo et al. 2017). Acclimation time was 30 min (Table 2.2).

Table 2.2 Summary of the methodology used during the critical swimming trials with larval (L) and juvenile (J) European sea bass. Abbreviations: BL, body length; C, cold treatment; W, warm treatment; A, Ambient PCO_2 (650 µatm); Δ500, ambient + 500 µatm CO_2; Δ1000, ambient + 1000 µatm CO_2.

Treatment	BL (mm)	Age (dph)	Total larvae tested (n)	Acclimation period	Water flow steps (cm s^{-1})	Time steps (min)
L C A	6.67 - 16.69	21 - 59	58	5 min at 0.8-1.6 cm s^{-1}	0.4 - 0.8 (0.5 Bls)	3
L C Δ500	7.45 - 16.83	21 - 58	51			
L C Δ1000	7.82 - 17.97	22 - 58	59			
L W A	7.55 - 16.66	17 - 46	54	5 min at 0.8-1.6 cm s^{-1}	0.4 - 0.8 (0.5 Bls)	3
L W Δ500	7.61 - 17.41	18 - 45	55			
L W Δ1000	6.35 - 17.35	18 - 45	54			
J C A	83.2±7.1	239 - 242	30	30 min at 7 cm s^{-1}	2.8	10
J C Δ500			30			
J W A	119.9±9.3	233 - 238	30	30 min at 7 cm s^{-1}	5.6	10
J W Δ500			30			

Fish were considered to be exhausted when they were up against the downstream grid for 5 consecutive seconds. Without interrupting the flow, these individuals were then removed from the tunnel, via a hatch located above the grid, anesthetized, measured (BL) and transferred to a recovery tank before being returned to their rearing tank where they were maintained for future research. For juveniles, BL is equivalent to standard length. The corresponding time and water velocity was recorded. The test was completed when all 5 fish were removed from the swim chamber.

2.2.2.3 U_{crit} measurement

The U_{crit} (cm s^{-1}) was calculated using the equation provided by Brett (1964), which adds the velocity of the most recently completed increment to the product of the incremental increase in velocity and the proportion of the final increment completed before fatigue. No correction for the solid blocking effect of the fish was considered, as the total cross-sectional area of the fish did not exceed 5% of that of the swimming chamber (Bell & Terhune 1970) (Table 2.2). Larvae which did not orientate themselves to start swimming during the acclimation period were removed from the test and dataset.

2.2.3 Statistical analysis

Differences in growth (in BL) across treatments were analyzed, in larvae, with a linear mixed model that included fixed effects (age, temperature, PCO_2) and random effects (tank). Similarly, differences in BH were also tested with a linear mixed model (BL, temperature and PCO_2 as fixed effects, tank as a random effect). When no effect of ocean acidification (OA) was observed, data were pooled across OA treatments and regressions were calculated on pooled data. In larvae, inter-individual variability in U_{crit} was large and increased with body size, thus, we used quantile regression to estimate the maximum U_{crit}–at-size (i.e. maximum swimming capacity) across treatments (Cade & Noon 2003). A backward model selection procedure was used to identify variables (e.g. size, temperature, PCO_2) influencing maximum U_{crit} starting with the most complex model (including all interactive effects among fixed factors) and ending with only significant factors. Models were fit to the upper 85 to 95% quantiles (in 1% steps) to ensure patterns were consistent, and treatment differences were tested with an ANOVA. Model residuals were tested for a potential effect of rearing tank with an ANOVA. All quantile regression analyses were done with the "quantreg" package in R. Normality and homoscedasticity of data were tested using Shapiro-Wilk and Levene tests, respectively. The effects of temperature and PCO_2 levels on the percentage (%) of larvae with the ability / choice to swim was tested using two-way ANOVAs after logit transformation of the data. Larvae not swimming already at acclimation speed were excluded from the other analysis. The effect of PCO_2 on juvenile U_{crit} was tested using one-way ANOVA when assumption of normality (Shapiro-Wilk) and homoscedasticity (Levene test) were met (case for 20°C-reared fish after log10 transformation). ANOVA included PCO_2 as a fixed effect and run as a random effects. When one or more of these assumptions was not met (for 15°C-reared fish), a generalized linear mixed-effects model (GLMM) was performed, including fixed effect (PCO_2) and random effects (run). All statistical analyses were performed using R (version 3.4.1, R Core Team 2014).

2.3 Results

2.3.1 Larval growth and development

The mean (± SE) growth rate of sea bass larvae at ambient, Δ500, Δ1000 (650, 1150, 1700 µatm of CO2 partial pressures (PCO_2); pH 8.0, 7.8 and 7.6) was, 0.17 (0.01), 0.17 (0.01) and 0.16 (0.01) mm d^{-1}, respectively, at 15°C and 0.21 (0.02), 0.21 (0.02) and 0.28 (0.03) mm d^{-1}, respectively, at 20°C. Larval growth rate was significantly higher at 20°C compared to 15°C ($p < 0.001$), but there was no significant effect of PCO_2 treatment ($p = 0.120$) (Figure 2.1).

Larval BH increased linearly with BL (Figure 2.2) and this relationship was significantly impacted by temperature ($p< 0.001$) but not PCO_2 ($p= 0.805$). For example, 10-mm larvae at 20°C had a 16% larger BH than those reared at 15°C (mean BH of 1.63 and 1.37 mm, respectively). These morphological differences were related to a faster development at the

warmer temperature. At 15°C, notochord flexion was completed between 9.0(±0.3; mean±SE) and 10.8(±0.1), 8.7(±0.2) and 11.1(±0.5), and 9.8(±0.2) and 11.4(±0.2) mm BL at ambient, Δ500, and Δ1000 PCO_2, respectively. At 20°C, notochord flexion was completed between 7.8(±0.1) and 9.6(±0.3), 7.9(±0.1) and 8.9(±0.1), and 7.0(±0.2) and 9.4(±0.1) mm BL in the ambient, Δ500 and Δ1000 PCO_2 treatments, respectively. Unfortunately, the sample size of <12 mm BL larvae was too small to conduct further analyses.

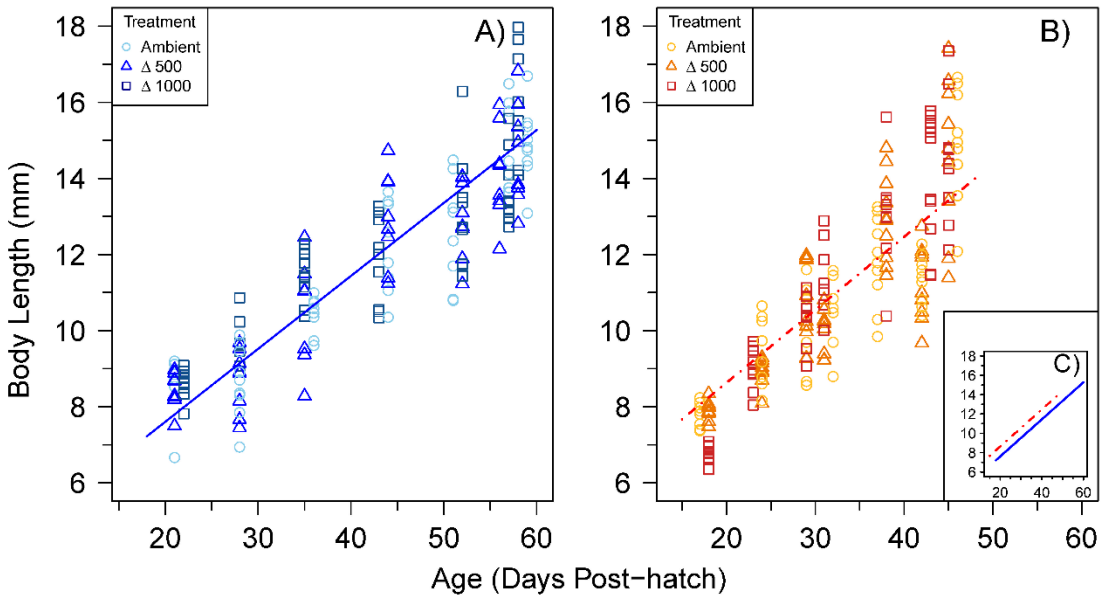


Figure 2.1 Body length (BL) with age, in days post-hatch, of European sea bass larvae reared at A) cold condition (15°C) and B) warm condition (20°C). Symbols and colors indicate the PCO_2 treatment (A, Ambient PCO_2 (650 µatm); Δ500, ambient + 500 µatm CO_2; Δ1000, ambient + 1000 µatm CO_2). Regression (mean ± SE parameter estimates) are included: 15°C, (n=180) BL = 017(0.01)*Age + 4.62(0.28), R^2 = 0.80, $p < 0.001$; 20°C (n=190) BL = 0.23(0.01)*Age + 3.44(0.36), R^2 = 0.73, $p < 0.001$). For clarity, both regression lines are compared in subpanel **C**) (insert).

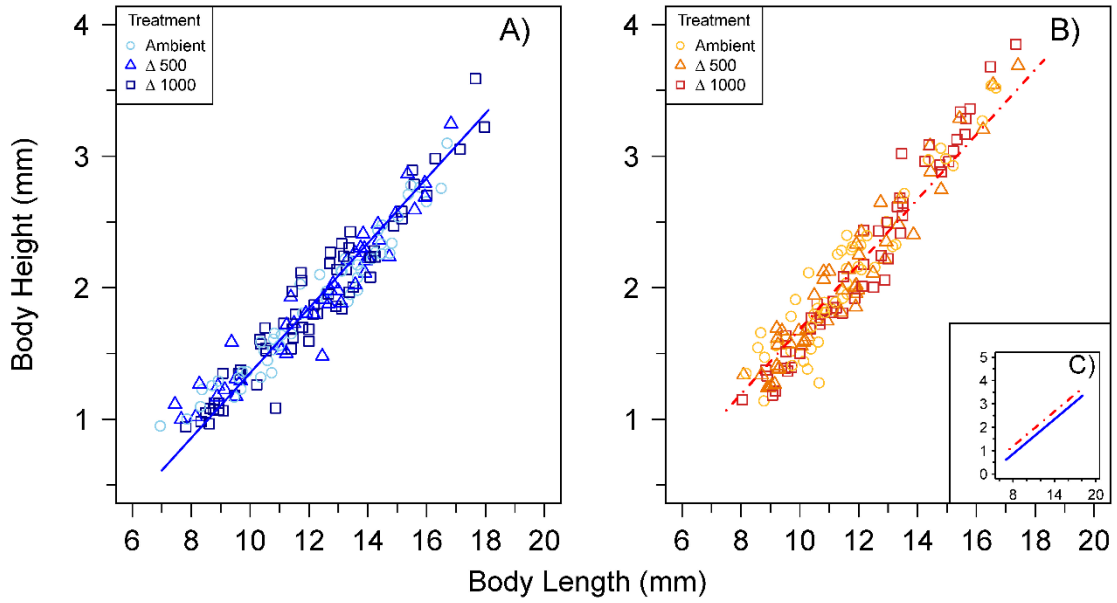


Figure 2.2 Body height (BH) versus body length (BL) of European sea bass larvae reared at A) cold condition (15°C) and B) warm condition (20°C). Symbols and colors indicate the PCO_2 treatment (A, Ambient PCO_2 (650 µatm); Δ500, ambient + 500 µatm CO_2; Δ1000, ambient + 1000 µatm CO_2). Regression (mean ± SE parameter estimates) are included: 15°C, (n=160) BH = 0.22(0.00)*BL - 0.83(0.06), $R^2 = 0.93$, $p < 0.001$; 20°C (n=190) BH = 0.28(0.01)*BL -1.14(0.07), $R^2 = 0.93$, $p < 0.001$). For clarity, both regression lines are compared in subpanel **C**) (insert).

2.3.2 Swimming capacity of larvae

The U_{crit} and inter-individual differences in U_{crit} of sea bass larvae increased with increasing BL (Figure 2.3). The final quantile regression model (90th percentile) reported significant effects of BL, temperature and their interaction (S2.4 Table), but no significant effect of PCO_2 (ANOVA; $p = 0.94$). There was no significant tank effect on the final model (ANOVA, $p = 0.402$). The U_{crit} increased faster with BL in cold (15°C) versus warm (20°C) larvae and, at the end of the larval stage (16 mm BL) was 15.1 and 12.2 cm s^{-1} at 15 and 20°C, respectively.

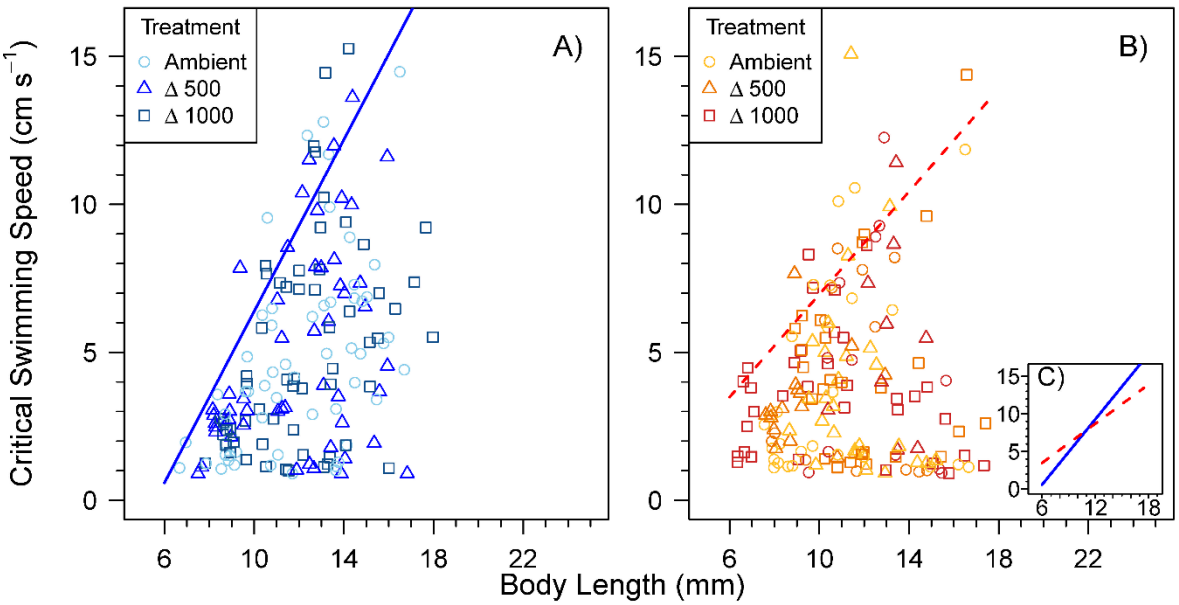


Figure 2.3 Ontogeny of critical swimming speed (U_{crit}, cm s $^{-1}$) in larvae of European sea bass reared at A) cold condition (15°C; n=168) and B) warm condition (20°C; n=163). Symbols and colors indicate PCO_2 levels treatment (A, Ambient PCO_2 (650 µatm); Δ500, ambient + 500 µatm CO_2; Δ1000, ambient + 1000 µatm CO_2). The solid blue (cold) and dashed red (warm) lines shows the maximum U_{crit} as defined by the 90th percentile (see text). For clarity, both lines are compared (see insert panel **C**).

In the 15°C treatment, the coefficient of variation (CV) of U_{crit} increased from 35.6 to 57.2% between notochord flexion (BL = 9.0 ± 0.2 mm) and post-flexion (BL = 12.9 ± 0.3 mm) stages. At 20°C, the same life stages (BL = 9.1 ± 0.1 and 12.6 ± 0.2, respectively) had CVs of 46.9 to 73.1%. Some larvae were not able (or chose not) to swim beyond the minimum water velocity used during the acclimation period (0.8 cm s^{-1} for 5 min). The percentage of larvae not swimming was significantly higher at 20°C compared to 15°C (ANOVA, p = 0.007, Figure 2.4).

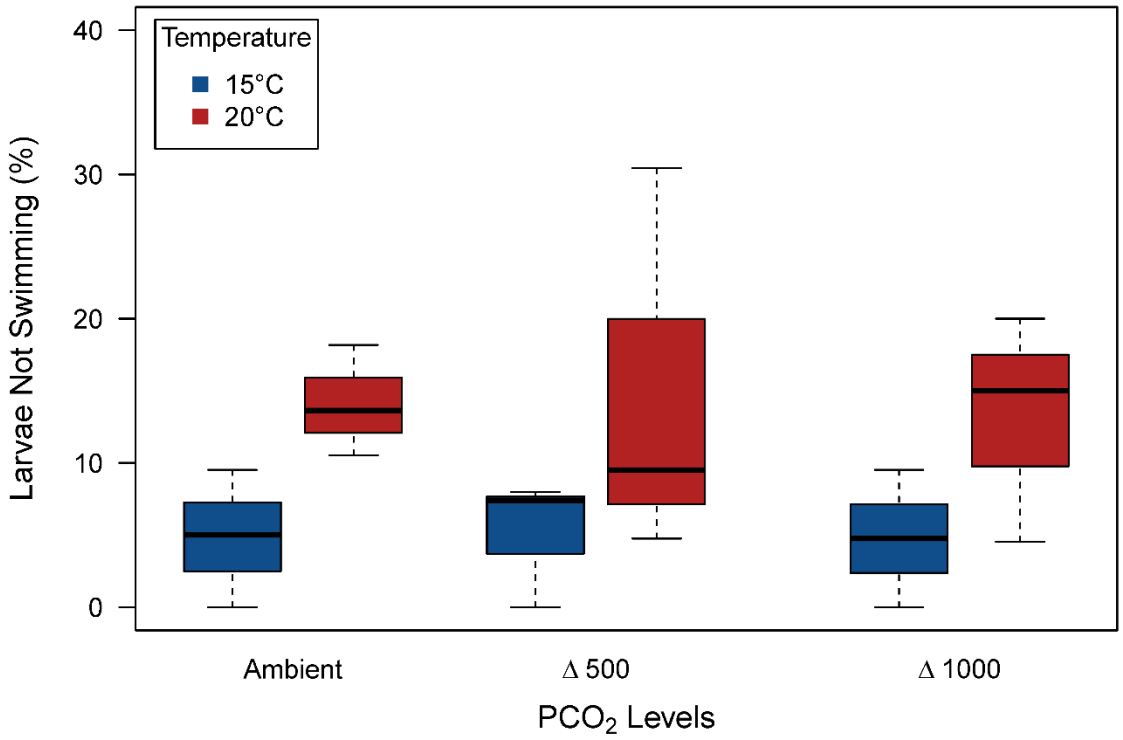


Figure 2.4 Proportion (in %) of European sea bass larvae not swimming during the U_{crit} trial in the cold (15°C) and warm (20°C) treatments at three PCO_2 levels (Ambient PCO_2 (650 µatm); Δ500 = ambient + 500 µatm CO_2; Δ1000, ambient + 1000 µatm CO_2).

2.3.3 Swimming capacity of juveniles

The mean (± SE) U_{crit} of juveniles reared in cold and warm conditions was 66.7 (0.7) and 79.2 (1.5) cm s^{-1}, respectively. There was no significant effect of PCO_2 treatment on U_{crit} measured in cold (GLMM, p = 0.562) and warm conditions, respectively (ANOVA, p = 0.518). Although a group of 5 fish was tested in each trial, individuals became exhausted at different water velocities and our protocol allowed us to collect individual-level data. Large inter-individual differences were observed in U_{crit}, particularly among juveniles reared at warmer temperatures. Body sizes of fish tested in the cold and warm conditions differed, precluding direct comparison between temperatures (Figure 2.5, S2.2 Figure).

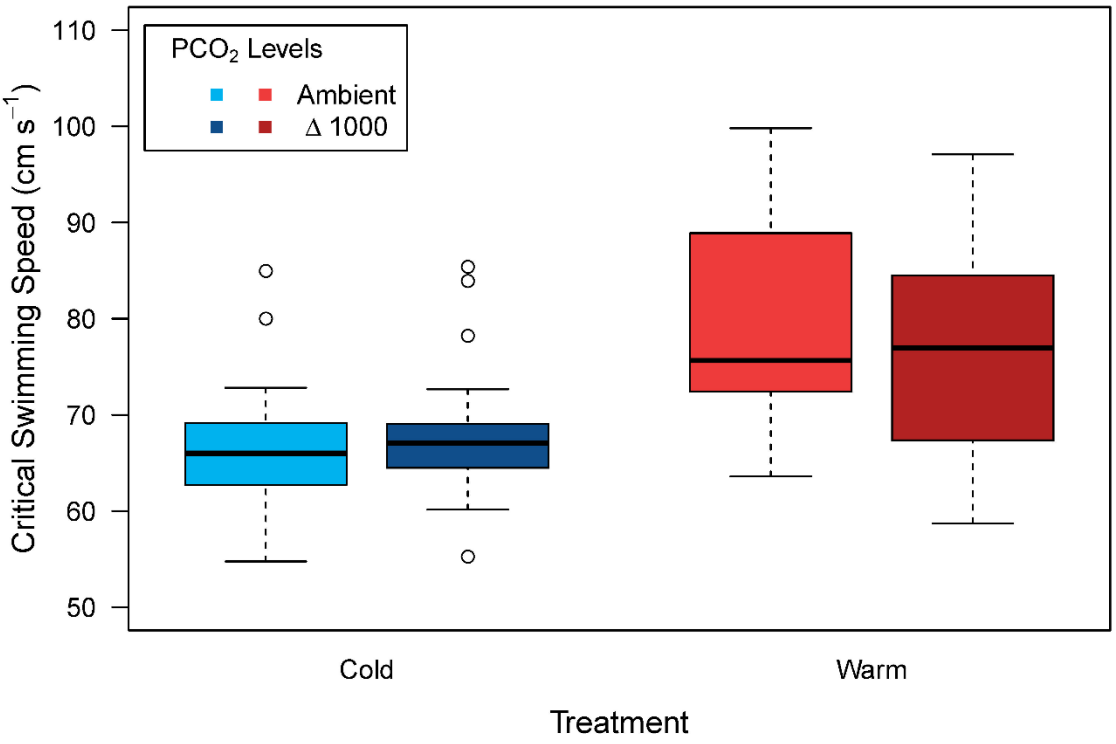


Figure 2.5 Critical swimming speed (U_{crit}, cm s^{-1}) in 233 to 242 day post-hatch juvenile European sea bass in the cold (15°C) and warm (20°C) treatments at two *P*CO_2 levels: Ambient *P*CO_2, 650 µatm (n=40 and n=30, for cold and warm treatment, respectively), and Δ1000, ambient + 1000 µatm CO_2) (n=33 and n=30, for cold and warm treatment, respectively). The raw data are shown in S2.2 Figure.

2.4 Discussion

Recent studies have examined the impact of ocean acidification (OA) and ocean acidification and warming (OAW) on early life stage of teleost growth and survival (Baumann et al. 2012; Flynn et al. 2015). Large gaps in knowledge, however, persist, particularly on how long-term exposure to OAW may influence the performance of fish larvae and young juveniles. We investigated the effect of OAW on growth, development and swimming ability of European sea bass throughout larval and early juvenile ontogeny. Our results suggest that temperature, but not elevated CO_2, influenced growth, development and swimming performance. Our results also revealed a trade-off between swimming capacity and fast growth at warm temperatures, casting new light on the determinants of larval survival, dispersal, settlement pattern and recruitment (Fisher et al. 2010; Leis & McCormick 2002; Sponaugle et al. 2002).

2.4.1 Effect of ocean acidification

Juvenile and adult European sea bass inhabit shallow estuaries where physicochemical parameters such as temperature and levels of partial pressure of CO_2 (PCO_2) strongly fluctuate over short (diel) and longer (seasonal) time scales (Ringwood & Keppler 2002). Thus, these life stages experience substantially more variation in water PCO_2 (e.g. daily variation up to 1 pH unit, Hofmann et al. (2011)) compared to early life stages. Measurements made between 1992 and 2004 within inner estuaries of the Loire and Gironde Rivers were 452 to 2780 µatm and 612 to 2829 µatm, respectively (Borges et al. 2006). On the other hand, sea bass larvae develop in offshore waters where PCO_2 levels are generally stable (annual variation of <0.1 pH units (Doney et al. 2009). Taking this into account, it was expected that increased levels of PCO_2 might negatively affect the growth and/or swimming ability of larvae due to poorly developed acid-base regulation and the need to partition energy between homeostasis-related mechanisms and ecologically important activities.

Contrary to our expectations, the results of the present study indicated that life-long exposure to OA had no significant effect on the somatic growth rate and swimming capacity (U_{crit}) of sea bass larvae. A previous study on sea bass larvae, using similar exposure, rearing protocols and OA levels (Ambient, Δ500 and Δ1000 (PCO_2 = 650, 1150 and 1700 µatm)), also found no effect of OA on somatic growth at 19°C (Mehrbach et al. 1973). These findings are in accordance with those reported in a recent meta-analysis conducted by (Cattano et al. 2018), highlighting no overall effects of high CO_2 on growth. A few studies, however, have reported impacts of OA on growth. For example, OA was associated with a higher growth rate in the clown anemonefish (*Amphiprion percula*) larvae (Munday et al. 2009) but slower growth rates were reported for gilthead seabream (*Sparus aurata*) (Pimentel et al. 2016) and decreased length-at-hatch for inland silversides (*Menidia beryllina*) (Baumann et al. 2012).

Similar to our findings for growth rate, we also found no significant effect of OA on swimming capacity in sea bass larvae and juveniles. These results are in line with those in a number of other studies testing routine swimming characteristics (Maneja et al. 2013; Maneja et al. 2015) or U_{crit} (Melzner et al. 2009; Munday et al. 2009; Silva et al. 2016) in marine fish larvae exposed to OA (Table 2.3). For example, (Munday et al. 2009) highlighted that swimming speed (U_{crit}) of the clown anemonefish (*Amphiprion percula*) larvae was unaffected by future OA scenarios. Similarly, Bignami et al. (2014) did not observed a significant effect of high acidification on the swimming performance (U_{crit} and mean routine swimming speed) of larval cobia (*Rachycentron canadum*) and mahi-mahi (*Coryphaena hippurus*). Although most studies on marine fish early life stages suggest that the physiological attributes related to swimming performance are not substantially impacted by levels of PCO_2 projected for the end of this century, some studies have reported impaired swimming duration and orientation (Pimentel et al. 2014) and reduced U_{crit} as well as the average speed in response to a stimuli (Watson et al. 2018) (Table 2.3). Indeed, U_{crit} was significantly lower, in juvenile yellowtail kingfish (*Seriola lalandi*) exposed to high (Δ500) PCO_2. This reduction in U_{crit} values may be linked to 1) reduced

motivation to swim and/or 2) reduced physiological performance, two traits that may differ according to individual variation (Watson et al. 2018). It is worth noting that high inter-individual variability in U_{crit} was observed across all treatments (CVs of 57 and 73% at 15 and 20°C, respectively, for 9 to 13 mm BL). This large inter-individual variability, however, is generally observed in swimming studies in sea bass (Claireaux & Lagardère 1999; Nelson and Claireaux 2005; Claireaux et al. 2007). One hypothesis is that these large differences in swimming ability (e.g. maximum 10-fold in 15 mm larvae) also reflect variation in other behavioral traits such as boldness or willingness to swim. Using U_{crit} as a performance test allowed us to compare swimming performance among treatments but also highlighted that results may not only reflect physiological limitation but also potentially inter-individual differences in behavior. Our results, together with these studies, highlight the species-specific nature of the responses to OA and the need for continued mechanistic (physiological-based) studies of potential impacts and the importance of publishing of studies that report no significant effects.

The fish used in the present study were the progeny of wild-caught adults acclimatized and maintained for about 5 years in the Aquastream aquaculture facility. These fish spawned at a pH of 7.6 which corresponds to our highest CO_2 treatment. A number of studies have reported that parental exposure to an elevated level of CO_2 may decreased the sensitivity of their progeny to OA (Munday 2014; Murray & Baumann 2018; Jarrold & Munday 2019). In our study, therefore, the absence of differences in growth and swimming capacity observed among the three PCO_2 treatments could be the result of transgenerational plasticity. According to Griffith & Gobler (2017), however, the exposure of parents to a stressor (such as low pH) can also increase the sensitivity of their offspring to that stressor and that transgenerational plasticity is highly species-specific.

Table 2.3 Summary of published studies investigating the impact of ocean warming (OW), ocean acidification (OA) and their combined effect (OAW) on swimming performance of early life stages (larvae, and juveniles) of marine fishes.

Species		Stressor	Measurement	Life stage	T (°C)		PCO_2 (µatm)		Results	Ref
Common name	Scientific name				Cont	Treat	Cont	Treat		
Anemonefish	*Amphiprion melanopus*	OW	U_{crit}	L	28	25	ambient	-	Colder T induced slower development of swimming capacity	(1)
		OAW	RSB	J	28.5	30, 31.5	420	530, 960	Elevated T alone reduced food consumption and foraging activity, combined with high PCO_2 these behaviors were increased	(2)
	Amphiprion percula	OA	U_{crit}	L	30	-	400	550, 750, 1030	No effect	(3)
Cobia	*Rachycentron canadum*	OA	RSB/ U_{crit}	L	ambient	-	400	3500, 5400	No effect	(4)
Dolphinfish	*Coryphaena hippurus*	OA	RSB/ U_{crit}	L	ambient	-	400	770 to 2100	No effect	(4)
		OA	RSB	L	26	-	457	1671	Swimming duration and vertical orientation frequency decreased with elevated PCO_2	(5)
Yellowtail kingfish	*Seriola lalandi*	OAW	RSB/ U_{crit}	J	19.5	21, 25	589.4	462, 538.3, 959.8, 1010.6	Ucrit and escape performances are enhanced by elevated T. High PCO_2 reduced U_{crit} and distance moved after stimuli	(6)

(1) Green & Fisher (2004)*, (2)* Nowicki et al. (2012)*, (3)* Munday et al. (2009)*, (4)* Bignami et al. (2013)*, (5)* Pimentel et al. 2014)*, (6)* Watson et al. (2018)

Species		Stressor	Measurement	Life stage	T (°C)		PCO_2 (µatm)		Results	Ref
Common name	Scientific name				Cont	Treat	Cont	Treat		
Meagre	*Argyrosomus regius*	OAW	RSB	L	20	24	350	1400	T increased the time spent swimming. Elevated PCO_2 decreased the time spent swimming and lower capture success	(7)
Gilthead seabream	*Sparus aurata*	OAW	RSB	L	18	22	350	1400	Elevated PCO_2 decreased the time spent swimming and lower capture success	(7)
Black seabream	*Spondyliosoma cantharus*	OA	RSB	L	23.7	-	356.8	777, 2051.5	Elevated PCO_2 decreased velocity and increased erratic swimming behaviors	(8)
Sand smelt	*Atherina presbyter*	OA	RSB	L	15.9	-	537.1	2080.6	No effect on routine swimming speed. Elevated PCO_2 increased the time to acquire shoaling behaviors and decreased laterization	(9)
		OA	U_{crit}	L	16.4	-	600	1000, 1800	No effect	(10)
Atlantic herring	*Clupea harengus*	OA	RSB	L	5 to 10	-	370	1800, 4200	No effect	(11)
Atlantic cod	*Gadus morhua*	OW	U_{crit}	L	6	10	ambient	-	U_{crit} decreased with elevated T before metamorphosis	(12)
		OA	RSB	L	7.2	-	370	1800, 4200	No effect	(13)
		OA	U_{crit}	J	5	-	528	3080, 5792	No effect	(14)

(7) Pimentel et al. (2016)*, (8)* Jiahuan et al. (2018)*, (9)* Lopes et al. (2016)*, (10)* Silva et al. (2016)*, (11)* Maneja et al. (2015)*, (12)* Guan et al. (2008)*, (13)* (Maneja et al. (2013)*, (14)* Melzner et al. (2009)

Species		Stressor	Measurement	Life stage	T (°C)		PCO_2 (µatm)		Results	Ref
Common name	Scientific name				Cont	Treat	Cont	Treat		
European sea bass	*Dicentrarchus labrax*	OW	U_{crit}	L	18	21	ambient	-	Swimming performance declined with temperature.	(15)
		OAW	U_{crit}	L, J	15	20	650	1150, 1700	U_{crit} decreased with elevated T in larvae. No effect of PCO_2.	This Study
Shorthorn sculpin	*Myoxocephalus scorpius*	OW	U_{crit}	L	3	6	ambient	-	U_{crit} increased with elevated T before metamorphosis	(12)

(15) Leis et al. (2012)*, (12)* Guan et al. (2008)

Abbreviations: T, temperature; U_{crit}, critical swimming speed; RSB, routine swimming behavior; L, larvae; J, juveniles; Cont, control; Treat, treatment.

2.4.2 Effect of temperature

Rearing at a warmer temperature (20 vs 15°C) resulted in shorter larval stage duration and accelerated growth rate. These results agree with previous studies suggesting that sea bass larvae fed ad libitum display increased growth as temperatures increase to 22°C (Koumoundouros et al. 2001; Vagner et al. 2007). Temperatures supporting the maximum growth rate in sea bass larvae have not been estimated, but they are close to 22 to 24°C in adults ~35 cm (Claireaux & Lagardère 1999).

The impacts of temperature on performance traits of developing larvae can be complex due to the potential for different thermal optima for different physiological activities (e.g. multiple performances – multiple optima (MPMO) hypothesis) (Clark et al. 2013) and/or changes in thermal tolerance/optima during ontogeny. Increases in U_{crit} with increasing temperature has been observed in the larvae of a variety of marine fish, including both tropical and temperate species, e.g. red and black anemonefish (*Amphiprion melanopus*) (Green & Fisher 2004), shorthorn sculpin (*Myoxocephalus Scorpius*) (Guan et al. 2008), yellowtail kingfish (*Seriola lalandi*) (Watson et al. 2018) and Atlantic herring (*Clupea harengus*) (Moyano et al. 2016). This increased swimming performance was linked to changes in morphology and developmental rates, as well as to a decrease in water viscosity at warmer temperatures, which is especially relevant for cold-temperate species (Fuiman & Batty 1997). An increase in U_{crit} with OW, however, is not expected to be universal but to be context- and species-specific (Lopes et al. 2016). This is the case with sea bass at the two temperatures tested in the present study. Just prior to metamorphosis and for the same body size, larvae reared at 15°C displayed better swimming ability than larvae reared at 20°C.

Thermal optima for performance measures such as U_{crit} may change during ontogeny. In larger juvenile and adult sea bass maximum scope for activity (MO_2max) is between 22 and 24°C, and coincides with optimal temperature for U_{cirt} (Claireaux & Lagardère 1999; Koumoundouros et al. 2002). Unfortunately no information is available on thermal performance curves for maximum scope for activity (MO_2max) nor U_{crit} in sea bass larvae, but this and previous studies suggest that it is below 20°C (Leis et al. 2012). Temperature-dependent shifts in swimming ability may reflect temperature-dependent changes in developmental physiology affecting body shape and skeletal structure and/or muscle characteristics (Johnston & Dunn 1987; Langerhans & Reznick 2009; Sfakianakis et al. 2013). In our study, for the same BL, larvae were more streamlined at 15 compared to 20°C. A streamlined body shape generally enhanced steady swimming while greater BH induced more irregular and more complex locomotor patterns (e.g. drastic changes in velocity or direction, fast-starts or rapid turns) (Langerhans & Reznick 2009) , the velocity of the 20°C-reared fish might then be affect by their rounder body shape. In addition, mitochondrial efficiency can decreased already at sub-lethal warm temperatures due to membrane associated problems (e.g. proton leak), with consequences for energy demanding processes like swimming. It might be that at 20°C, sea bass larvae are already exposed to a sub-lethal warm temperature likely

to affect mitochondrial structure and thus performance. Although, in a companion study (Howald et al. submitted), mitochondrial capacities for aerobic ATP production of permeabilized heart fibers were increased in juvenile sea bass reared at the warm versus the cold life condition. Juveniles have generally higher thermal optima than larvae, and this would indicate a rearrangement of biochemical pathways during ontogeny towards evolutionary thermal optima (colder for larvae than for juveniles).

2.4.3 Trade-off between growth and swimming performance

Faster-growing individuals have better chances of survival by spending less time in life stages particularly vulnerable to mortality due to predators or starvation (Anderson 1988; Bailey & Houde 1989; Pepin 1991; Ferron & Leggett 1994). Most fish, however, occur within habitats that are somewhat cooler than temperatures supporting maximum rates of growth (Sandblom et al. 2016). This could be due to the need for a thermal-safety margin to survive sudden warming such as heat waves (Sunday et al. 2014) or due to physiological trade-offs related to fast growth at relatively warm temperatures. For young larvae, rapid growth can lead to decrements in other fitness-related traits such as resistance to pathogens (Kirpichnikov et al. 1993; Fuiman & Batty 1997), longevity (Jonsson et al. 1991), energy storage (Forsman & Lindell 1991), or locomotion (Moyano et al. 2016). In our study, sea bass larvae grew faster at 20°C but displayed poorer swimming ability than larvae reared at 15°C. The existence of a trade-off between growth and swimming ability has been reported in an increasing number of studies. Studies on fathead minnow (*Pimephales promelas*) and Atlantic silversides (*Menidia menidia*) reported a negative correlation between growth rate and locomotory performance (Kolok & Oris 1995; Billerbeck et al. 2001). A negative correlation between growth (compensatory after food deprivation) and sprint speed was reported for juvenile sea bass (Handelsman et al. 2010; Killen et al. 2014).

The mechanisms behind the trade-off between fast growth and swimming ability have been poorly investigated, although several hypotheses have been proposed. First, the trade-off is based on the principle of energy allocation (Sibly & Calow 1986; Billerbeck et al. 2001). Rapid growth mobilizes more aerobic capacity leaving less energy dissipating capacity for locomotion (Conover & Schultz 1997; Billerbeck et al. 2001). Early developmental stages have a relatively low aerobic capacity with factorial metabolic scopes around 1.5 and, thus, may be especially vulnerable to energy allocation conflicts (Conover & Schultz 1997; Killen et al. 2007). Second, rapid growth in fish can influence muscle characteristics including changes in the cellular structure and composition of muscle fibers (Johnston et al. 2002) leading to poor locomotor performance. This may contribute to their higher swimming performance. Third, the relationship (and potential trade-off) between growth and swimming performance may have a genetic basis and can, therefore, be population-specific (Lankford et al. 2001). It would be interesting to study additional populations that inhabit different thermal regimes to test if trade-offs differ due to local adaptation.

Our results indicate that, when fed *ad libitum*, European sea bass larvae are not impacted by projected future increases in levels of PCO_2 thanks to the use of physiological mechanisms allowing them to maintain growth and swimming performance. Life-time rearing at +5°C above ambient influenced larval growth, development and swimming performance. Individuals reared at warmer condition grew faster but showed reduced swimming ability at metamorphosis. While growing faster reduced the duration of the larval phase, this reduction happened at the expense of swimming performance. Although the potential for local adaptation (adaptive capacity) is not known, our findings suggest that sea bass larvae of this Atlantic population may be negatively impacted by projected climate-driven warming (but not OA) under a business-as-usual (RCP8.5) scenario. These impacts appear to be due to physiological trade-offs between growth rate and U_{crit}. The physiological mechanism (i.e. limits in aerobic capacity and/or changes in energy partitioning) is unclear. The results of this and several other studies demonstrate that physiologically optimal thermal windows are stage-specific and appear more narrow (with a colder optimum) in larvae compared to juveniles. Finally, our study adds to the growing number of studies reporting no effects of OA or OAW on the swimming performance of marine fish larvae. Our review of this literature, however, also indicates that the impact of OA and/or OAW is species-specific.

2.5 Supplementary Information

2.5.1 Supplementary Tables

S2.1 Table. Light intensity during larval phase. Abbreviation: dph, days post-hatch.

Age (dph)	2	7	9	12	16	20	27	31	36	44
Light intensity (lux)	0	0-1	1	2	5	7	10	31	59	96

S2.2 Table. Larval mortality (%) in the different larval rearing tanks. Abbreviations: A, Ambient PCO_2; Δ500, ambient + 500 µatm CO_2; Δ1000, ambient + 1000 µatm CO_2; T, temperature, Rep, replicate tank.

	A			**Δ500**			**Δ1000**		
T (°C)	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
15	31.5	37.3	25.9	78.6	21.9	33.3	35.6	11.3	16.8
20	43.5	29.4	30.5	46.6	33.6	34.9	39.6	26.7	35.7

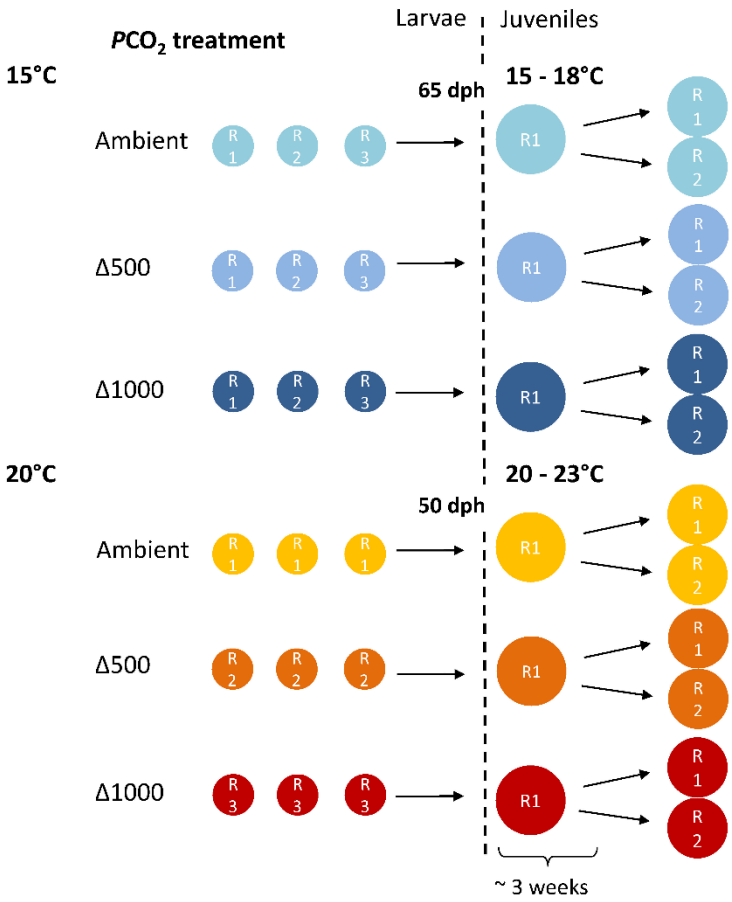
S2.3 Table. Juvenile mortality in % in the different tanks. Abbreviations: A, Ambient PCO_2; Δ500, ambient + 500 µatm CO_2; Δ1000, ambient + 1000 µatm CO_2; T, temperature.

T (°C)	**A**	**Δ500**	**Δ1000**
15	24.8	43.4	29.7
20	35.2	41.7	38.2

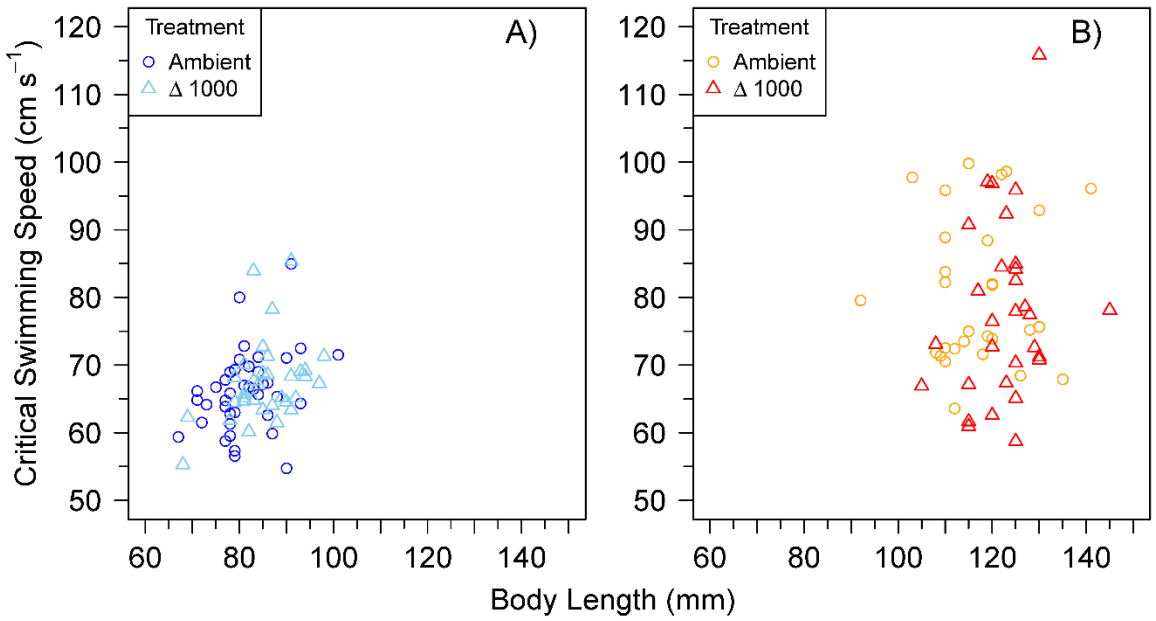
S2.4 Table. Significance of terms for the 90% quantile regression model on the impact of water temperature and body length (BL) on the critical swimming speed (U_{crit}) in European sea bass larvae. Abbreviation: DF, degrees of freedom.

	DF	**Value (± s.e.m.)**	**p-value**
Intercept	1	-27.25 (± 7.94)	<0.01
Body Length	1	3.19 (± 0.84)	<0.01
Temperature	2	1.28 (± 0.42)	<0.01
Body Length : Temperature	2	-0.12 (± 0.04)	<0.01

2.5.2 Supplementary Figures



S2.1 Figure. Experimental design and tanks transfer from larvae to juvenile stage. Abbreviation: dph, days post-hatch.



S2.2 Figure. Critical swimming speed (U_{crit}, cm s^{-1}) in juvenile European sea bass reared at A) cold condition (15°C; n=73; 242 days post-hatch (dph)) and B) warm condition (20°C; n=60; 233 dph). Symbols and colors indicate by PCO_2 levels treatment (A, Ambient PCO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2).

CHAPTER 3: Acidification and warming alter fish internal development

Combined effects of ocean acidification and temperature alter the calcification of inner body structures in larval European sea bass (*Dicentrarchus labrax*)

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Ocean acidification and warming are known to have far reaching consequences on marine organisms, especially calcifiers. While a number of studies have highlighted the negative impacts of temperature and acidification on the calcification rates of shell and reef building in marine invertebrates, dataset regarding potential effects on teleost fish is still scarce. Starting at 2 days post-hatch (dph), European sea bass (*Dicentrarchus labrax*) larvae were exposed to one of three levels of PCO_2 (650, 1150, 1700 µatm; pH 8.0, 7.8, 7.6) at either a cold (15°C) or warm (20°C) temperature. Otoliths morphometrics (i.e. area, perimeter, maximum diameter and circularity), skeleton calcification and the proportion of malformation were repeatedly assessed as sea bass grew from early postflexion stage to early juvenile. Exposure to different levels of PCO_2 alone had no significant effect on otolith and skeleton formation. Temperature, however, and the combined effects of temperature and elevated PCO_2 altered the elongation of the otoliths, increase the rate of ossification and the occurrence of vertebral malformation. Overall, this study suggests that teleost pelagic fish, just like invertebrate, are affected by the complex effect of acidification and warming with probable deleterious consequences on their ecology.

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3.1 Introduction

There is a growing realization that, in addition to climate and ocean warming (OW), anthropogenic activities have contributed to ocean acidification (OA). About 30% of the excess of all the anthropogenic CO_2 emitted in the atmosphere has been absorbed by the ocean (Quéré et al. 2015) causing a decline in pH and leading to OA (Caldeira & Wickett 2005). The interaction between OA and warming (OAW) is expected to have deleterious impacts on marine biota (Kroeker et al. 2013). OA is accompanied by a reduction in the saturation state of carbonate ions and the availability of calcium carbonate (CaCO3) polymorphs (aragonite, calcite and vaterite) (Doney et al. 2009) needed to build or even maintain shells and exoskeletons of marine calcifiers (Kroeker et al. 2013). Fish are internal calcifiers, and have several regulatory mechanisms to cope with changes in CO2 partial pressures (PCO_2). For example, juveniles and adults are capable of 1) precipitating CaCO3 in the intestinal lumen for water absorption and osmoregulation, and 2) actively regulating their acid-base balance through bicarbonate accumulation and ion exchange across the gills under hypercapnic conditions (Melzner et al. 2009). However, early life stages may be more sensitive to variations in PCO_2, as their physiological homeostasis is not fully developed (Pörtner and Peck 2010).

Early life stages of marine fish undergo drastic changes in their tissues and organs as they develop into juveniles, including the calcification of skeletal elements (critical for structure and swimming ability) and changes in the size and shape of otoliths (important for equilibrium and sound detection) (Checkley et al. 2009; Munday et al. 2011b). While the potential influence of OA on morphometric characteristics of otoliths has been fairly well studied (reviewed by Holmberg et al. 2019), relatively few studies have evaluated how OA and/or OAW may affect skeleton mineralization. These latter studies report contradictory results, from no effect (Munday et al. 2011b) to positive effects such as increased density and decreased malformations (Crespel et al. 2017; Di Santo Valentina 2019). This study investigated the single and interactive effects of OA, OW and OAW on the structural development (somatic and otolith growth, otolith morphology, skeleton ossification) of larvae and young juveniles of a temperate marine fish, the European sea bass (*Dicentrarchus labrax*).

3.2 Materials and methods

The present experiment was conducted at the Ifremer-Center facilities (agreement number: B29-212-05) and was approved by the local ethics committee of Brittany, France (Comité d'Ethique Finistérien en Experimentation Animal, CEFEA, registering code C2EA-74) (Authorization APAFIS 4341.03, permit number 2016120211505680.v3).

3.2.1 Animals and experimental conditions

Sea bass larvae were obtained from a wild broodstock kept at 13°C (Aquastream, northern France). Spawners included four females (mean wet weight = 4.5 kg) and 10 males (mean wet weight = 2.4 kg). Starting at 3 days post-hatch (dph), larvae were reared within one of the four ocean acidification and warming (OAW) conditions at the Ifremer facilities (Brest, France). The OAW treatments were "Ambient" corresponding to today's current situation in the Bay of Brest (approximately 650 µatm (cf. Pope et al. 2014) and high, "Δ 1000" µatm (approx. 1700 µatm; IPCC 2014) at both 15 and 20°C. The CO_2 partial pressure (PCO_2) treatments were based on current levels in European estuaries and high levels projected to become more frequent in the future (Frankignoulle et al. 1998; Melzner et al. 2013). Each treatment was replicated three times.

Sea water supply and quality was maintained as described by Crespel et al. (2017). The PCO_2 levels were established in the seawater in head tanks before flowing into the rearing tanks (flow rate: 0.18 L min^{-1}, corresponding to a water exchange of 30% hr^{-1}). The PCO_2 was regulated using an IKS Aquastar system (IKS Computer Systeme GmbH, Germany). Water temperature and pH were measured daily (WTW 3110 pH meter, Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW Sentix 41, NIST scale) and total alkalinity (TA) was measured weekly following Anderson & Robinson (1946) and Strickland & Parsons (1972) (Table 3.1). The pH meter and IKS Aquastar system were calibrated with NIST certified WTW technical buffers pH 4.01 and pH 7.00 (Xylem Analytics Germany, Weilheim, Germany).

At 3 dph, ~ 5000 larvae were distributed to each replicate tanks (35-l). The PCO_2 level was immediately established after transferring larvae into the treatment tanks. Changes in temperature, feeding regime and light regime were based on Gourtay et al. (2018) (Table S3.1). From 7 to 33 dph, larvae were fed with live brine shrimp (*Artemia salina*) nauplii, hatched from High HUFA Premium cysts (Catvis, AE's-Hertogenbosch, Netherlands) and enriched with cod liver oil and dry yeast for 24 h (>33 dph). Mean larval mortality (n = 18 tanks) was 30%, (S3.2 Table). After metamorphosis (50 dph at 15°C, 65 dph at 20°C), juveniles were randomly transferred to two replicate 670-l tanks per treatment. Although the reduction in replication limited our ability to estimate variation, randomly loading the tanks removed any effect of previous larval rearing tank. Up to 40% mortality was associated with this juvenile transfer (S3.3 Table). Juveniles were fed ad libitum rations of commercial fish food (Neo Start, Le Gouessant, Lamballe, France) using automatic feeders. Water flow rates maintained oxygen saturation levels above 90%. The light regime was adjusted each week to mimic natural conditions.

3.2.2 Sampling

Seven fish per tank (n=21 per treatment) were sampled at ca. 430, 905, 1200 and 1550 degree-days (dd, day × temperature). Larvae were anaesthetized with tricaine methanesulfonate (MS-222; dose adapted to water temperature and fish mass, typically 0.2 g l^{-1}) and stored for ~6 months in a solution of 4% formalin prior to measurement of ossification and skeletal malformation.

Ossification was based on (Darias et al. 2010) First, larvae were bleached and stained following the alcian blue-alizarin red double staining method. Alician blue stains cartilaginous tissues while alizarin red stained the bony structures (Crespel et al. 2017; Darias et al. 2010). Next, larvae were stored in 100% glycerol. Individuals were digitally scanned at a 3200 dpi (Epson Perfection 4990 Photo, Epson America, Long Beach, CA, USA) and images were analyzed using Photoshop CS based on pixel color. An ossification ratio was calculated for each larva based on the ratio of mineralized structures to cartilage components. Images were also used to determine body length (BL) (somatic growth) and skeletal malformation based on macroscopic observations of images of individuals >900 dd for i) visible distortion of the vertebral column (i.e., lordosis, scoliosis and kyphosis), ii) vertebral compression and fusion and iii) disproportion of the mouth such as prominence or retraction of the lower jaw.

Otoliths were obtained from the larvae studied for ossification. The left and right sagitta were dissected and photographed using an optical microscope equipped with a camera (model, etc). Otolith images were analyzed using ImageJ (Rasband 2014). The otolith area (μm^2), the perimeter (µm), the longest diameter (µm) and the circularity index ($4\pi \times (area/perimeter^2)$) were measured. Circularity values ranged from 0 to 1 with 1 indicating a perfect circle and 0 indicating an irregular shape.

3.2.3 Statistical analysis

Data normality (Shapiro-Wilk test) and homoscedasticity (Levene or Fligner-Killeen test) was tested. Growth data were checked for outliers using Grubbs. Linear regression (GLM) was used to estimate growth rate. The effects of temperature and PCO_2 levels were tested by comparing regression slopes; when slopes were homogeneous, an ANCOVA compared intercepts. Kruskal Wallis tested for treatments effects on otolith metrics and skeleton ossification and, when required, was followed by pairwise comparisons (Wilcoxon tests). Macroscopic deformities were calculated as a percentage for each treatment. Homogeneity of variance in the proportion of malformation was evaluated using Chi square test. The effect of rearing treatment and stage of development on jaw and vertebral malformation was tested using two-way ANOVAs after logit transformation of the data. Differences were considered significant at $\alpha = 0.05$. Statistical analyses were conducted using R (ver.3.3.3; R Development Core Team).

3.3 Results and discussion

The mean (± SE) growth rate of sea bass larvae in the ambient and Δ1000 treatments (650 and 1700 µatm CO2 partial pressures (PCO_2); pH 8.0, 7.6) at 15°C was 0.18 (0.01) and 0.21 (0.01) mm d^{-1}, respectively, and 0.35 (0.02) and 0.29 (0.01) mm d^{-1}, respectively, at 20°C (Figure 3.1). Growth rate was impacted by age (linear mixed model lm; $p < 0.001$), temperature (lm; $p < 0.001$), and the interaction between age and temperature (lm; $p < 0.001$), age and PCO_2 levels (lm; $p = 0.001$), temperature and PCO_2 levels (lm; $p = 0.035$) and the interaction between the three (lm; $p = 0.001$). While growth rates of individuals reared at 15°C were similar to findings from Chapter 2, growth values observed for 20°C-reared fish were almost 40% higher. This difference might arise from the dissimilarity in the sampling patterns (i.e. longer time between sample points, fewer individuals), as well as the time window of samplings. In Chapter 2 larval growth was assess only prior metamorphosis and in this study growth measurement were still conducted in juveniles (37 and 46 days after metamorphosis in the 15°C- and 20°C-reared fish, respectively). It is possible that growth accelerate in early juveniles and that the phenomenon is enhanced at warm condition.

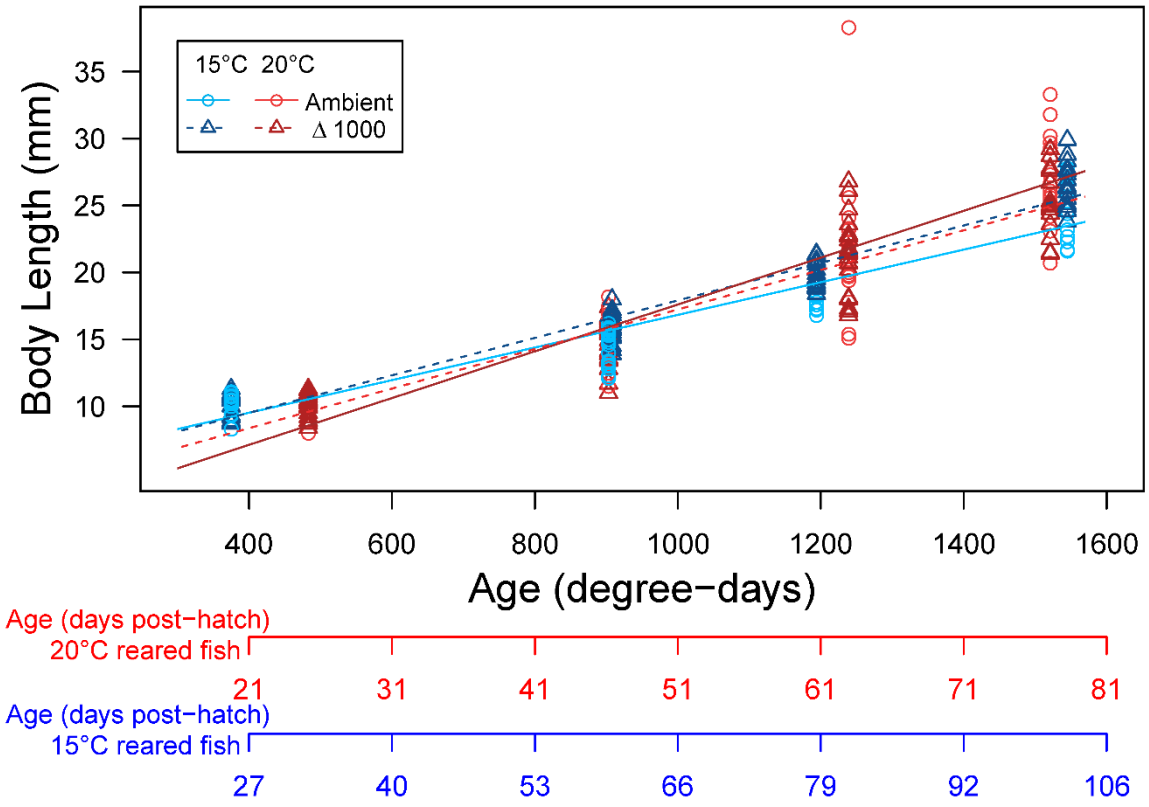


Figure 3.1 Relationship between body length (mm) and age (days post-hatch) of European sea bass early stages, color coded according to rearing temperature (15°C and 20°C). The different symbols represent the PCO_2 treatment (Ambient PCO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2). Regression for each condition are included and equations are shown in S3.4 Table.

No differences were observed in otolith morphology (i.e. area, perimeter, diameter, circularity) between the left and right sagittae. Although those findings agree with previous studies (Munday et al. 2011; Mu et al. 2015; Martins 2017), other studies have reported asymmetry in shape and size in teleost larvae exposed to ocean acidification (OA) (reviewed Holmberg et al. 2019). Otolith area was significantly impacted by temperature (Kruskal Wallis, $p<0.001$), age, and the interaction of temperature and PCO_2 (Kruskal Wallis, $p=0.015$) and of temperature, PCO_2 level and age (Kruskal Wallis, $p<0.001$) (Figure 3.2). Fish reared at 20°C had larger otoliths than those at 15°C, and this difference increased with age. Also, fish at high PCO_2 had larger otoliths than those at ambient, but only at 15°C. Such an increase in otolith area in response to OA has been reported in several studies (e.g. Coll-Lladó et al. 2018; Pimentel et al. 2014) but see (Mu et al. 2015).

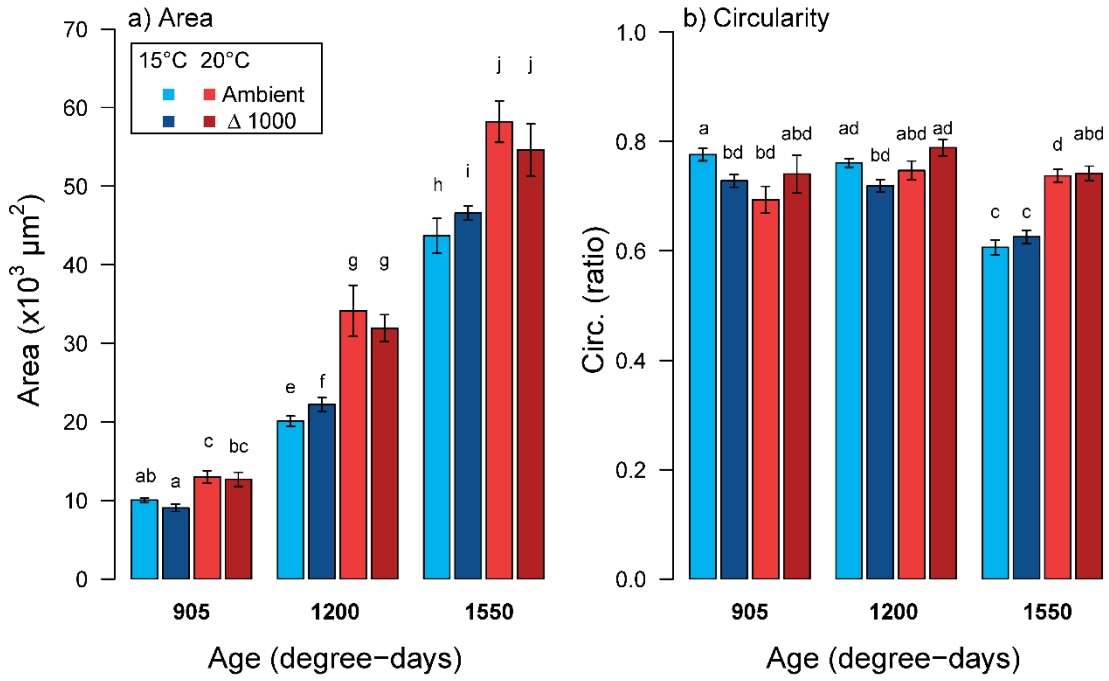


Figure 3.2 Mean (±SE; 18<n<23 per condition) of otolith metrics of European early stages reared at two temperature (15°C and 20°C) and two PCO_2 levels (Ambient PCO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2). Data include a) Area, and b) Circularity. Different letters denote significant differences (Pairwise Wilcoxon test, $p<0.05$) between each condition.

In nature, otolith shape changes with age, from round in young larvae to an elongate spheroid in juveniles and adults, as observed in anchovy (*Engraulis encrasicolus*) (Aldanondo et al. 2011). In the present study, the shape of sea bass otoliths was significantly influenced by physiological age (Kruskal Wallis, $p<0.001$) but significantly different ontogenetic patterns were observed between the two temperatures (Kruskal Wallis, $p<0.001$). For the 20°C-reared fish, otoliths area increased with fish BL and otoliths remained round (high index of circularity)

even after 1550 dd. For fish at 15°C, however, the circularity index decreased after 1200 dd (Figure 3.2; S3.1 Figure). In agreement with previous larval fish studies (e.g. Munday et al. 2011; Holmberg et al. 2019), PCO_2 treatment alone did not affect circularity of sea bass otoliths (Kruskal Wallis, p=0.875). There was, however, a significant effect of the interaction between PCO_2 and temperature on otolith shape (Kruskal Wallis , p<0.001), suggesting a synergistic effect of ocean acidification and warming (OAW) on otolith shape.

Ossification significantly increased with temperature and age (degree-days) as sea bass larvae grew into juveniles (Kruskal Wallis, p<0.001) (Figure 3.3 (a)). PCO_2 level alone did not significantly influence the ossification ratio (Kruskal Wallis, p=0.7684), but the following interactions were significant: PCO_2 and age (Kruskal Wallis, p<0.001), PCO_2 and temperature (Kruskal Wallis, p<0.001), PCO_2, temperature and age. Larvae exposed to Δ1000 PCO_2 thus had a higher ossification ratio compared to ambient that increased with age and that was specially marked in 20°C-reared fish. A previous study working with the same species at 19°C reported a much greater impact of elevated PCO_2 levels on skeletogenesis: ossification ratio was 35% higher in larvae (~850 dd) exposed to 1520 µatm compared to those at 590 µatm (Crespel et al. 2017). Other studies also suggest species-specific responses on the effects of PCO_2 on skeletal formation. For example, no effect of PCO_2 (850 µatm) was observed on size of skeletal structures of spiny damselfish (*Acanthochromis polyacanthus*) (Munday et al. 2011). On the other hand, Atlantic cod larvae had more ossified vertebrae and gills under high PCO_2 (Stiasny et al. 2019), and the elasmobranch (*Leucoraja erinaca*) showed an increased in density and cartilage mineralization in some skeletal part such as the jaw or the crus (Di Santo Valentina 2019).

Skeletal malformations in the jaw were not impacted by warming or PCO_2 (ANOVA; temperature: p = 0.729; PCO_2 levels: p = 0.275), but did significantly decrease with age (ANOVA; p = 0.001) (Figure 3.3 (b)). On the other hand, the percentage of deformities in the vertebral column was impacted by temperature (ANOVA; p = 0.042) but not PCO_2 (ANOVA; p = 0.987) (Figure 3.3 (c)). These results were mainly driven by the increase in distorted vertebrae that were impacted by temperature (ANOVA; p = 0.003) and by the interaction between temperature and stage of development (degree-days) (ANOVA; p = 0.008). Skeletal deformities may appear due to environmental etiologic reasons, as well as environmental stressors (Abdel et al. 2004). Increased temperature is known to modify in embryonic development and larval organogenesis, potentially leading to increased malformations. High temperatures (≥ 19°C) lead to higher growth rates, but at the cost of inducing a larger number of skeleton anomalies in European sea bass (Abdel et al. 2004; Boglione et al. 1989) and other warm-temperate species such as gilthead seabream (*Sparus aurata*) (Georgakopoulou et al. 2010) or the Senegalese sole (*Solea senegalensis*) (Dionísio et al. 2012 ; Pimentel et al. 2014). The frequency of vertebral deformities increased over time at high temperatures, suggesting a chronic stress (Slooff 1982). On the other hand, jaw malformations decreased over time in all treatment, suggesting selective survival against deformed individuals.

Despite the well-known decreased skeleton ossification in invertebrates due to OA (Byrne 2011), not much is known on the potential effect of OA on the skeleton development in fish. Previous studies have reported a reduction (Crespel et al. 2017), increase (Chambers et al. 2014; Pimentel et al. 2014) or no change (Di Santo Valentina 2019) in the skeletal malformations of larvae from different teleost species under high PCO_2. One hypothesis that may explain higher malformation frequency with elevated PCO_2, is the existence of a potential trade-off between skeleton growth and bones stiffness. Although skeleton development occur faster with OA, bone density decreases making them likely to broke or suffer from malformation (Kim et al. 2015).

Overall, this study provides an insight into the combined effects of OAW on the developmental ontogeny of European sea bass. Results suggest that, when fed *ad libitum*, European sea bass larvae and young juveniles responses to OA are temperature-dependent. OA led to bigger otoliths at colder temperatures, and higher ossification ratio at warmer temperatures. Warming (20°C) also increased the frequency of vertebral deformities, suggesting chronic stress in early life stages of sea bass. Otoliths are involved in balance, motion detection and hearing (Popper et al. 2005; Réveillac et al. 2015) and changes in traits such as circularity or asymmetry could have drastic impact on swimming performance or behavior and/or the ability of larvae to detect or escape from predators (Gagliano et al. 2008). Similarly, vertebral deformities will likely impact fish locomotion and individual performance (Bignami et al. 2013). Therefore the combined effect of warming and OA may lead to substantial physical impairment and consequently decreases in the fitness of sea bass in the wild.

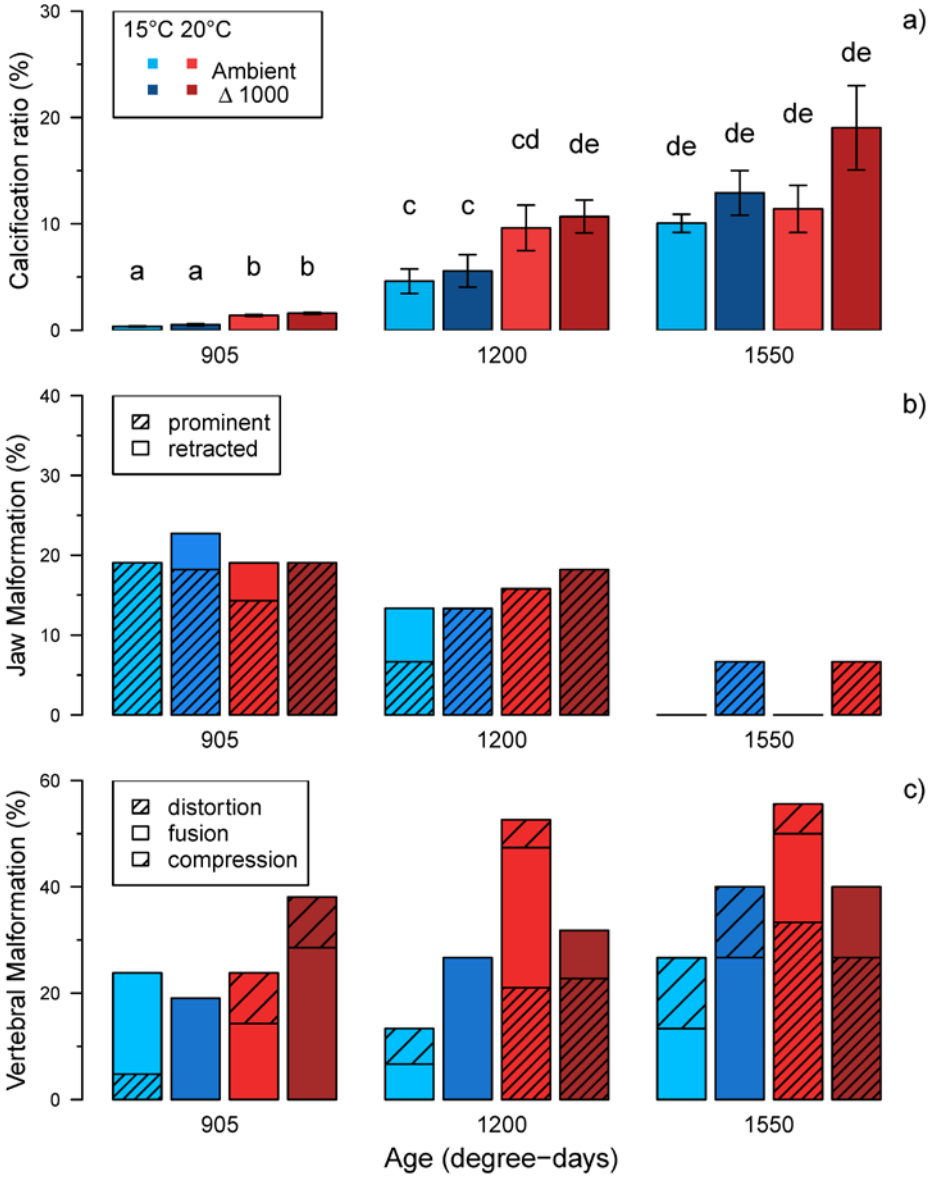


Figure 3.3 a) Evolution of ossification ratio, relative proportion of mineralized bones against cartilages components, of European early stages reared at two temperature (15°C and 20°C) and two PCO_2 levels (Ambient PCO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2). Higher mean (±SE) values suggest larger proportion of mineralized bones. The letters indicate significant differences among conditions ($\alpha = 0.05$). **Percentage (%) of macroscopically deformities of b) the jaw (prominent or retracted lower jaw) and c) the vertebral column (distortion, fusion and compression) in European early stages reared at 15°C and 20°C, and two PCO_2 levels (Ambient PCO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2).**

3.4 Supplementary Information

3.4.1 Supplementary Tables

S2.1 Table. Light intensity during larval phase. Abbreviation: dph, days post-hatch.

Age (dph)	2	7	9	12	16	20	27	31	36	44
Light intensity (lux)	0	0-1	1	2	5	7	10	31	59	96

S2.2 Table. Larval mortality (%) in the different larval rearing tanks. Abbreviations: A, Ambient PCO_2 (650 µatm); Δ500, ambient + 500 µatm CO_2; Δ1000, ambient + 1000 µatm CO_2; T, temperature, Rep, replicate tank.

	A			**Δ500**			**Δ1000**		
T (°C)	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
15	31.5	37.3	25.9	78.6	21.9	33.3	35.6	11.3	16.8
20	43.5	29.4	30.5	46.6	33.6	34.9	39.6	26.7	35.7

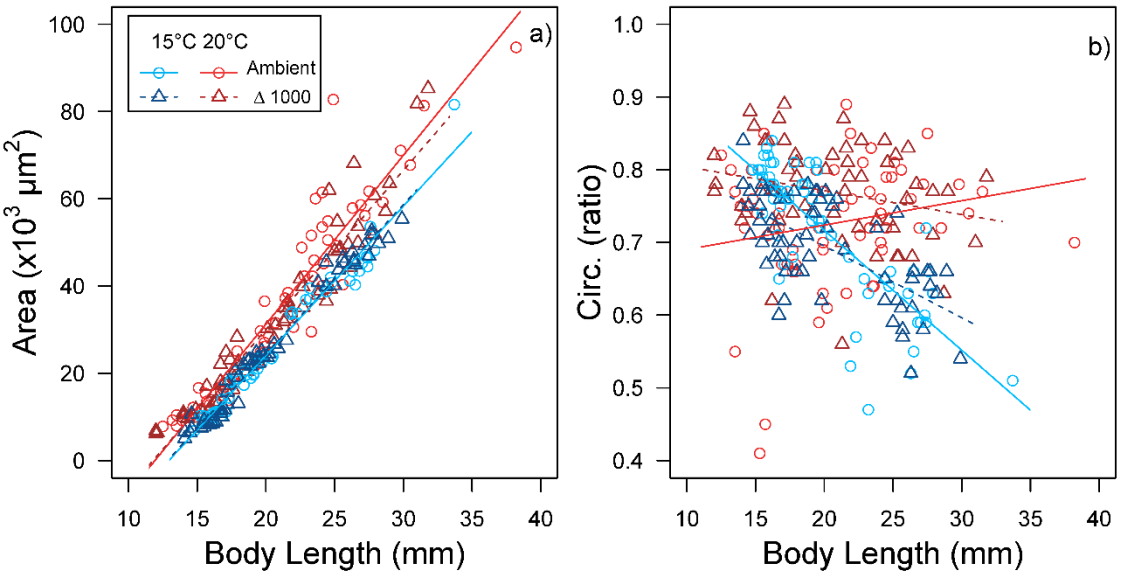
S2.3 Table. Juvenile mortality in % in the different tanks. Abbreviations: A, Ambient PCO_2 (650 µatm); Δ500, ambient + 500 µatm CO_2; Δ1000, ambient + 1000 µatm CO_2; T, temperature.

T (°C)	**A**	**Δ500**	**Δ1000**
15	24.8	43.4	29.7
20	35.2	41.7	38.2

S2.4 Table. Linear regressions for morphometric measurements for European sea bass larvae reared at four different conditions: two temperature (15°C and 20°C) and two PCO_2 levels (Ambient PCO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2). Mean values (±SE). Abbreviations: dph, days post-hatch; BL, body length.

Temperature (°C)	**PCO_2 level**	**n**	**Age (dph)**	**R^2**	**Regression**
15	Ambient	66	25 - 102	0.89	BL = 4.64±0.58 + 0.01±0.00 * Age
15	Δ1000	67	25 - 102	0.94	BL = 3.91±0.49 + 0.01±0.00 * Age
20	Ambient	75	25 - 77	0.79	BL = 0.13±1.19 + 0.02±0.00 * Age
20	Δ1000	80	25 - 77	0.87	BL =2.45±0.68 + 0.01±0.00 * Age

3.4.2 Supplementary Figures



S3.1 Figure. Allometric relationship between otolith area (panel a), otolith diameter (b) and fish body length (mm) in European sea bass early life stages reared at 15°C or 20°C and at either ambient (650 µatm) or ambient + 1000 µatm (Δ1000) PCO_2.

CHAPTER 4: Acidification and warming minimized by food supply

Food availability modulates the combined effects of ocean acidification and warming on fish growth

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When organisms are unable to feed *ad libitum* they may be more susceptible to negative effects of environmental stressors such as ocean acidification and warming (OAW). We reared sea bass (*Dicentrarchus labrax*) at 15 or 20°C and at ambient or high PCO_2 (650 versus 1750 µatm PCO_2; pH = 8.1 or 7.6) at *ad libitum* feeding and observed no discernible effect of PCO_2 on the size-at-age of juveniles after 277 (20°C) and 367 (15°C) days. Feeding trials were then conducted including a restricted ration (25% *ad libitum*). At 15°C, growth rate increased with ration but was unaffected by PCO_2. At 20°C, acidification and warming acted antagonistically and low feeding level enhanced PCO_2 effects. Differences in growth were not merely a consequence of lower food intake but also linked to changes in digestive efficiency. The specific activity of digestive enzymes (amylase, trypsin, phosphatase alkaline and aminopeptidase N) at 20°C was lower when the PCO_2 level was higher. Our study highlights the importance of incorporating restricted feeding into experimental designs examining OAW and suggests that *ad libitum* feeding used in the majority of the studies to date may not have been suitable to detect impacts of ecological significance.

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Submitted to Scientific Reports

4.1 Introduction

An amalgam of abiotic and biotic factors interact in nature to impact the vital rates of marine organisms (Breitburg et al. 1998; Frost et al. 1999; Schindler 2001) and understanding the cumulative effect of multiple stressors on marine organisms is currently one of the top priorities for ecologists (Hodgson & Halpern 2018). Unfortunately, the effect of multiple stressors is challenging to predict because their interaction can be either additive (the combined response is the sum of responses to individual factors), synergistic (the combined response is greater than the sum of responses to the independent factors) or antagonistic (the combined response is smaller than the response to either single) (Vinebrooke et al. 2004). For example, the projected increase of the concentration of carbon dioxide (CO_2) in the atmosphere by 2100 (from 280-410 ppm to 730-1020 ppm (Meehl et al. 2007; IPCC 2014) is expected to cause both ocean acidification (OA), decrease in pH by 0.3 to 0.5 units (Meehl et al. 2007)) and continued global warming (0.2°C increase per decade in the past 30 years (Doney et al., 2012). Research efforts are underway to understand how ocean acidification and warming (OAW) will combine to impact on the vital rates of marine biota (Harvey et al. 2013; Lefevre 2016).

When consumers in marine food webs have been exposed to OAW, a range of changes affecting growth responses have been reported (Kroeker et al. 2013). The level of OAW projected for 2100 caused significant reductions in the growth of mollusks and echinoderms, but a variety of responses has been reported in fish. For example, larval sea bass (*Dicentrarchus labrax*) incubated in four treatment groups (17 and 19°C, 600 and 1000 µatm CO2 partial pressures (PCO_2)) grew significantly faster in the warmer and higher PCO_2 treatment (Pope et al. 2014) whereas growth of Senegalese sole (*Solea senegalensis*) larvae increased with temperature but decreased with increasing PCO_2 (Pimentel et al., 2014b). A main conclusion to be drawn from the mixed results reported for fish is that the effect of OA and OAW can be life stage- and species-specific, and warming could either offset or aggravate any impacts of OA (McCulloch et al. 2012). An important caveat is that the vast majority of studies investigating the effects of OA or OAW, on fish or other marine animals, has been performed on individuals fed *ad libitum*. *Ad libitum* rations may provide ample energy allowing organisms to compensate for potential negative impacts of sub-optimal levels of temperature and/or PCO_2 on energy acquisition, dissipation and allocation. For instance, invertebrates such as corals, mussels and oysters maintained on restricted rations displayed more deleterious effects to OAW than well-fed conspecifics (Cohen & Michael 2009; Hettinger et al. 2013; Thomsen et al. 2013; Towle et al. 2015). In fish, only very recent studies have examined the influence of the interaction between CO_2 and food ration on larval growth and development (Gobler et al. 2018; Hurst et al. 2017; Sswat et al. 2018; Stiasny et al. 2019). They showed either no supplementary effect with food restriction (Gobler et al. 2018; Hurst et al. 2017) or observed larger individuals but with important organ damages (Stiasny et al. 2019).

Covering obligatory maintenance costs (standard metabolic rate) is generally the first priority when organisms allocate available energy. When additional food resources are available, however, the corresponding energy allocation to discretionary activities is based on fine-tuned trade-offs that depend on the organisms' activities, physiological state and environment. For instance, during long-term food restriction, energy is not available to fuel the production of digestive enzymes which inevitably impairs digestive capacity and reduces rates of growth and protein synthesis in fish (Cahu & Infante 1994). Environmental changes might also impact energy allocated for digestion and consequently for growth. For example, 15 months after European sea bass larvae were exposed to an 8-day hypoxic episode , their growth rates and protein digestive capacity (lower trypsin activity in the pancreas and aminopeptidase N and alkaline activity in the intestine) were still lower than siblings maintained in normoxia (Zambonino-Infante et al. 2017). Information on how OA will impact the digestive function of marine organisms is relatively scarce (Stumpp et al. 2013). The hypothesis is that OA will act as a metabolic stressor, similar to hypoxia, causing reduced digestive capacity. If OA impaired acid base regulation, more energy might be allocated to buttress this homeostasis (or others defense mechanisms) at the cost of digestive efficiency. Although there is no evidence yet that digestive function might be affected, (Strobel et al. 2012) demonstrated that in an Antarctic fish exposed to a 2000 µatm regulation of acid-base balance was enhanced at the detriment of other processes such as calcification or osmoregulation likely due to changes in energy allocation.

We examined the growth rate and digestive capacity of juvenile sea bass (*Dicentrarchus labrax*) fed *ad libitum* or restricted (25% of *ad libitum*) rations at an ambient and an elevated (Δ1000 µatm) level of PCO_2. Two trials were conducted using juveniles that had been reared for nearly a year under OA conditions since the early larval stage. The first and second trial was performed on fish reared at 20 and 15°C, respectively. The second trial was conducted 2 months after the first trial to allow the 15°C fish to grow to a body size more comparable to that of the warm-acclimated fish at the start of the first trial. We focused on understanding the underlying mechanisms of potential impacts of OA on growth including feed conversion efficiency (FCE), stomach pH and the activity of key digestive enzymes. Although OA had no discernable impact on size-at-age of sea bass feeding at *ad libitum*, expectations were that elevated PCO_2 combined with restricted feeding would cause decrements in growth performance in these fish, particularly at the warmer temperature. Incorporating feeding level treatments in a long-term exposure to OAW, this study reveal that the elevated temperature and elevated PCO_2 levels acted antagonistically on juvenile fish growth and highlights the need to re-examine how experiments are designed to test "real world" effects of climate-driven changes in abiotic factors.

4.2 Materials and Methods

The present work was performed within Ifremer-Centre de Bretagne facilities (agreement number: B29-212-05). Experiments were conducted according to the ethics and guideline of the French law and legislated by the local ethics committee (Comité d'Ethique Finistérien en Experimentation Animal, CEFEA, registering code C2EA-74) (Authorization APAFIS 4341.03, permit number 2016120211505680.v3).

4.2.1 Animals and experimental conditions

4.2.1.1 Water parameters

Sea bass used in the present experiments were reared since 3 days post-hatch (dph), under one of 4 different ocean acidification and warming (OAW) treatments including two different levels of partial pressure of CO_2 (PCO_2) (ambient and high (Δ1000)) and two thermal treatments (15°C and 20°C). The ambient PCO_2 was approx. 650 µatm. This is equal to today's situation for coastal waters of Brittany (Cameron & Iwama 1987; Pimentel et al. 2016) where, in 2014, the annual mean PCO_2 level was 603 µatm (range 284-888 µatm) in the Bay of Brest (Salt et al. 2016). The IPCC Representative Concentration Pathway (RCP) 8.5 scenario projected an increase of ~500 µatm above current values by the end of the century (IPCC 2014). The PCO_2 level in coastal areas and estuaries, habitats where sea bass juveniles and adults are encountered, however, is much higher (Pickett & Pawson 1994; Lonthair et al. 2017)). In these shallow water coastal systems, PCO_2 levels often above 2000 µatm have been reported (Wallace et al. 2014; Melzner et al. 2013). In accordance with these and additional PCO_2 levels in European estuaries reported by Frankignoulle et al. (1998), the second treatment was fixed at Δ1000 µatm above the ambient level (labelled Δ1000, approx. 1700 µatm). The 15°C treatment included larval rearing at 15°C while juveniles experienced naturally fluctuating thermal conditions between 15 and 18°C (natural, seasonal differences reflecting ambient summer conditions in the Bay of Brest (Chauvaud et al. 2001); (de Pontual et al., 2019), (http://marc.ifremer.fr/en/results/temperatureandsalinity/mars3d channelbay ofbiscaymode/(typevisu)/map/(zoneid)/sudbzh#appTop). The 20°C treatment included larval rearing at 20°C while juveniles experienced 20 to 23°C (5°C increase relative to ambient temperature). The 5°C increased was defined based of the 'business-as-usual' (RCP 8.5) scenario as predicted by the Global Climate Models (GCMs) by 2100 (IPCC 2007). Constant temperature were applied for the larval stage which experience relatively stable temperature offshore while juveniles reach the estuaries in the late spring and are then exposed to seasonal change in temperature (Vinagre et al. 2012; Anastasiadi et al. 2017).

Sea water was pumped in from the Bay of Brest from a depth of 20 m approximately 500 m from the coastline, passed through a sand filter (~500 µm), heated (tungsten, Plate Heat Exchanger, Vicarb, Sweden), degassed using a column, filtered using a 2 µm membrane and

finally UV sterilized (PZ50, 75W, Ocene, France) assuring high water quality. Replicate treatment tanks (n = 3 for larval rearing and n = 2 for juveniles rearing) were supplied with sea water via header tanks where water PCO_2 was controlled using IKS Aquastar system (IKS Computer Systeme GmbH, Germany). This system continuously measured water pH and was equipped with a solenoid valve that regulated the flow of CO_2 from the gas cylinder using feedback from the pH electrode. The valve was turned on and off according to the electrode measurement. This valve, therefore, controlled the amount of CO_2 injected in the water flowing through the header tank into the fish rearing tank (flow rate: 0.18 L min-1, corresponding to a water exchange of 30% per hour). Temperature and pH were checked ((WTW 3110 pH meter, Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW Sentix 41, NIST scale) each morning before feeding. The pH meter as well as the IKS Aquastar system were calibrated daily with NIST certified WTW technical buffers pH 4.01 and pH 7.00 (Xylem Analytics Germany, Weilheim, Germany). Total alkalinity was measured once a week following the protocol of Anderson & Robinson (1946) and Strickland & Parsons (1972): 50 ml of filtered tank water (200 µm nylon mesh) were mixed with 15 ml HCl (0.01 M) and pH was measured immediately. Total alkalinity was then calculated with the following formula:

$$TA = \frac{V_{HCl} \cdot c_{HCl}}{V_{sample}} - \frac{\left(V_{HCl} + V_{sample}\right)}{V_{sample}} \cdot \frac{\{H^+\}}{\gamma_{H^+}} \quad \left[\frac{mol}{l}\right]$$

With: TA – total alkalinity [mol * l^{-1}], V_{HCl} – volume HCl [l], c_{HCl} – concentration HCl [mol * l^{-1}], V_{sample} – volume of sample [l], H^+ – hydrogen activity (10^{-pH}), γ^{H+} – hydrogen activity coefficient (here $\gamma^{H+} = 0.758$).

The Microsoft Excel macro CO2sys (Lewis et al. 1998) was used to calculate seawater carbonate chemistry, the constants after Mehrbach et al. (1973) (as cited in CO2sys) refit by Dickson & Millero (1987), were employed. Using the CO2sys, daily pH (NIST) values were calculated in respective pH (free) values. Oxygen saturation (WTW Oxi 340, Xylem Analytics Germany, Weilheim, Germany) and salinity (WTW LF325, Xylem Analytics Germany, Weilheim, Germany) were measured once a week together with total alkalinity, from juvenile stage onwards, see all water parameters in Table 4.1.

4.2.1.2 Larval and juvenile rearing

Larvae used in this experiment were the progeny of wild brood stock fish caught off Morbihan, France, and kept at an aquaculture facility (Aquastream, Ploemeur-Lorient, France). Four females (mean mass 4.5 kg) were crossed with ten males (mean mass 2.4 kg), which spawned naturally using photothermal manipulation. At 2 dph, larvae were transferred to the Ifremer-Centre de Bretagne. Larval rearing was performed in a temperature-controlled room using black, 35-L tanks. Temperature shift, feeding regime and photoperiod was implemented as described by Gourtay et al. (2018) until the juvenile stage. Juvenile were moved to 670-L tanks at 50 dph and 65 dph for fish reared at 20°C and 15°C, respectively. There were randomly allocated to two treatment tanks. Having only two replicates limited our ability to estimate

variation but dividing the fish randomly will remove any potential tank effect during larval rearing. Prior to trials, during the rearing of juveniles, mortality was between 24.8 and 43.4% per tank. Juveniles were fed *ad libitum* daily rations of commercial fish food (Neo Start, Le Gouessant, Lamballe, France) using automatic feeders. Photoperiod was adjusted to natural conditions once a week. The tanks were cleaned daily after pH-measurements. Water flow rates maintained oxygen saturation levels above 90%.

Table 4.2 Water parameters during larval and juvenile phase of batch 2016: Larval period until 04.03.2016 (46 dph) and 18.03.2016 (60 dph) for 20°C and 15°C treatment respectively, for the juveniles until 24.10.2016 (280 dph) and 08.02.2017 (387 dph) for 20°C and 15°C treatment respectively. Means (±SE) over all replicate tanks per condition. Temperature (Temp.) and pH (free scale) were measured daily; salinity and total alkalinity (TA) weekly, oxygen was measured weekly during juvenile rearing; PCO_2 was calculated with CO2sys; sea water (SW) measurements were conducted during 2017 and 2018; A – Ambient PCO_2, Δ1000 – ambient + 1000 µatm CO_2, L – Larvae, J – Juveniles, 15°C – 15°C treatment, 20°C – 20°C treatment.

Treatment	pH [-]	Temp. [°C]	Salinity [psu]	O2 [% airsat.]	TA []	PCO_2 [µatm]
L 15°C A	7.95±0.01	15.3±0.0	33.0±0.1	-	2364±17	656±16
L 15°C Δ1000	7.58±0.00	15.3±0.0	33.0±0.1	-	2394±26	1682±26
L 20°C A	7.88±0.01	20.0±0.1	33.1±0.1	-	2369±21	832±13
L 20°C Δ1000	7.60±0.01	20.0±0.1	33.1±0.1	-	2380±23	1672±33
J 15°C A	7.97±0.01	16.0±0.2	34.2±0.1	90.9±0.5	2396±18	655±18
J 15°C Δ1000	7.55±0.01	16.1±0.2	34.2±0.1	90.9±0.6	2399±19	1841±40
J 20°C A	7.92±0.01	21.9±0.2	35.0±0.2	90.2±0.9	2418±12	788±22
J 20°C Δ1000	7.59±0.01	21.9±0.2	35.0±0.2	91.3±0.6	2423±12	1808±65
SW 15°C	8.05±0.01	14.5±0.5	33.0±0.2	101.2±0.6	2434±21	522±18
SW 20°C	7.95±0.02	21.2±0.4	32.7±0.1	102.3±1.4	2433±28	723±33

4.2.2 Feeding-growth trial

At 8 and 11 months post-hatch, for the 20°C and the 15°C rearing condition, respectively, fish between 10 and 100 g were selected for the feeding trials (about 90% of all juveniles). Fish were subcutaneously tagged (Passive integrated transponder; Pit-tag) for individual identification and randomly allocated among 12 indoor, 500-L tanks supplied with filtered and aerated natural seawater. Fish were excluded that i) were < 10 g since these were too small to be tagged, ii) had any morphological deformities, and iii) were > 100 g. Fish were allocated (maintaining PCO_2 history) so that there was a similar mean and variance of fish sizes and, hence, similar total biomass in each replicate tank (mean ± SE; 1876.72 ± 30.94 g (~33 fish) and 1287.30 ± 14.87 g (~35 fish), for 20°C and 15°C trials, respectively). Feeding-growth trials commenced after a > 7-day acclimation period to the tanks (S4.1 Figure). Juveniles were 303

and 399 dph at the start of the 20°C and 15°C trials, respectively, and had a mean (± SE) wet mass of 52.13 (0.62) and 31.08 (0.42) g, respectively. Three replicate tanks for each PCO_2 treatment were randomly assigned to *ad libitum* and "restricted" feeding treatments. Feed was administrated during daylight hours. In the *ad libitum* treatment, fish were fed three times a day (at 09:00, 13:00 and 17:00). A known initial mass of food (30 and 50 g for 15 and 20°C fish, respectively) was partially distributed to each tank three times a day (09:00, 13:00 and 17:00). Food was delivered by hand making sure that no food was left uneaten. The mass of food not distributed to each tank was determined. The mass fed (consumed by fish) was the difference between the final and initial masses of feed for a tank on that day. The mean value for the three replicate *ad libitum* tanks was determined and 25% of that value was set as the ration for the restricted feeding group the next day (starting at 9:00 and distributed using an automatic feeder). The restricted ration was fed using an automatic feeder starting at 9:00. Food consumption of *ad libitum*-fed fish showed daily variation (reported in the S4.1 Figure). The 20 and 15°C trial lasted 18 and 38 days, respectively. At the start and end of the trial, every fish was slightly anesthetized with tricaine methanesulfonate (MS-222; dose adapted to water temperature and fish mass, typically 0.2 g l^{-1}) and wet mass (WM) was measured (Cubis MSE12201S-000-D0, Sartorius, Germany; d=0.1g). Specific growth rate (SGR, % d^{-1}) and feed conversion efficiency (FCE, %) were calculated according to the following formulas:

$$SGR = 100(\ln[WM_{final}] - \ln[WM_{initial}]) / \text{Number of Days of Feeding}$$

$$FCE = \text{Biomassgain (g)} / \text{Total Mass of Food Consumed in the Tank (g)}$$

With Biomassgain corresponding to the final wet biomass minus the initial wet biomass in the tank.

4.2.3 Determination of digestive enzymes

Fish were sampled for digestive enzymes twice, once one day before the start of the trial (after the acclimation period) and one week after final weighing, while keeping them on the two rations levels (experimental day 29 at 20°C, and 49 at 15°C, see S4.2 Figure). Fish were starved for 48h prior to both samplings and each time 8 to 9 individuals were randomly sampled per treatment. Fish were dissected on ice, the abdominal cavity was opened and the intestine was separated from the rest of the gut. For each fish, the mucosa of the digestive track was collected by scraping the anterior of the intestine, put directly in 1.5-ml microtubes and stored at -80°C. To purify brush border membranes, intestinal mucosa was homogenized according to a method described by Crane et al. (1979). This included homogenizing the intestinal mucosa for 20 s (ultra turax, Poltron PT2100, Kinematica AG, Switzerland) at maximum speed with a mix solution of Mannitol and Tris-HCl, collecting 1 ml of homogenate, adding $CaCl_2$, centrifuging at 9,000 x g for 10 min, removing the supernatant and centrifuging at 3,400 x g for 20 min. The pellet was resuspended in Tris–Hepesbuffer and used for enzymatic assays.

Trypsin and amylase activities were assayed according to Holm et al. 81988) and Métais and Bieth (1968), respectively. Enzymes of the brush border membrane, alkaline phosphatase (AP) and aminopeptidase N were assayed according to Bessey et al. (1946) and Maroux et al. (1973), respectively. Proteins were determined according to the Bradford (1976) procedure. Enzyme activities were expressed in milliunits of specific activity (i.e. mU mg protein^{-1}) and units of total activity (i.e. U segment^{-1}).

4.2.4 Determination of kinetic of stomach pH following ingestion

On experimental day 35 at 20°C and 55 at 15°C, fish were fasted for 48-h and then re-fed based on their treatment. Stomach pH was measured at the end of the fasting period, 30 min post feeding and then regularly during the digestion process (see S4.1 Figure). For each measurement, 8 individuals were randomly sampled within each replicate tank, anaesthetized with MS-222, and the stomach immediately dissected out. A pH electrode (WTW Inolab 720 pH meter, Xylem Analytics Germany, Weilheim, Germany) was then inserted in the stomach and maintained in the anterior portion. While dissections took place on ice, the pH was measured at room temperature and the electrode was calibrated every three measurements.

4.2.5 Statistical analysis

Normality for SGR data was first assumed according to the central limit theorem and verified visually via a q-plot of the raw data and residuals. Differences in SGR and FCE were tested using two-way ANOVAs. The overall effect of temperature and PCO_2 level on food intake was examined using a two-way, nested ANOVA. Significant ANOVAs were followed by a Student-Newman-Keuls multiple comparison test to determine differences among experimental groups. The effect of time, PCO_2 ratios and feeding level on stomach pH was tested with a linear mixed-effects model (LME models), with time being considered as random effect and PCO_2 ratios and feeding level as fixed effects. Differences were considered significant at $\alpha = 0.05$. Enzymes activities (specific and total), of each enzymes, were first tested for a temperature effect via a one-way ANOVA. Differences between PCO_2 and feeding treatments were masked by high response of activity found at 20°C, so potential effects were test separately between the two temperatures using two-way ANOVAs. Differences in enzyme activity were considered significant at $\alpha = 0.01$. All statistical analyses were performed with R (ver.3.3.3; R Development Core Team).

4.3 Results

4.3.1 Growth performance

Prior to the trial, for each temperature treatment, no significant differences, were observed in the mass-at-age of fish reared since larvae at different levels of partial pressure of CO_2 (PCO_2) (measurements conducted at 277 dph and 367 dph for 20°C and 15°C, respectively; see S4.3 Figure). No mortalities occurred during the trials and individual SGR (specific growth rate) ranged from -0.53 to 1.30 % d^{-1} at 15°C and from -0.99 to 2.62 % d^{-1} at 20°C (Figure 4.1). Growth appeared to be similar across all body sizes, from relatively small to large fish at 15°C (S4.4 Figure) and 20°C (S4.5 Figure). At 15°C, SGR was not affected by PCO_2 and, not unexpectedly, fish fed *ad libitum* grew significantly faster than those fed restricted rations (ANOVA, $p<0.001$). A different pattern emerged at 20°C, where SGR was significantly affected not only by ration level but also by PCO_2, as indicated by a significant interaction (ANOVA, $p<0.001$). For fish fed *ad libitum,* the SGR of ambient fish was 110% higher than fish from the elevated (Δ1000 µatm) treatment. In restricted feeding condition, the difference was even more pronounced, as the Δ1000-acclimated fish lost mass, while the groups of fish reared at ambient PCO_2 had a mean SGR of 0.5 % d^{-1}.

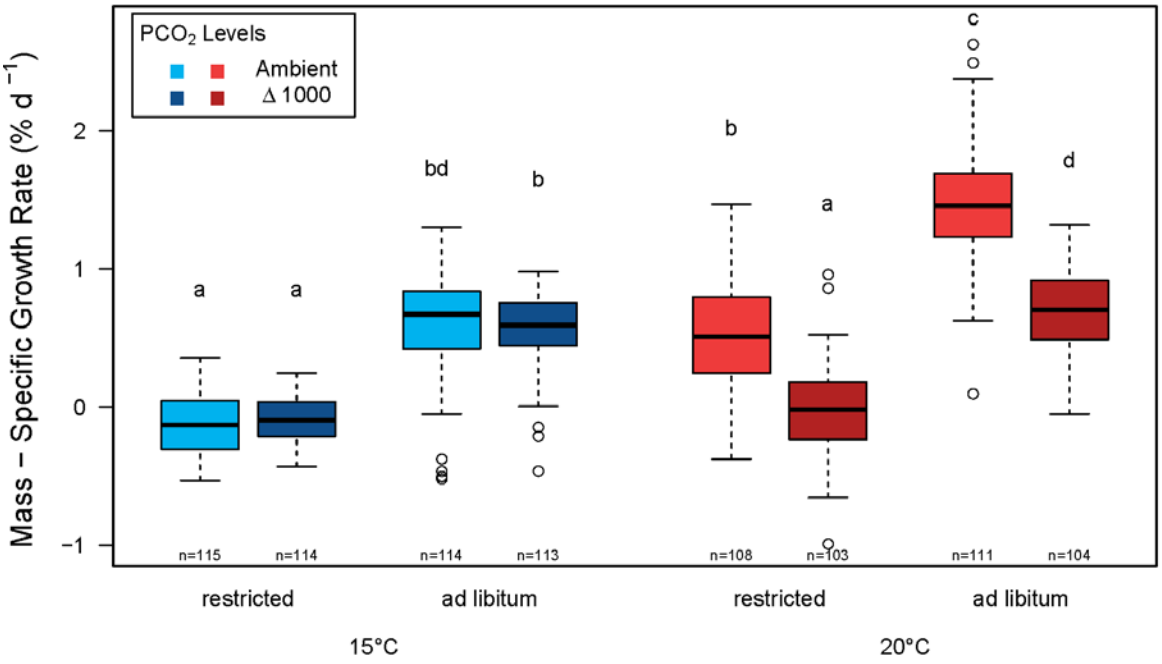


Figure 4.1 Box and whisker plots of mass-specific growth rates (SGR) of individually tagged juveniles sea bass reared at two temperatures (15 and 20°C), two PCO_2 levels (Ambient PCO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2) and two feeding levels. Different letters denote significant differences (Student-Newman-Keuls test, $p<0.05$) between each condition. The whiskers denote the 10th and 90th percentiles, the box denotes the 25th and 75th percentiles, the median value is shown (horizontal line) as well as outliers (points).

Mass-specific rates of food consumption in the *ad libitum* treatment were significantly lower at 15°C compared to 20°C (nested ANOVA p<0.001, Figure 2). In the *ad libitum* treatment at 20°C, the amount of food consumed by fish in the high PCO_2 treatment was lower than that consumed by fish in the ambient treatment on 13 of the 18 days (S4.1 Figure). At both temperatures and PCO_2 levels, day-to-day feeding patterns of fish in the *ad libitum* treatments were variable and lacked any regular periodicity (S4.1 Figure).

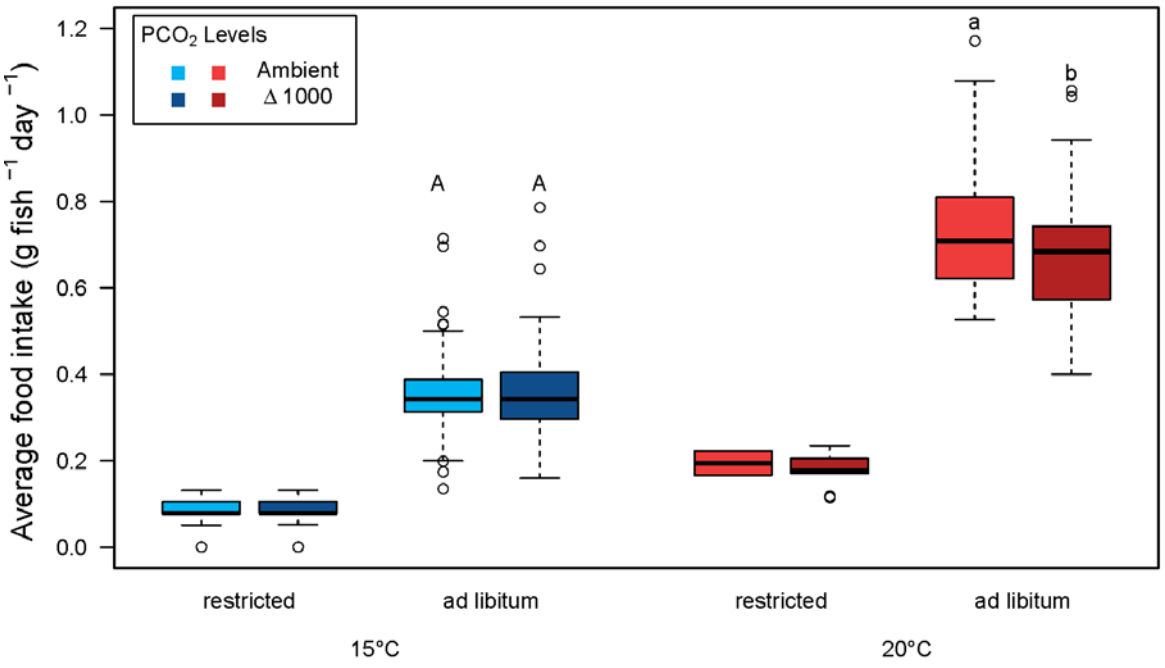


Figure 4.2 Box and whisker plots of the food consumption rate (g food fish-1 d-1) at two temperatures (15 and 20°C,) two PCO_2 levels (Ambient PCO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2) and two feeding levels. Differences in food intake was tested between ad libitum PCO_2 groups separately for each temperature treatment. Different capital letters denote significant differences (nested ANOVA, $p < 0.05$) between ad libitum PCO_2 groups at 15°C. Different lowercase letters denote significant differences (nested ANOVA, $p < 0.05$) between ad libitum PCO_2 groups at 20°C. The whiskers denote the 10^{th} and 90^{th} percentiles, the box denotes the 25^{th} and 75^{th} percentiles, the median value is shown (horizontal line) as well as outliers (points).

The FCE (feed conversion efficiency) was highest for fish in the ambient PCO_2 treatment at 20°C (>1.0) and was reduced by almost half (<0.6) in fish at 20°C in the Δ1000 treatment. At 20°C ambient PCO_2, the FCE of fish fed restricted and ad libitum rations was not significantly different. In contrast, FCE in fish in the Δ1000 and restricted ration treatment was negative at both temperatures (mean (± SE); -0.35(0.08) and -0.09(0.10) for 15°C and 20°C, respectively). The level of PCO_2 had a significant effect on FCE at 20°C (ANOVA, p<0.001) but not at 15°C. The largest difference in FCE between the ambient and Δ1000 PCO_2 treatments was observed at both feeding levels at 20°C (Figure 4.3).

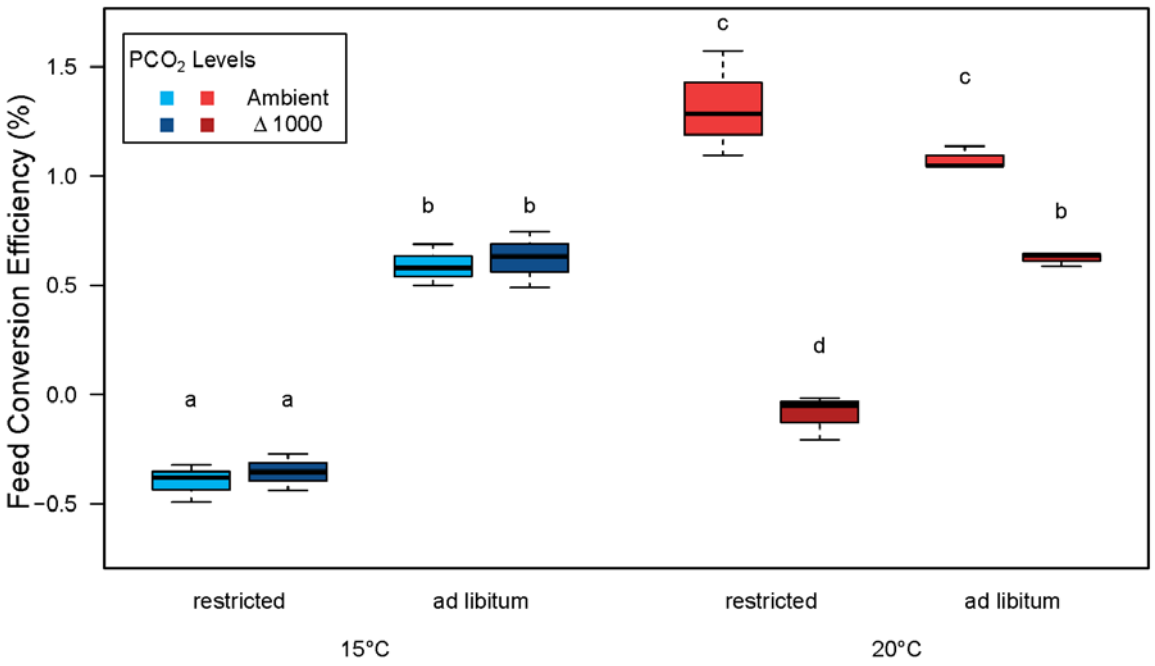


Figure 4.3 Box and whisker plots of feed conversion efficiency (FCE) of juvenile sea bass reared at two temperatures, two PCO_2 levels (Ambient PCO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2) and at two feeding levels. Different letters denote significant differences (Student-Newman-Keuls test, $p<0.05$) between each treatment group. The whiskers denote the 10th and 90th percentiles, the box denotes the 25th and 75th percentiles, the median value is shown (horizontal line) as well as outliers (points).

4.3.2 Kinetics of stomach pH

Prior to feeding, mean (±SE) stomach pH ranged from 6.17 (1.15) to 7.15 (0.18) and was similar at both temperatures, PCO_2 levels and feeding levels. In all treatments, acid was rapidly secreted after feeding and pH rapidly declined to reach a minimum value about 8 h post-feeding. After this initial decrease, the time course of stomach pH depended on the rearing treatment. At 15°C, time needed for stomach pH to return above a standard value (5.5) was significantly slower for fish fed restricted feeding levels (LME, $p < 0.001$) (Figure 4.4). At 20°C, the kinetics of stomach pH of fish from the restricted feeding + Δ1000 µatm treatment were significantly different (LME, $p < 0.001$) compared to the *ad libitum* one. Fish fed restricted rations at Δ1000 µatm took nearly twice as long to return to pre-feeding pH values (e.g. 48 versus 26 hrs).

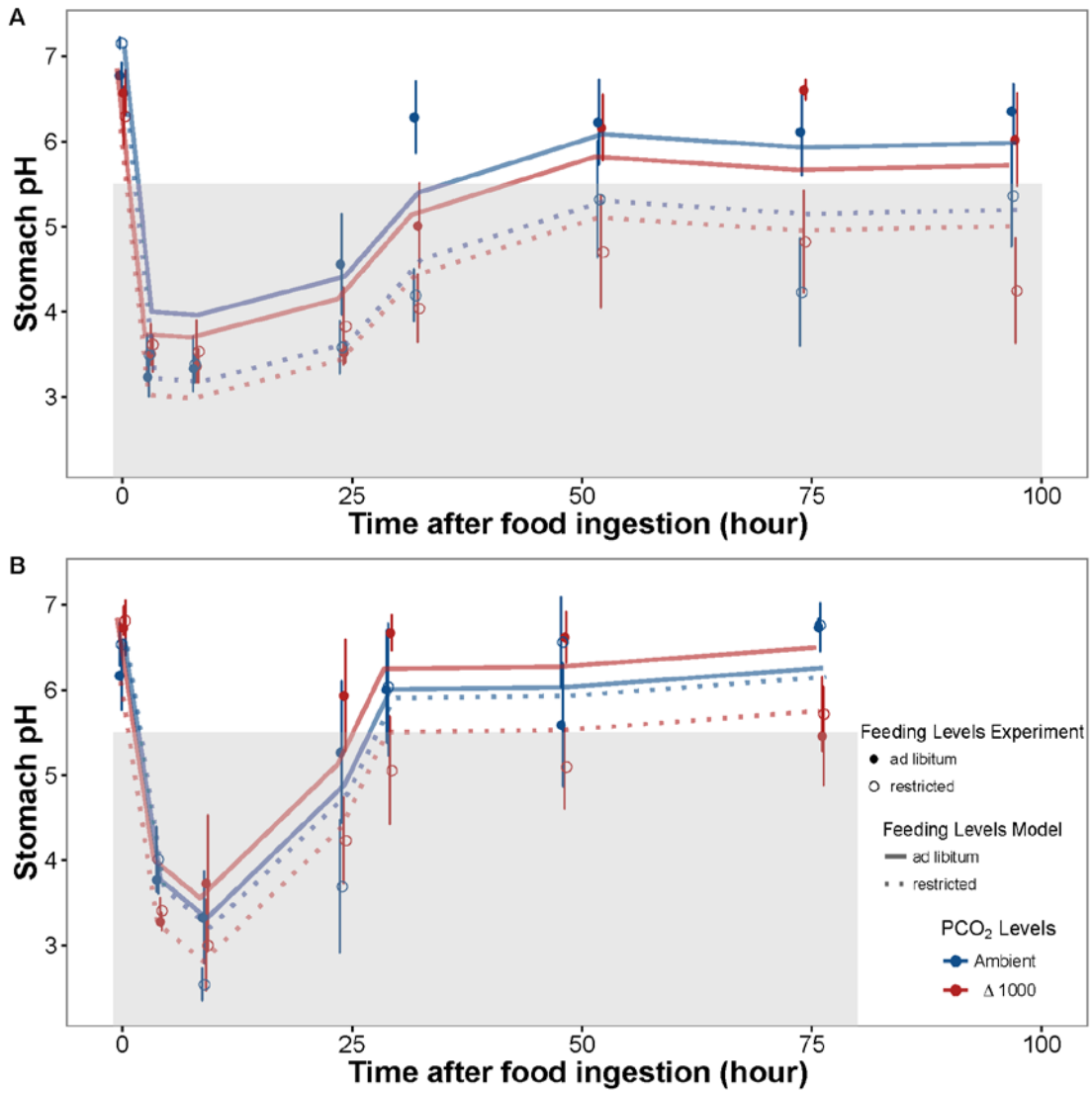


Figure 4.4 Post-prandial kinetics of stomach pH in juvenile sea bass. Symbols display the mean (±SE, n=8) for fish reared at two *P*CO_2 levels (Ambient *P*CO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2) (colors) and two feeding levels (shape) at either (A) 15°C or (B) 20°C. In both panels, the lines are model predictions (LME).

4.2.3 Enzyme measurement

The total activity of each of the four enzymes was significantly lower at the colder compared to the warmer temperature (ANOVA, $p < 0.001$). Total enzyme activity also tended to be higher in fish fed *ad libitum* versus restricted ration (Figure 4.5). At 15°C, the total activity of AP (alkaline phosphatase) was significantly higher for fish on the *ad libitum* versus the restricted ration (ANOVA, $p < 0.001$; Figure 4.5, G). At 20°C, the total activity of trypsin was significantly higher for fish on the *ad libitum* versus restricted rations (ANOVA, $p = 0.005$) (Figure 4.5, D).

Significant reductions in the specific activities of AP (ANOVA, $p < 0.001$) and aminopeptidase N (ANOVA, $p = 0.007$) were observed in fish at 15°C compared to 20°C. At 15°C, AP wassignificantly lower in fish fed restricted versus *ad libitum* rations (ANOVA, $p < 0.001$; Figure 4.5, E). In contrast, at 20°C the specific activity of AP was higher in fish fed restricted rations (Figure 4.5, F). At 15°C, despite a tendency for the specific activity of all four enzymes to be higher at the high versus the ambient PCO_2 level, no significant differences were found (ANOVA, $p > 0.05$) (Figure 4.5). At 20°C, in contrast, the specific activity of AP tended to decline with increasing PCO_2 and that for trypsin was significantly lower at high PCO_2 (ANOVA, $p = 0.009$). Indeed, the specific activity of trypsin in fish at Δ1000 PCO_2 at 20°C was ≈ 70% that of fish in the ambient treatment (Figure 4.5, B).

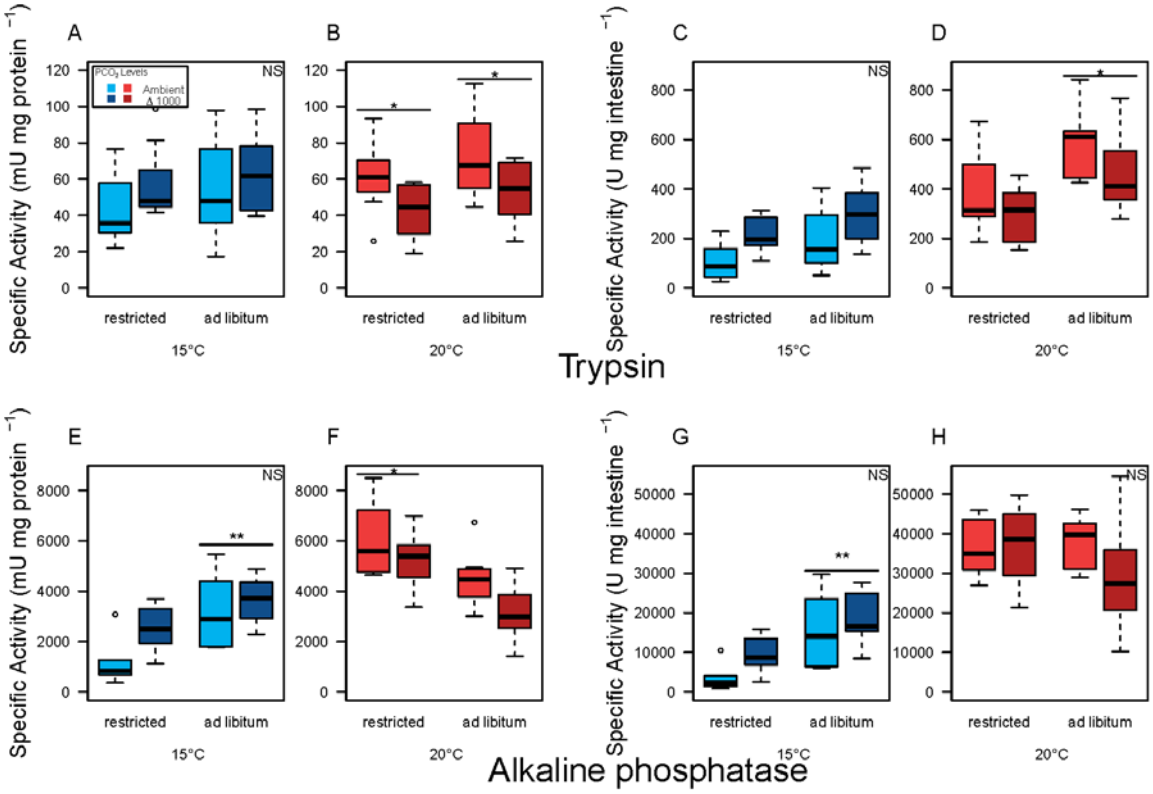


Figure 4.5 Box and whisker plots (n = 8) of specific activities of digestive enzymes of fish reared at two temperatures according to PCO_2 levels (Ambient PCO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2) and feeding levels. Results are given in milli-units per mg of protein (mU.mg protein^{-1}) for specific activity and in units per mg of intestine segment (U mg intestine^{-1}) for total activity. Stars denote significant differences (ANOVAs, * <0.01, ** <0.001). The whiskers denote the 10th and 90th percentiles, the box denotes the 25th and 75th percentiles, the median value is shown (horizontal line) as well as outliers (points).

4.4 Discussion

Knowledge on the combined effect of multiple stressors on the ecology of species and communities is still relatively scarce (Hodgson & Halpern 2018; Gunderson et al. 2016) and there remains an urgent need to conduct experiments incorporating such interactions. Ocean acidification and warming (OAW) appears to induce a variety of responses in well-fed marine organisms (Harvey et al. 2013). The results of the present study suggest that different responses may have been observed in those studies if restricted feeding levels had been employed. In the present study, although no differences were observed in sea bass growth after nearly one year of rearing under OAW, obvious and significant differences in various aspects of growth physiology were observed due to ocean acidification (OA) when restricted feeding levels were applied in the feeding-growth trials.

Although the effect of OA on food intake and growth of marine organisms appears to be taxon-, species- and life stage-specific, the results of the growing number of studies suggest that potentially deleterious effects of OA may be offset if ample food is available (Melzner et al. 2011; Ramajo et al. 2016). In calcifying organisms, for instance, it has been shown that food supply modulated the impact of low pH on growth (Thomsen et al. 2013; Melzner et al. 2011; Ramajo et al., 2016). Along that line, work on the eastern oyster (*Crassostrea virginica*) indicated that food availability partially offset the impacts of OA on larval growth and development (Hettinger et al. 2013). Similarly, examination of the growth dynamics of polyps of two jellyfish species suggested that potential climate-driven changes in prey stoichiometry were more important in determining mass-gain than OA per se (Lesniowski et al. 2015). On the contrary, no differences were observed in the growth rate and skeletal development of cod (*Gadus morhua*) larvae reared at high CO_2 and provided either a low or high food concentration (Stiasny et al. 2019). To maintain similar rates of growth, however, those authors suggested that compensatory energy (re-)allocation occurred since larvae reared at low prey concentrations also displayed organ damage, especially to the liver. Although the growth performance of fish, particularly juveniles, is considered to be largely unaffected under high levels of partial pressure of CO_2 (PCO_2) (Cattano et al. 2018; Foss et al., 2006), energy limitation imposed by restricted feeding may cause increased sensitivity to environmental stressors (Stiasny et al. 2019; Pimentel et al. 2016).

Prior to the feeding trial, no difference in growth was observed regarding PCO_2 treatment, while after the trial a difference in growth was observed between ambient and Δ1000 PCO_2, at 20°C. During long-term rearing, growth measurements were made on a subsample of fish from each tank while growth information was available for each (individual tagged) fish during the feeding trial. Moreover, while no mortalities occurred during the trials, ~40% mortality occurred during rearing after larvae were transferred to juvenile tanks. European sea bass are known to be cannibalistic and it is possible that a larger number of small individuals were eaten in the Δ1000 treatment during long-term rearing which would have biased (increased) the growth rates calculated for fish in this treatment (Hatziathanasiou et al. 2002). The mean

growth rate of *ad libitum* fed individuals in this study was 1.4% d^{-1} at 20°C which is similar to values (0.6 to 1.5% d^{-1}) reported for sea bass reared at comparable feeding levels and temperature (Benhaïm et al. 2011; Gardeur et al. 2001). Given that optimal temperatures for growth in juvenile sea bass were reported to be 22 to 24°C (Claireaux & Lagardèr, 1999), faster growth was expected at 20°C compared to 15°C in the present study. Although this was the case for fish in the ambient PCO_2 treatment, mean growth rates of well-fed fish were similar at 20 and 15°C in the high PCO_2 treatment.

The individuals used in the present study originated from wild-caught adults acclimatized and reared (~5 years) in the aquaculture facility. The adults spawned at 13°C at a pH of 7.6 corresponding to our elevated condition of acidification. Previous studies have reported that the sensitivity of offspring to acidification might decrease with parental conditioning to high PCO_2 (Munday 2014; Murray et al. 2014; Jarrold & Munday 2019). Therefore, the absence of differences in growth observed here at 15°C might be a consequence of transgenerational plasticity (TGP). This potential TGP, however, was absent at 20°C. We are unaware of studies suggesting that TGP is expressed in only a narrow range of parental temperatures and OA conditions. (Griffith & Gobler 2017) highlighted that TGP is likely species-specific and, to our best knowledge, no studies have investigated TGP in sea bass.

Previous studies have indicated that PCO_2 may alter rates of feeding (Nowicki et al. 2012; Vargas et al. 2013). For example, feeding and foraging activities of juvenile anemonefish (*Amphiprion melanopus*) were depressed at moderate levels of PCO_2 (530 µatm) but enhanced at a higher level of PCO_2 (960 µatm) (Nowicki et al. 2012). On the contrary, reduced feeding by Chilean abalone (*Concholepas concholepas*) larvae at high PCO_2 were reported (Vargas et al. 2013). Similarly, in the present study, daily *ad libitum* feeding rate was lower in fish in the high PCO_2 treatment at 20°C (but not 15°C) compared to the normocapnic treatment. Differences in SGR (specific growth rate) also existed, however, between ambient and high PCO_2 in fish maintained at a similar restricted ration level, suggesting that differences in growth were driven by differences in feed conversion efficiency (FCE) and not food consumption rate. The FCE depends on several, interacting factors such as feeding level and water temperature (Buentello et al. 2000; Handeland et al. 2008; Imsland et al. 2006). The relatively low FCE at 15°C (i.e. ~0.5) further highlight that this is a sub-optimal temperature for the growth of juvenile sea bass. The highest feed efficiency however, occurs when fish are fed slightly below satiation (Zoccarato et al. 1994), which agrees with the pattern observed between the ration levels at 20°C at ambient PCO_2. At 20°C and high PCO_2, a drastic reduction in FCE was observed for fish on restricted rations compared to fish fed *ad libitum*. It is possible that high PCO_2 increases maintenance or activity costs and/or reduces digestive capacity, and consequently reduces growth. Substantial level of feeding providing sufficient energy income, however, could counteract those effects.

Values and changes in gastric pH as functions of stomach fullness and digestive stage have been well characterized in fish (Deguara et al. 2003; Getachew 1989; Kuz'mina 1996).

Although some teleosts constantly secrete acid to maintain low stomach pH, even in absence of food (Bucking & Wood 2009), other teleosts, such as sea bass, only secrete acid in response to food ingestion. As a result, pH values in empty stomach are generally less acidic (> pH 5) in these species (Solovyev & Gisbert 2016). Post-prandial pH values between 2.5 and 5.5 were found here and in a previous study on the same species (Nikolopoulou et al. 2011). Post-prandial changes in stomach pH observed in the present study are similar to those reported for other temperate and sub-tropical teleosts such as gilthead sea bream and the white sea bream (*Diplodus sargus*) (Deguara et al. 2003; Yúfera et al. 2012), but are faster than those from a previous study on larger sea bass at 26°C (Nikolopoulou et al. 2011). The maximum activity of this protease has been measured at pH values between 2.0 and 3.0 in various species (Kuz'mina 1996; Solovyev & Gisbert 2016; Nikolopoulou et al. 2011), values which were obtained within 30 min post-feeding across all treatments. Although not measured here, pepsin is the most important enzyme in the digestion of proteins in the stomach.

In the present study, treatment-specific differences in the kinetics of stomach pH (time needed for pH values to return above 5.5) was influenced by meal size and PCO_2. Ingesting large amounts of food leads to larger stomach distension (Nikolopoulou et al. 2011) that promotes stronger and more frequent peristaltic contraction and increased rates of food evacuation (Jobling 1981). Results showed that, except for 20°C- *ad libitum* fish, high PCO_2 led to a slower post-prandial return of stomach pH to more neutral, pre-feeding levels. Based on changes in oxygen consumption rate, a similar prolongation in digestion time was observed in Atlantic cod exposed to elevated CO_2 (Tirsgaard et al. 2015). Tirsgaard et al. (2015) assumed that an extended digestion time and slower stomach clearance might lower food intake. In fish fed *ad libitum* at 20°C, this slow return of stomach pH to pre-fed levels was observed in fish in the -Δ1000 µatm treatment but not for fish in the ambient treatment, which may explain why the latter group consumed more food than the former group. As the alimentary bolus enters the intestine, a rapid buffering takes place through intense bicarbonate secretion into the intestine lumen (Taylor & Grosell 2006). We did not examine the time course of this process but it would be interesting to do so given the contribution of bicarbonate to maintaining acid-base homeostasis under hypercapnic conditions.

Higher values of enzyme activity were expected when feeding fish *ad libitum* rations. Surprisingly, at 20°C, the specific activity of AP was higher when animals were feed-restricted. The potential preservation or increase in AP activity under dietary restriction is, to our knowledge, a unique finding in fish but similar results were reported in mice where restricted energy intake led to a significant increase in intestinal AP (Dao et al. 1989). A second unexpected finding was the lack of a significant effect of feeding level (at both temperatures) on the specific activities of amylase and aminopeptidase N. A lower activity of both enzymes was expected with ration restriction (Cyrino et al. 2008; Infante et al. 1996). The reason for this response is unknown.

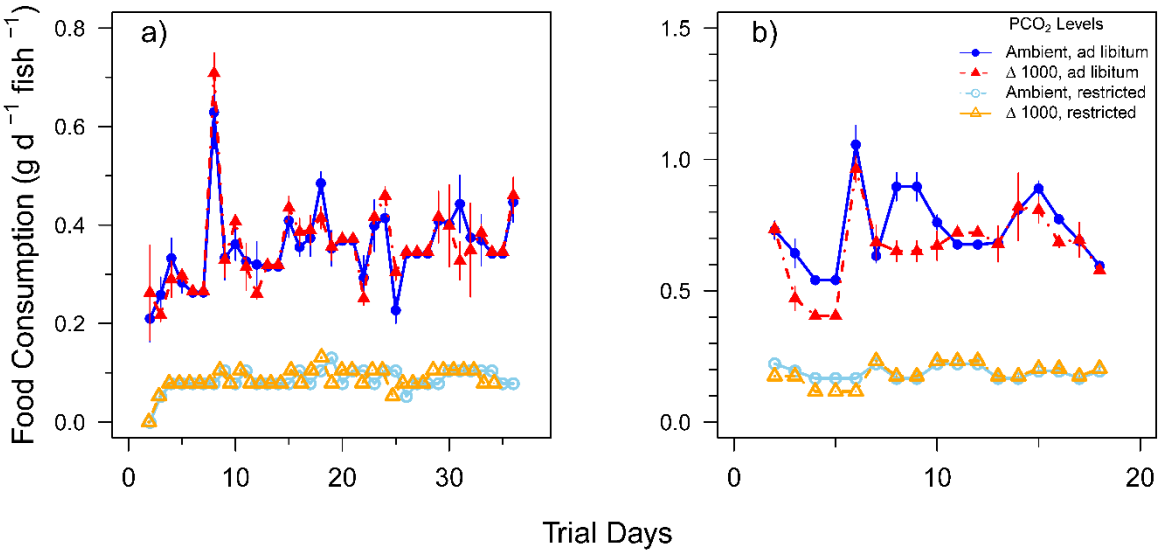
Recent studies have shown that exposure to high PCO_2 can trigger an increase in the secretion of intestinal bicarbonate (HCO_3^-) (Heuer et al. 2012) which, in turn, enhances AP activity (Akiba et al. 2007). In normocapnia, bicarbonate is secreted in the proximal intestine in response to low intestinal pH (Gregório et al. 2019). This secretion makes the intestine more alkaline, bringing pH closer to the optimum value for enzyme activity, such as AP (Fraisse et al. 1981; Lallès 2010). Such regulation of HCO_3^- secretion in response to low intestinal pH would explain why there was a tendency for higher enzymes activities in fish from the Δ1000 compared to the ambient PCO_2 treatment at 15°C. This tendency, however, was absent when individuals were reared at a warmer (+5°C) temperature. Indeed, antagonistic effects (PCO_2 x temperature) were found for trypsin and AP activity. Thus, enzymatic activities were reduced at Δ1000 compared to ambient PCO_2. Similar antagonistic patterns in digestive enzymes have been reported in newly born bamboo sharks (*Chiloscyllium punctatum*) experiencing OAW (+4°C x ~1400 µatm PCO_2) (Rosa et al. 2016).

The decrease in the specific activity of AP and trypsin under hypercapnia observed in the present study are similar to those (AP) reported in Senegalese sole (*Solea senegalensis*) larvae (Pimentel et al., 2015). Several studies have suggested that reduced activity of trypsin is an important mechanism limiting growth rate. A hypothesis is that energy invested in the ion exchange and the release of bicarbonate is not invested in trypsin synthesis. Trypsin is necessary for protein hydrolysis but it is extremely energetically costly to synthetize and, thus, lowering its synthesis rate during persistent food restriction would allow animals to allocate resources elsewhere. Such reductions in trypsin activity have been observed in juvenile sea bass exposed to hypoxia early in life (Zambonino-Infante et al. 2017). It must be noted, however, that we measured the specific activity of trypsin in the lumen of the intestine, and that measurements in the pancreas would be necessary to verify this hypothesis. Moreover, reductions in digestive capacity could also result from direct damage to gut tissues as was demonstrated in young Atlantic cod reared under high PCO_2 conditions (Frommel et al. 2012).

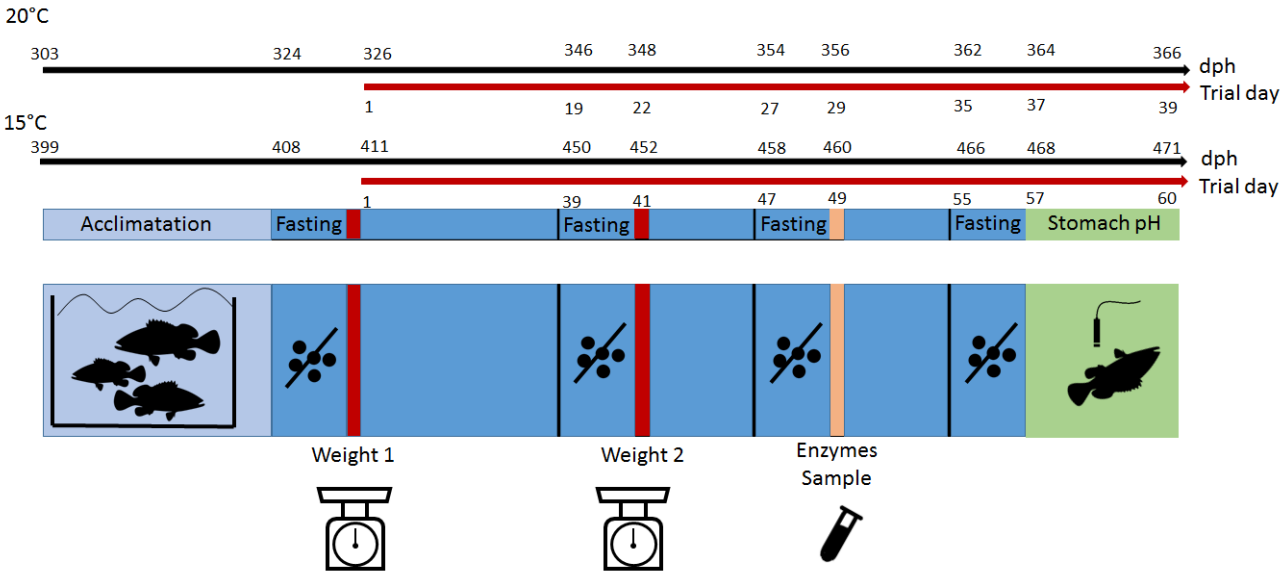
Overall, using a long-term exposure to OAW throughout the first year of life and examining the mechanisms of growth performance in a marine fish, the present study demonstrates how high feeding levels can reduce the impact of OAW. High PCO_2 reduced the growth of juvenile sea bass reared at 20°C and these effects were exacerbated in fish fed restricted rations. Reduction in growth was not merely due to reduced food intake but also related to processes decreasing feed conversion efficiency such as digestive capacity (e.g. via reductions in the specific activity of digestive enzymes). Many of these deleterious impacts of PCO_2 on sea bass were not observed at 15°C, a sub-optimal temperature for the growth of juveniles of this species. In this study, the focus was on the impact of OAW combined with food availability. It is important to note that nutritional requirements of sea bass may differ under OA compared to ambient (present-day) conditions; consequently, fish on restricted rations may have not only experienced decreased caloric / energy intake but also an additional impact of poor nutrition. Changes in global nutritional requirements under OA would be interesting to examine in the context of energy allocation and digestive efficiency. Our study emphasizes

the need to integrate different, ecologically relevant feeding levels in laboratory experiments assessing effect of OAW on marine organisms and suggests that previous studies that have used *ad libitum* feeding may underestimated the deleterious impacts of OAW.

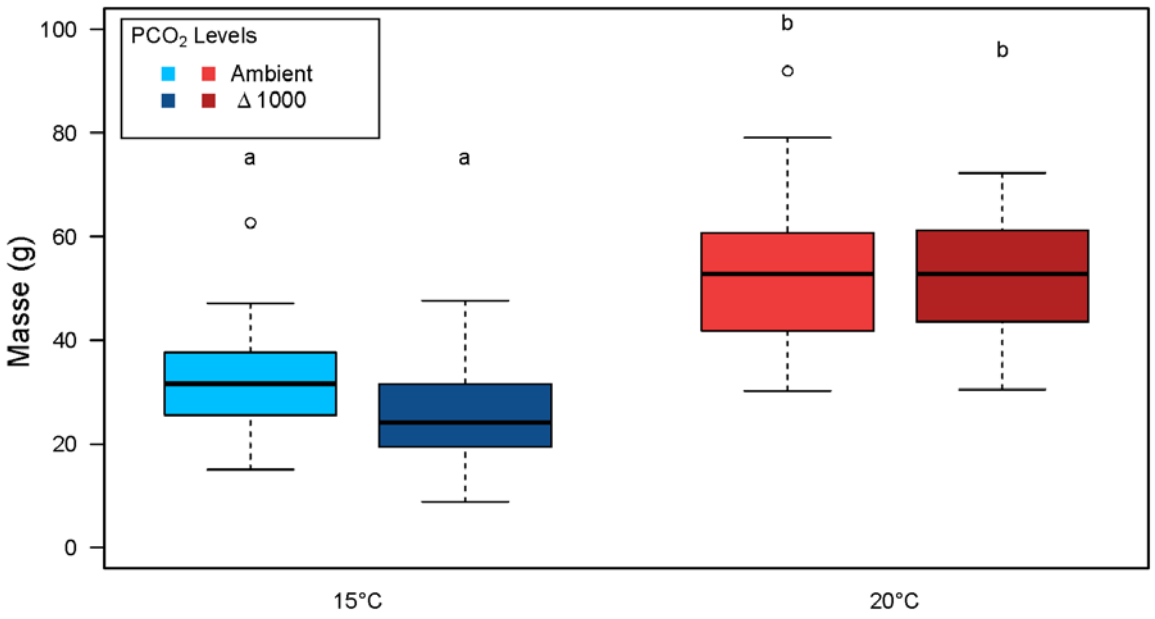
4.5 Supplementary Information



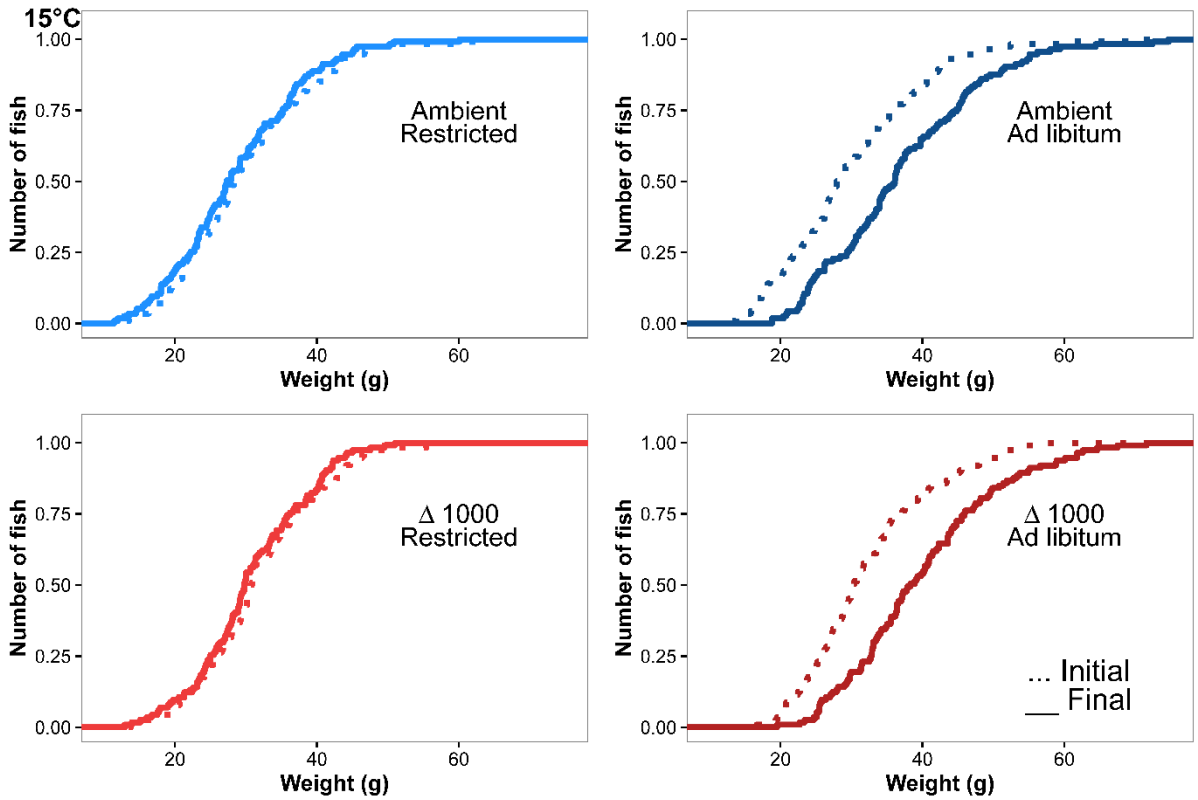
S4.1 Figure. Mean (± SE, n=3) food consumed by juvenile sea bass at two *P*CO_2 (Ambient *P*CO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2) and ration levels during each of two feeding-growth trials (a) 15°C and b) 20°C). In each trial, *P*CO_2 levels were ambient (circles, 650 µatm) and Δ1000 (triangles, 1700 µatm) (see text) and ration levels were *ad libitum* (filled symbols) or restricted (25% *ad libitum*, unfilled symbols).



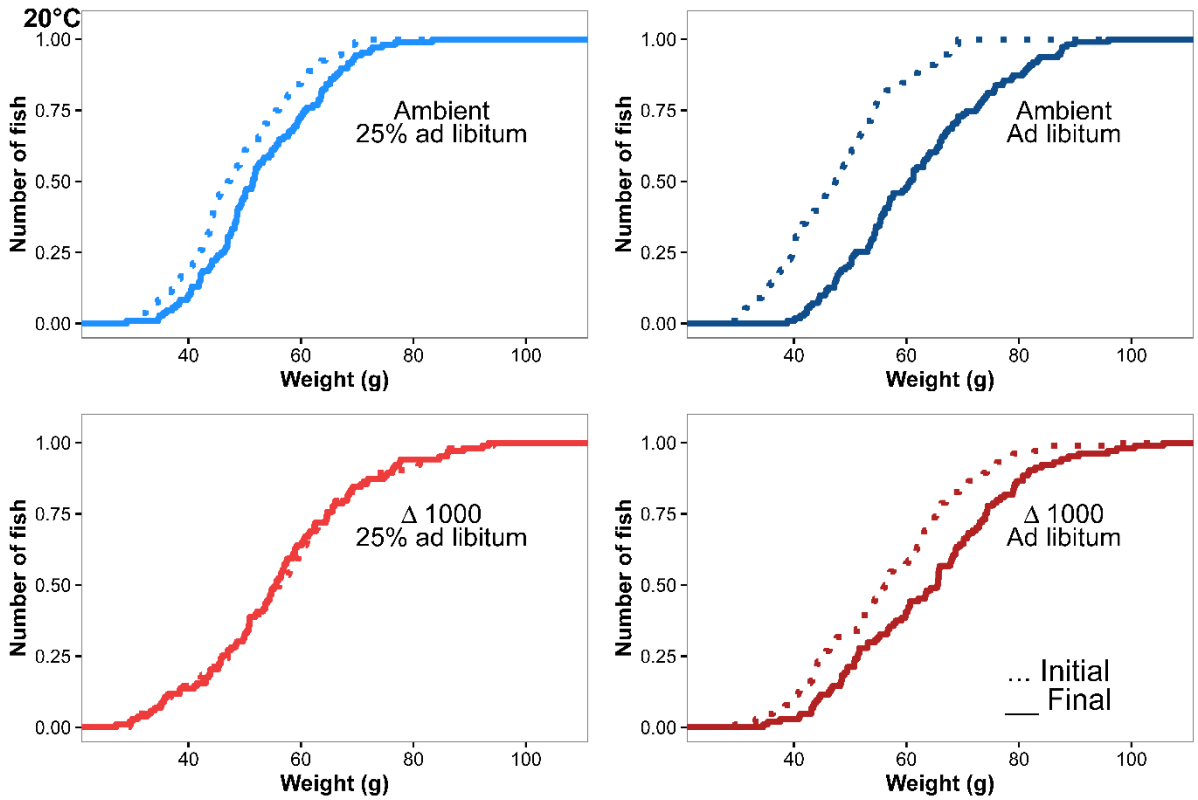
S4.2 Figure. Proceeding of the feeding trial at two temperature regimes. Dph: days post-hatch. Fish icons (CC) by Adam Zubin, MV.



S4.3 Figure. Box and whisker plots of wet mass of fish reared at two temperature regimes and two *P*CO_2 levels (Ambient *P*CO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2) at 367 dph and 277 dph at 15°C and 20°C, respectively. The whiskers denote the 10th and 90th percentiles, the box denotes the 25th and 75th percentiles, the median value is shown (horizontal line) as well as outliers (points).



S4.4 Figure. Wet mass cumulative distribution of fish reared at 15°C from the first weighing (Initial) to the second weighing (Final) for each condition.



S4.5 Figure. Wet mass cumulative distribution of fish reared at 20°C from the first weighing (Initial) to the second weighing (Final) for each condition.

CHAPTER 5: Transgenerational responses

Transgenerational tolerance to the effects of ocean acidification and warming in larval European sea bass

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Rising CO_2 levels in the oceans are predicted to have serious impacts on the physiological performance of marine organisms especially when combined with the effects of global warming. Yet the potential for marine organisms to acclimate to ocean acidification and warming (OAW) over successive generations is poorly characterized, particularly in large pelagic fish. The goal of this study was to assess the transgenerational effects of ocean acidification and warming in the European sea bass (*Dicentrarchus labrax*). From 2 days post-hatch until maturity, adults were conditioned to ambient (~590 µatm) and high levels of partial pressure of CO_2 (PCO_2) (~1520 µatm) and during larval development, they experienced a warm temperature (19°C). The progeny were exposed to the corresponding PCO_2 treatments in combination with two temperature treatments, cold (15°C) and warm (20°C). Swimming performance in offspring was unaffected by ocean acidification, whereas the negative impact of warm temperature, on first exposed individuals, was offset when parents were previously conditioned to a short warm episode at larval stage. The results suggested that transgenerational effects can alter the physiological response of marine fish to warming conditions.

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5.1 Introduction

Over the past 150 years, anthropogenic activities have caused a rise in atmospheric carbon dioxide (CO_2) levels form approximately 280 to 410 ppm, with further increase predicted by the end of the century (730 to 1020 ppm; Meehl et al. 2007; IPCC 2014). Increasing atmospheric concentrations of CO_2 have contributed to a greenhouse gas-driven warming of the globe of ~0.2°C every ten years in the last 30 years (Hansen et al. 2006). As a major carbon sink, the ocean has absorbed 30% of the excess of all the anthropogenic CO_2 emitted (Quéré et al. 2015) causing a decrease in water surface pH by 0.1 units during the 20th century; a further reduction of 0.3-0.5 pH units is expected by the end of 2100 (Caldeira & Wickett 2005). Changes in partial pressures of CO_2 (PCO_2) and temperature and, thus, ocean acidification (OA) and warming (OAW), together, represent a threat for marine ecosystems (Doney et al. 2012; Kroeker et al. 2013; Nagelkerken & Connell 2015).

The OAW induced by anthropogenic activities is occurring faster than ever in the earth's history and the increase in PCO_2 and temperature will occur over several generations for the majority of marine organisms (Doney et al. 2012). Until now, work investigating OAW impacts were mostly short-term experiments assessing one life stage. Long-term transgenerational studies are needed to investigate the responses of organisms over successive generations to shifts in environmental conditions and to understand potential mechanisms allowing organisms to acclimate or adapt (Donelson et al. 2012; Salinas et al. 2013). When the capacity for organisms to acclimate or adapt to changes in abiotic conditions was incorporated in models projecting biological impacts, Bell (2013) and Logan et al. (2014) demonstrated that the effects of climate change (warming and OA) may be less severe. Generally, adaptation occurs over many generations and involves genetic selection while acclimation can happen within a generation due to non-genetic inheritance from parents (Salinas & Munch, 2012; Bonduriansky et al. 2012) via nutritional, somatic, cytoplasmic or epigenetic transmission (Mousseau & Fox 1998; Marshall and Keough 2006). This latter phenomenon is known as parental conditioning or transgenerational plasticity (TGP).

In the last decade, a growing number of studies on marine species has investigated the potential for increased performance (survival, growth, development) of offspring after parental conditioning to future climate conditions. When parents of the spiny damselfish (*Acanthochromis polyacanthus*) were acclimated to Δ1.5 °C, the aerobic scope of their progeny remained high compared to progeny from parents in controls (Donelson et al. 2012). Similarly, young sheepshead minnow (*Cyprinodon variegatus*) grew faster when their parents were first acclimated to warmer temperatures (Salinas & Munch, 2012). In terms of PCO_2 acclimation, exposure to intermediate PCO_2 levels (~850µatm) reduced rates of survival, growth and calcification in Sydney rock oysters (*Saccostrea glomerata*) but those effects disappeared once their parents has been conditioned to these conditions during reproduction (Parker et al. 2012). Similar results were reported in another calcifier, the blue mussel (*Mytilus*

edulis) (Thomsen et al. 2017). Although elevated PCO_2 levels decreased shell formation in the larvae, this effect also disappeared in the second generation. In teleosts, transgenerational plasticity (TGP) in the effects of PCO_2 has also been reported such work on anemonefish (Amphiprion melanopus) (Miller et al. 2012; Jarrold & Munday 2019) and Atlantic silversides (Murray et al. 2014). The latter study exposed parents to seasonal oscillations in OAW and demonstrated that progeny produced from delayed spawners (later in spring when OA was more severe) had higher rates of survival when exposed to acidified condition than offspring from early spawners (produced at lower levels of PCO_2) (Murray et al. 2014). Altogether, these results suggest that acclimation, through parental conditioning, may be a key mechanism for marine organisms to adjust to OAW. The benefits of TGP, however, have not been observed in all organisms. For instance, negative parental effects of elevated PCO_2 were reported on juvenile settlement success and survival in green sea urchins (*Strongylocentrotus droebachiensis*) (Dupont et al. 2012). Therefore potential benefits of TGP may be species-specific (Griffith & Gobler 2017). In addition, the magnitude and the time of exposure to environmental stressors likely influences the potential for TGP.

The goal of the present study was to assess the potential for TGP in the performance European sea bass, a teleost with a relatively long life span. We used swimming ability as a key performance measure because of the fundamental importance of swimming for food acquisition, predator avoidance and habitat connectivity (Leis 2006). From a practical standpoint, the critical swimming speed (U_{crit}; Brett 1964) is widely used to assess the potential impact of environmental factors such as temperature, dissolved oxygen or the presence of pathogens on physiological performance in fish (Beamish 1978). Little is known on the potential for TGP in swimming performance. A sister study reported negative effects of warm temperature but no effect of OA on the U_{crit} of European sea bass larvae reared under OAW from 3 days-post-hatch (Cominassi et al. 2019). Those larvae were the progeny of wild caught adults maintained in an aquaculture facility where pH decreases to 7.6 and temperature is ~ 14°C during the spawning period. In contrast, the present study examined the offspring of sea bass reared since the early larval period at different levels of *P*CO2 and 19°C. Using the same rearing, exposure treatments and measurement protocol as Cominassi et al. (2019), we tested whether parental lifetime exposure to PCO2 and warm temperature would impact the effects of OAW on larval performance. Results will contribute to our understanding of TGP and yield further insight on the potential for longer-lived teleosts to cope with climate-driven changes in their environment.

5.2 Materials and methods

5.2.1 Rearing and reproduction

Parental origin, and the rearing of broodstock during their larval stage have been previously described in a sister study (Crespel et al., 2017a). Starting at two days post-hatch, fish were

exposed to two different levels of partial pressure of CO_2 (PCO_2) (ambient PCO_2: ~ 590 µatm; high acidification: ~ 1520 µatm) at a temperature of 19°C. Animals from ambient and high PCO_2 level were kept in those conditions throughout the juvenile phase until the adult stage and maintained at ambient temperature. Fish were pit tagged as juveniles. At about four years, prior to the reproductive period, males and females with the highest sex steroid plasma concentration during the previous reproductive season, were placed in separate tanks. Once the water temperature reached 13°C, tanks with females were checked daily for eggs. After the first batch was observed, females were injected with GnRHa (10 µg kg^{-1} female) to accelerate oocyte maturation. After 72h animals were strip spawned (10 ml of eggs were mixed with sea water and milt (0.05 ml milt L^{-1} seawater) for ideal fertilization conditions (Parazo et al. 1998). Ten females (mean (±SD) wet weight 1556.1(243.7) kg) were crossed with 18 males (1068.8(164.0) kg) at the ambient PCO_2 condition and 11 females (1276.3(296.1) kg) were crossed with 19 males (990.3(192.9) kg) at Δ1000 µatm. Fertilized eggs were then incubated for four days at 14°C in 40-L tanks at the PCO_2 parental conditions.

After hatching, animals were separated among four treatment groups. Fish from parents reared at ambient PCO_2 condition were reared at either 15°C or 20°C at the "Ambient" PCO_2 treatment corresponding to the present day situation in the Bay of Brest (approximately 650 µatm (cf. Pope et al. 2014; Duteil et al. 2016). The progeny of the broodstock from high acidification were also reared at 15 and 20°C at the high, "Δ 1000" µatm (approx. 1700 µatm; IPCC 2014). There were three tanks of fish at each treatment. Sea water pumped from the Bay of Brest was treated (i.e. degassed, filtered, sterilized) (Cominassi et al. 2019) before reaching larval tank. Elevated PCO_2 level was controlled using an IKS Aquastar system (IKS Computer Systeme GmbH, Germany). Water temperature and pH were measured daily (WTW 3110 pH meter, Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW Sentix 41, NIST scale) while measurements of total alkalinity (TA) were conducted weekly following Anderson & Robinson (1946) and Strickland & Parsons (1972) (Table 4.1). The pH meter and IKS Aquastar system were calibrated with NIST certified WTW technical buffers pH 4.01 and pH 7.00 (Xylem Analytics Germany, Weilheim, Germany). Temperature, feeding regime and photoperiod was implemented as described by Gourtay et al. (2018). At 7-33 dph, larvae were fed with live brine shrimp (*Artemia salina*) nauplii, hatched from High HUFA Premium cysts (Catvis, AE's-Hertogenbosch, Netherlands). From 33 dph and until the end of the larval stage, larvae were given nauplii previously enriched with cod liver oil and dry yeast for 24 h.

5.2.2 Larval trials

Two sets of swimming trials were conducted. The first trials measured the critical swimming speed (U_{crit}) starting with larvae at 14 dph and every 3 to 5 days until the juvenile stage, when all individuals completed the formation of the caudal fin (at ~ 45 dph and ~60 dph for the 20°C and 15°C-reared fish, respectively). A minimum of 5 trials were conducted for individuals in

each of four (PCO_2 x T) treatments. In each trial, 10 larvae per treatment were tested, 3 to 4 larvae randomly selected from each of the three replicate tanks.

The U_{crit} trials were conducted in a Loligo® Systems swim tunnel (Denmark), customized for work on larvae. The Brett-type flume was composed of two lanes (46 x 4.5 x 7 cm). Swimming measurements were performed on an individual larva tested in each lane. Larvae were tested at their treatment conditions. The temperature was controlled using a cooling/heating system (Tr10, TECO, Italy) and PCO_2 was maintained by a gas diffuser directly injecting CO_2 into the water of the head tank of the swim tunnel. Water speed was calibrated with a vane-wheel flowmeter (HFA, Höntzsch GmbH, Germany), and controlled by an AC motor (Santo et al. 2017). Laminar flow was ensured by adding a honeycomb section (length ~10 cm) upstream of the lane. Pilot trials using dye confirmed a homogeneous water velocity through the lane. One larva was introduced into each lane and acclimation for 5 min at the lowest water velocity. The water speed was then increased at a rate of 0.5 body lengths (BLs) s^{-1} every 3 min until exhaustion (i.e. the larva was not capable of maintaining its position in the lane and drifted downstream against a mesh screen). Prior to each trial, larvae average length were assessed for each condition to establish the standard (0.5 BL) velocity increment. Larvae maintained position in the middle of the lane suggesting minimal or no wall effects. Once the larva reach exhaustion, the animal was removed from the tunnel and euthanized with an overdose of Tricaine methane-sulfonate MS222, (PharmaQ Limited, Hampshire, United Kingdom) as prescribed by European legislation to minimize fish stress. Some individuals showed the incapacity or reluctance to swim at the lowest water velocity implement during the acclimation period (0.8 cm s^{-1} for 5 min). All larvae were digitally photographed using a stereomicroscope (Leica MZ 16, Wetzlar, Germany) and stored in 4% formalin. The BL was measured using ImageJ (Rasband, 1997) from the tip of the snout to the end of the notochord for preflexion larvae (notochord length) and as the distance from the tip of the snout to the posterior end of the hypural plate (for flexion and postflexion larvae).

The second test was a temperature challenge performed at the “sustained swimming speed”. This challenge was conducted only in progeny maintained at the ambient PCO_2 treatment. Trials were conducted at three developmental stages, early postflexion (~410 degree-days pos hatch (ddph)), postflexion (~820 ddph), and early juvenile stage (~1330 ddph) (21, 38, 70 dph and 28, 60, 84 dph for 20°C- and 15°C-reared fish, respectively). About 30 (~ 10 per replicate tank) individuals were tested in each trial using the swim tunnel from the first set of tests. The methods were the same except i) the water level was decreased by half, ii) 10 randomly selected individuals per replicate tank were swum together, and iii) only one lane was used which was 46 cm long, 7 cm in depth and either 4.5 or 14 cm wide depending on fish size. The time spent in the tunnel and water velocity were used to calculate a U_{crit} for each individuals and a mean U_{crit} (U_{crit} at which 50% were removed after sign of exhaustion or sustainable swimming speed). The temperature trial was then performed on another 10 random selected fish. Once the mean U_{crit} from the previous run was reached, water velocity was held constant and water temperature was rapidly increased from ambient to 26°C and then slowly increased

(6°C h^{-1}) until all fish lost their ability to maintain equilibrium. Temperature to loss of equilibrium (TLOE) and time to exhaustion (TE) were recorded. Water temperature was controlled using a 3000 W heater (Profi heater, Netherlands) and 100% air saturation was maintained by bubbling a controlled mixture of oxygen and air into the head tank. The challenge was repeated for each replicate tank in each treatment. Once removed, fish were euthanized with an overdose of MS222) and their BL measured using the methods in trial 1.

5.2.3 Statistical analysis

The U_{crit} across treatments was estimated using linear regression (GLM). Variables (e.g. BL, temperature, PCO_2) affecting U_{crit} were identified via the Akaike Information Criteria (AIC) (Burnham & Anderson 2002). Differences among treatments as well as model residuals related to any potential tank effect were then tested using an ANOVA. Normality and homoscedasticity of data were tested using Shapiro-Wilk and Fligner Killeen tests, respectively. Larvae which did not start to swim at acclimation speed were removed from the analysis. The effect of treatment on the ability (or willingness) to swim at the lowest velocity was tested using two-way ANOVAs after logit transformation of the data. To compare swimming probability at the temperature test, a Kaplan-Meier survival analysis was conducted. Swimming probability with increasing temperature among temperature and developmental stage condition was assessed using a log-rank test. Difference in temperature to loss equilibrium (TLOE) between temperature treatments was tested using Wilcoxon test, while U_{crit} distribution and time to exhaustion (TE) was estimated via multiple comparisons test (Pairwise Wilcoxon test). Values are presented as mean(±SE) and significant difference was accepted at $p < 0.05$. All statistical analyses were performed using R (R Development Core Team, 2008).

5.3 Results

The U_{cirt} was significantly influenced by body length (BL) (ANOVA, $p < 0.001$), temperature (ANOVA, $p < 0.001$) but not their interaction (ANOVA, $p = 0.384$) (Figure 5.1). The the critical swimming speed (U_{crit}) increased with BL and, at the same BL, was higher at 20 compared to 15°C with 7.09 cm s^{-1} at 8 mm and 18.90 cm s^{-1} at 16 mm, compare to 15°C reared-fish (2.81 and 13.73 cm s^{-1} at 8 and 16 mm, respectively). The levels of partial pressure of CO_2 (PCO_2) had no significant effect on U_{crit} (ANOVA, $p = 0.632$). U_{crit} values were also tested for tank effect, no significant effect of tank were found (ANOVA; $p = 0.406$). The mean proportion of larvae that could not (chose not) to swim was ~11%. The proportion of larvae not swimming was not unaffected by either temperature (ANOVA, $p = 0.606$) or PCO_2 levels (ANOVA, $p = 0.349$) (S5.1 Figure).

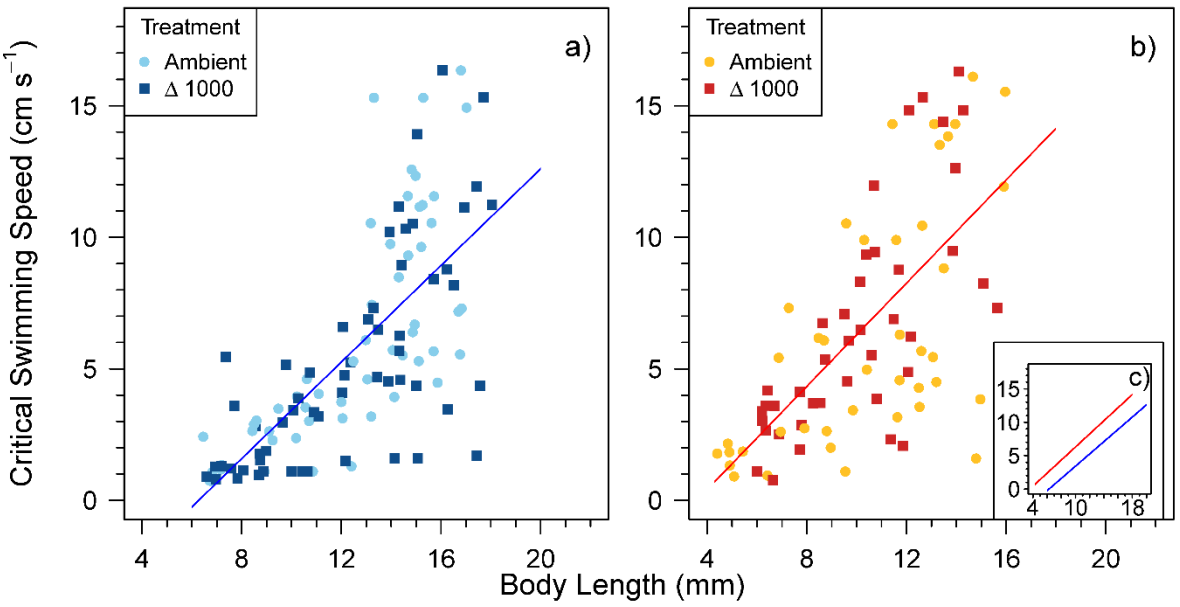


Figure 5.1 Ontogeny of critical swimming speed (U_{crit}, cm s^{-1}) in larvae of European sea bass reared at 15°C (a) and at 20°C (b). Symbols and colors indicate PCO_2 treatment (Ambient PCO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2) – see text. Regression (mean ± SE parameter estimates) are included: 15°C, (n=114) U_{crit} = -5.76(1.09)*BL + 0.92(0.08), R^2 = 0.51, $p < 0.001$; 20°C (n=83) U_{crit} = -3.51(1.31)*BL + 0.98(0.12), R^2 = 0.44, $p < 0.001$). For clarity, both regression lines are compared in subpanel **c)** (insert).

When three developmental stages were tested, the sustained swimming speed was higher in 20°C-reared larvae compare to 15°C-reared fish at the first developmental stage (Pairwise Wilcoxon; p = 0.003) (Figure 5.2). Mean (±SE) sustained swimming speed at early postflexion stage was 2.96 ± 0.11 cm s^{-1} and 2.53 ± 0.07 cm s^{-1} in warm and cold condition, respectively. No significant difference were observed in swimming performance between fish group of cold and warm condition at postflexion (Pairwise Wilcoxon; p = 0.207), with mean values of 5.50 ± 0.35 cm s^{-1} (cold) and 4.77 ± 0.42 cm s^{-1} (warm), and in early juveniles (Pairwise Wilcoxon; p = 0.905). In early juveniles, sustained swimming speed was 11.38 ± 1.00 cm s^{-1} and 11.75 ± 1.31 cm s^{-1}.

After a threshold temperature was reached, the probability of larval swimming decreased with increasing temperature and both the threshold temperature and the rate of decline depended on temperature (Figure 5.3 (a)). Rearing temperature and developmental stage significantly affected the temperature to loss of equilibrium (TLOE) (Log-rank; $p < 0.001$). At early postflexion stage, mean(±SE) TLOE was 16.89(0.3) and 23.4(0.4) °C for the 15 and the 20°C-reared fish, respectively (Figure 5.3 (b)). Mean time to exhaustion (TE) was also greater for fish reared at 20°C at the first developmental stage (Pairwise Wilcoxon; $p < 0.05$) (Figure 5.4). Similar results were observed in later developmental stage in TLOE. At postflexion, mean(±SE) TLOE was 21.91 ± 0.75 °C and 29.30 ± 0.28 °C for the 15°C- and the 20°C-reared fish, respectively (Figure 5.3 (c)), while no significant differences were observed between TE of the cold and warm condition at this physiological developmental stage (Pairwise Wilcoxon; $p = 0.084$). Finally in early juveniles, mean(±SE) TLOE was significantly higher at warm condition (log-rank; $p < 0.001$; Wilcoxon; $p < 0.001$) with 26.55 ± 0.47 °C and 33.15 ± 0.15 °C for the 15°C- and the 20°C-reared fish, respectively (Figure 5.3 (d)) (TE = 61.46 ± 3.48 min for 15°C-reared fish; TE = 70.22 ± 1.45 min for 20°C-reared fish).

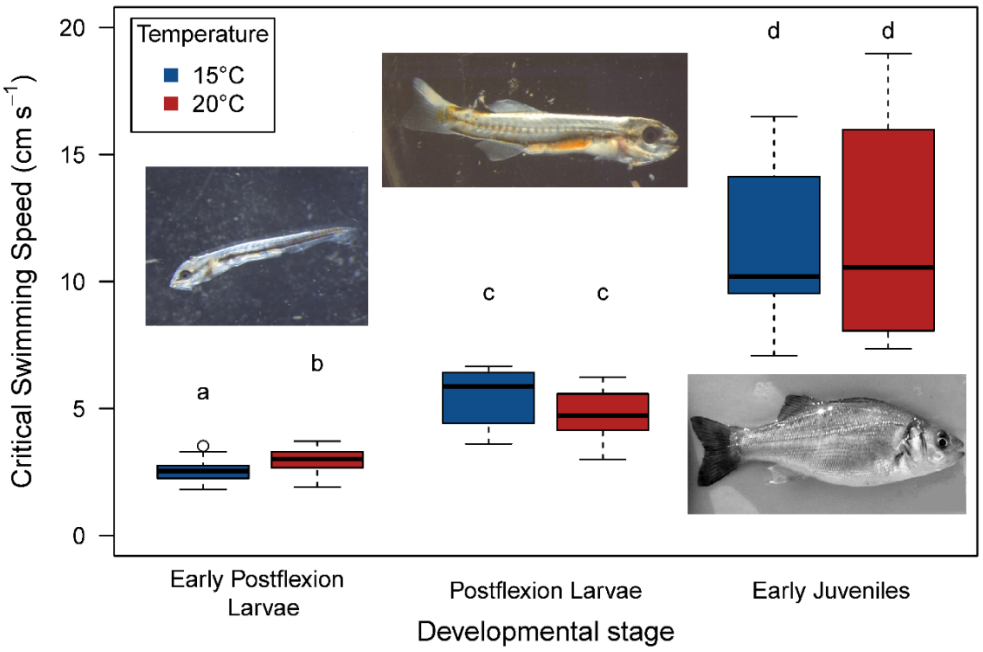


Figure 5.2 Distribution of critical swimming speed (cm s^{-1}) at three developmental stage early postflexion, postflexion and early juvenile stage in European sea bass reared at cold condition (blue) and warm condition (red). Different letters denote significant differences (Pairwise Wilcoxon, $p<0.05$) between each condition.

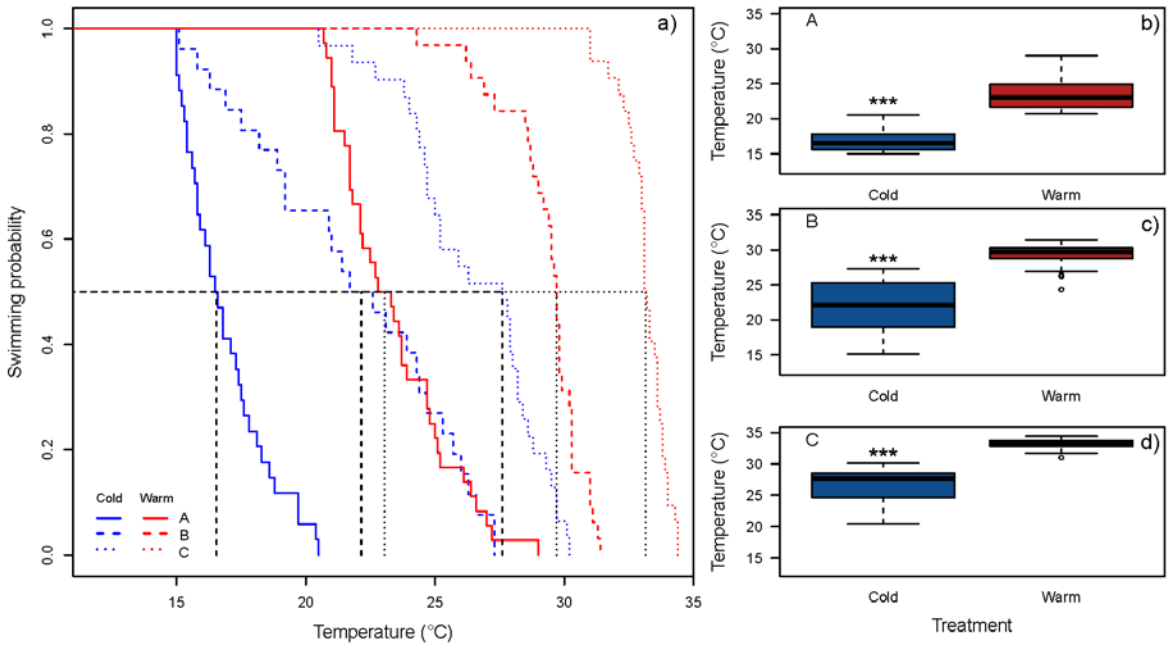


Figure 5.3 Probability of fish still swimming with increasing temperature (in °C) (a), in A early postflexion larvae (b), B postflexion larvae (c) and C early juveniles (d) European sea bass reared at cold condition (blue) and warm condition (red). Box and whisker plots correspond to the distribution of temperature to loss equilibrium. The whiskers denote the 10th and 90th percentiles, the box denotes the 25th and 75th percentiles, the median value is shown (horizontal line) as well as outliers (points).Stars denote significant differences (Wilcoxon, $p<0.05$) between temperature condition.

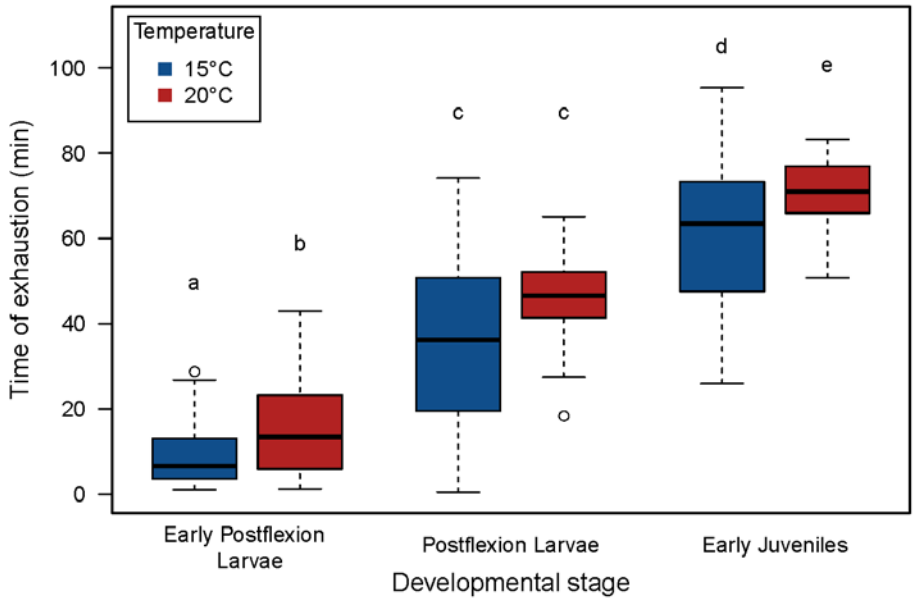


Figure 5.4 Box and whisker plots of the distribution of time to exhaustion. The whiskers denote the 10th and 90th percentiles, the box denotes the 25th and 75th percentiles, the median value is shown (horizontal line) as well as outliers (points). Letters denote significant differences (Pairwise Wilcoxon test, $p<0.05$) between temperature condition.

5.4 Discussion

Swimming performance of sea bass larvae is not impacted by ocean acidification (OA) conditions even when exposed at early sensitive stages. While juvenile and adult European sea bass can experience high levels of partial pressure of CO_2 (PCO_2) in coastal areas and estuaries (Borges et al. 2006), embryos and early life stages are usually exposed to lower and more stable levels of acidification (Doney et al. 2009). In addition of being unaccustomed to elevated and variable PCO_2 levels, early life stages, such as embryos and larvae, are particularly sensible to changes in hypercapnic condition due to their lack of ionic balance (Pörtner & Peck 2010; Hurst et al. 2013). In this study, a negative effects of OA was expected considering that individuals where conditioned just after fertilization at the earliest stage including cleavage which, according to Melzner et al. (2009), represent the true bottleneck for teleosts to cope with future OA conditions. Results, however, reported no significant difference in critical swimming speed (U_{crit}) between sea bass larvae exposed to ambient or Δ1000 PCO_2 level. Although a few experiments have highlighted some impairment in swimming duration or orientation (Pimentel et al., 2014) and a reduction in U_{crit} and mean speed after a stimuli (Watson et al. 2018), the majority of the studies found similarly to our results no effect of elevated PCO_2 levels on swimming performance. Indeed, Cominassi et al. (2019) already reported no effects of OA on swimming performance in sea bass larvae conditioned at the beginning of the larval stage (3 dph). Munday et al. (2009b) noted that high acidification did not affect U_{crit} of larvae of the clown anemonefish (*Amphiprion percula*) and Bignami et al. (2014) reported that swimming speed (U_{crit}) and mean routine swimming were unaffected by future OA conditions in larval of both cobia (*Rachycentron canadum*) and mahi-mahi (*Coryphaena hippurus*). While the upkeep of the swimming performance might be due to the species tolerance, it is also possible that it results from a transgenerational acclimation. In this study, swimming test were conducted in larvae from parents already conditioned to these levels of PCO_2. The negative effects of elevated levels of PCO_2 on growth and survival in offsprings anemonefish (*Amphiprion melanopus*) (Miller et al. 2012) or survival in Atlantic silverside (Murray et al., 2014) have been reported to be offset when parents were first exposed to OA conditions. The question is thus if this work add to the body of literature reporting no effect of PCO_2 on swimming capacity or if the absence of vulnerability is due to potential transgenerational plasticity (TGP).

In Cominassi et al. (2019), larvae swimming capacity was unaffected by PCO_2 levels but significant differences were observed with rearing temperature. Before reaching metamorphosis, at equivalent body length (BL), better U_{crit} was observed in 15°C-reared larvae than in 20°C-reared ones. In this study, U_{crit} was also impacted by temperature, however, U_{crit} was high in the 20°C-reared fish throughout the larval, i.e. from 14 dph until metamorphosis. The U_{crit} is a fairly easy trait to measure in most species, and the methodology allowed to get data analog from one species to another and among developmental stages (Nelson et al. 2002; Plaut 2001). In both studies, U_{crit} has been measured from early larval stage until metamorphosis in European sea bass. It is important to note, however, that even through fish

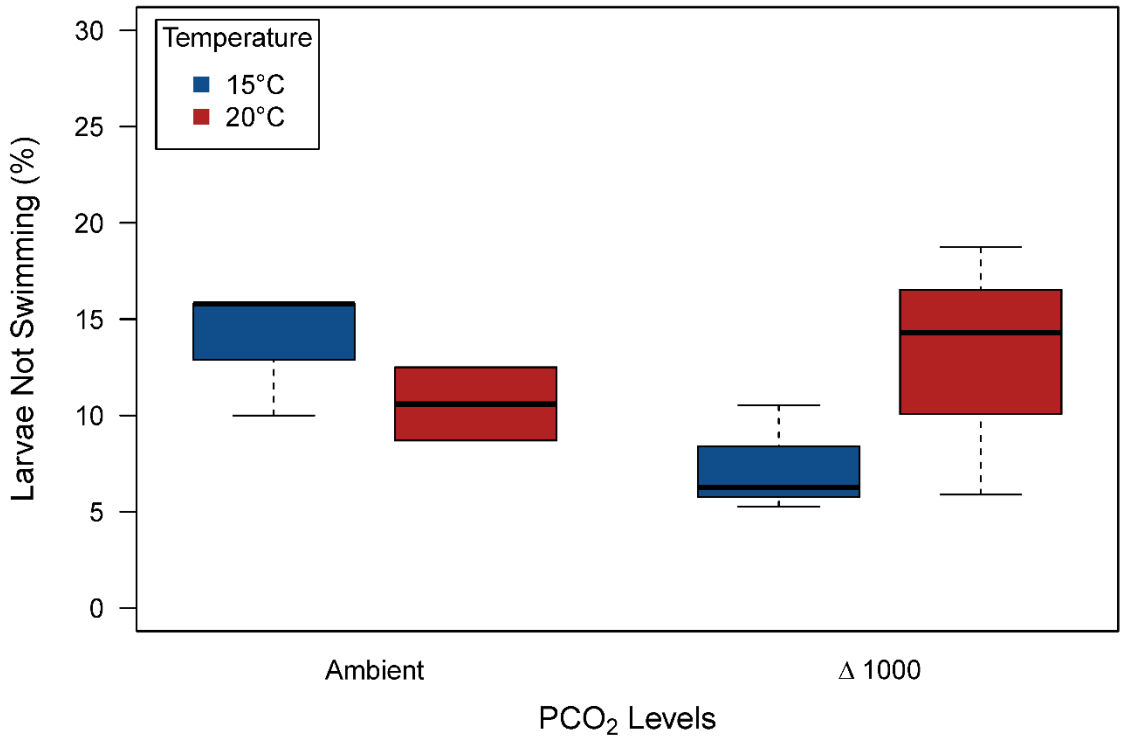
were measured at the same time of day with Brett-type flume (Stobutzki & Bellwood 1997), the tunnel used was not the same. While larval rearing and methodology for U_{crit} measurements were similar, parental conditioning differed between the two experiments. Parent's form Cominassi et al. (2019) study came from the wild and as such experienced ambient temperature from early stages (open sea) until maturing adult (coastal temperature from the Morbihan bay), whereas in this work parents were exposed to a warmer temperature of 19°C during their larval phase before being maintained at ambient temperature (see Crespel et al. 2017). The absence of reduced performance observed in 20°C-reared fish prior to metamorphosis might be due to TGP. Donelson et al. (2012) had showed that fish impairments with warming could be set-off when previous generations where first exposed to the same elevated temperatures. An increase of the water temperature of 3°C led to a decline in aerobic scope in spiny damselfish (*Acanthochromis polyacanthus*) but when two previous generations where exposed, offspring had their aerobic scope restored, suggesting a total acclimation to high temperature. Similarly, Salinas & Munch (2012), found that 30 days of parental conditioning change the response to temperature in offspring sheepshead minnow (*Cyprinodon variegatus*). Offspring from parents reared at low temperature grow better at low temperature and offspring from parents exposed to high temperature showed better grow at high temperature, which suggest an adaptive response. In these two studies parents were exposed either throughout all their lives to the environmental changes or just before fertilization, while in our study parents were exposed to the warming condition only during early development. It has been reported that hypoxia and warming in the European sea bass (Cadiz et al. 2017) and hypoxia alone, in zebrafish (*Danio rerio*) (Robertson et al. 2014), encounter at early stages can have long-lasting impact on fish metabolism and consequently on their history traits. Thus, the experience of a chronic warming event during early stage would allow for TGP in a key performance such as swimming speed. Furthermore, while Cominassi et al. (2019) reported higher proportion of individuals unwilling to swim, here no difference were observed between the two temperatures. While it might imply that temperature-dependent TGP could lead to offset the negative behavior display by the parents after being exposed to warm condition, further investigation are needed. Potential for TGP in fish behavior have been explored in response to OA (Welch et al. 2014) but, as we know of, never to warming.

It appears that temperature-dependent TGP lead to better swimming performance at higher temperature in sea bass larvae but one can wonder if the acclimation induced a shift in thermal optimum, thermal window and general temperature tolerate by the species at larval stage. In Cominassi et al. (2019), no information was provided concerning thermal performance curves for maximum scope for activity (MO2max) nor U_{crit} in sea bass larvae. It was suggested, however, that the optimum was below 20°C, 20°C being likely a subletal warm temperature for the species at larval stage (see Chapter 2). Thus, a challenge test was conducted to assess if parental conditioning might modify the upper temperature tolerated, or critical thermal maximum (CTmax). To do so, we first determined a speed of sustainable swimming to be assure to measure the temperature of loss equilibrium (TOLE) and not a

fatigue state. U_{crit} swimming is support by the red oxidative muscles and is a measure of aerobic endurance performance (Marras et al. 2013). In an ideal situation of oxygen concentration and nutrients supply, the aerobic metabolism is able to support these muscles indefinitely when confronted to slow to moderate speeds. In this study, the mean swimming speed was used as a sustainable swimming speed at three developmental stages. Mean swimming speed was significantly higher at the first developmental stage in 20°C-reared larvae and were equivalent for both temperature in later stage. Results of the temperature test showed that while time of exhaustion (TE) was similar at both rearing temperature, 20°C-reared larvae support highest temperature compare to 15°C-reared larvae. Those values, however, are lower than the one reported by Moyano et al. (2017), using the dynamic method (ramping assay) and warming rate of 0.5 to 9°C h^{-1}. Mean CTmax was 32.8°C for postflexion sea bass larvae reared at 20°C and originated from the same experiment as Cominassi et al. (2019). While the part for potential TGP temperature-dependent cannot be completely overlooked, results from Moyan et al. (2017) suggest that it is likely that upper temperature tolerate in our 20°C-reared fish were due to larval conditioning alone.

Overall, this study highlighted the potential for TGP to influence a key fitness trait such as locomotion in early stages of the European sea bass. Conditioning the parents to even a short warming episode during their early development increase the resilience of the offspring to a +5°C temperature increase. This study also providedan example of technic to assess fish thermal maximum. Maximum temperature supported by the individuals was higher in 20°C-reared fish compared to 15°C reared-fish. Whether the increase in thermal maximum is tie with larval conditioning alone or transgenerational effect is not clear, however, higher thermal maximum can be beneficial for early juveniles as they might encounter heat waves when reaching coastal areas.

5.5 Supplementary Information



S5.1 Figure. Proportion (in %) of European sea bass larvae not swimming during the U_{crit} trial in the cold (15°C) and warm (20°C) treatments at two *P*CO_2 levels (Ambient *P*CO_2 (650 µatm); Δ1000, ambient + 1000 µatm CO_2).

CHAPTER 6: General Discussion

Anthropogenic activities have intensified global climate changes, and new stressor have emerged and reached unprecedented levels, challenging marine organisms (IPCC 2014; Carroll et al. 2007). Many of these stressors, including increasing temperature, ocean acidification, coastal hypoxia and ocean deoxygenation co-occur in time and space (Harley et al. 2006; Doney et al. 2012). This may lead to a reduction in ocean productivity, changes in food web dynamics, modified community structures or shifts in species distributions (Hoegh-Guldberg et al. 2015). To limit such effects of climate change, it is essential to identify the stressors with the potential to negatively impact the resilience of ecosystems (Peterson et al. 1998). Physiological responses of marine species exposed to a single-factor stressor have been largely examined, while the influence of combined stressors has yet to be fully investigated (Vinebrooke et al. 2004). Although organisms can be resilient to a single stressor, adding another stressor can significantly increase the complexity of the responses of organisms due to potential interactions between the stressors or their effects. Therefore, experiments involving only one stressor might over- or underestimate the sensitivity of organisms to environmental changes. The goal of this thesis was to assess the potential of one temperate species of teleost, the European sea bass (*Dicentrarchus labrax*), to cope with projected future levels of partial pressure of CO_2 (PCO_2) and future temperatures. The combined effect of these two stressors, ocean acidification (OA) and ocean warming (OW), was investigated via a full factorial experiment producing original data regarding the species physiological responses on a number of traits across generations.

6.1 Impacts of acidification and warming (OAW) on European sea bass larvae and their ecological implications

Ocean acidification and warming (OAW) alter essential ecologic traits in sea bass larvae. Future warming conditions elicited a growth increase in sea bass larvae (Chapter 2). Larval growth was almost twice higher in individuals exposed to future temperature (+5°C) compared to larvae maintained in present day conditions (15°C), which subsequently results in a shorter larval stage duration of almost one third in the former. While temperature strongly influenced growth, no significant differences were revealed by elevated levels of acidification, and no interaction between high levels of partial pressure of CO_2 (PCO_2) and warm condition was

detected. In early life stages (eggs to young juveniles), faster growth rates are expected to heighten the chances of survival by decreasing the time spent in life stages particularly vulnerable to mortality through predation and starvation (Anderson 1988; Bailey & Houde 1989; Ferron & Leggett 1994; Pepin 1991). For example, Takasuka et al. (2004) observed that slower-growing larvae were more likely to be consumed by predators than faster-growing larvae of the same body size suggesting that predator avoidance was reduced in slower growers. That and a variety of other studies have documented the selective mortality of slower growing individuals, although evidence from these same studies also suggest that the relationship between growth and survival is complex and context-specific (i.e. depending on year, cohort, predators) (e.g. Meekan & Fortier 1996; Robert et al. 2007; Takasuka et al. 2004). Life-time rearing at +5°C above ambient, determined in the present study, appears thus beneficial for sea bass larvae, reducing the time spent in a particularly vulnerable stage.

While ocean warming (OW) positively affects a key fitness trait in sea bass larvae, i.e. growth, its effect alone or in combination with ocean acidification (OA) appears either deleterious or debatable in other ontogenetic characteristics, such as vertebral development. Elevated temperature alone and in synergy with OA increased skeleton ossification (Chapter 3). Highest calcification rates during development were observed at the elevated PCO_2 level in combination with elevated temperature. While it remains unclear whether faster ossification in teleost early stages is advantageous or not, it was accompanied by an increase in vertebral malformation frequencies. An increase in occurrence of vertebral deformities is likely to induce changes in locomotor abilities, feeding efficiency and in the general individual performance (Powell et al. 2009; Bignami et al. 2013) and subsequently to reduce the chance for larvae to survive. Similarly, otolith size and shape appeared to be unaffected by PCO_2 levels alone but were affected by increased temperature and the interaction between the two stressors (Chapter 3). The findings from Chapter 3 revealed that OA and OW operated together, increasing otolith formation in sea bass early stages and preventing otolith elongation with growth. Otoliths are implicated in the sense of orientation and hearing and alterations in otolith size and shape evolution may modify the sense of balance or direct motion detection in the larvae. Further, investigation focusing on potential changes in behavior are required to fully understand the implications of otoliths alterations.

Swimming performance responses to ocean acidification and warming (OAW) lead to the conclusion that the combined stressors impair the overall health of the species at early life stage. The critical swimming speed (U_{crit}), was negatively affected by elevated temperature in European sea bass larvae (Chapter 2). Reaching metamorphosis, larvae exposed to warm condition display a reduction in U_{crit} of about 20% compared to larvae exposed to cold condition. Locomotor performance is considered as a fundamental character for survival in the majority of marine fish species because it influences the capacity of individuals to forage, to avoid predators and to mediate dispersal towards nursery habitats (Leis et al. 2004). This fitness trait is even considered as an indicator of functional integrity and species health (Fry 1947; McKenzie et al. 2007; Claireaux et al. 2013). To date, animal health is defined as the

ability of individuals to adapt or respond to life challenges and environmental changes (Frankish et al. 2001). Thus, measuring whether or not fish swimming performance changes in the new environment gives an insight into the potential of the organism to cope with the altered conditions (Somero, 2005). In this study, although elevated levels of PCO_2 did not affect swimming performance in sea bass larvae, swimming capacity was reduced at elevated temperature suggesting that sea bass larvae are unable to cope with the future environment.

6.2 Specificity of the physiological response

6.2.1 The Impacts of OAW are traits-specific.

The effects of ocean acidification and warming (OAW) vary among performance and fitness traits (Chapters 2 and 3). In the previous section I demonstrated that OAW increased growth and calcification rates but reduced swimming capacity in sea bass larvae. A number of hypothesis can be emitted to try to elucidate why the responses differ among traits. First it might be link with the 'multiple performances – multiple optima' (MPMO) hypothesis. This hypothesis supposes that the thermal optimum differs among each physiological activity (Clark et al. 2013). Therefore, while a +5°C increase from ambient temperature might be optimal for growth it may not be a thermal optimum for other performances such as swimming capacity (Chapter 2).

Still investigating growth and swimming performance responses, I assumed secondly that the divergence between the two responses of the performance, i.e. positive growth and reduced swimming, might be due to a trade-off. The energy normally allocated to one fitness trait is redirected to another key performance leading to a reduction in the former (Sibly & Calow 1986). Literature already reported that rapid growth in early life fish can lead to decrements in other fitness-related traits including swimming performance as demonstrated for fathead minnow (*Pimephales promelas*) and Atlantic silversides (*Menidia menidia*) (Billerbeck et al. 2001; Kolok & Oris 1995). In this study, the existence of a trade-off between growth and swimming capacity suggests that while the increase in growth with warmer temperature first appears beneficial for the species by shortening the duration of the larval phase, it occurs at the detriment of another major fitness trait and therefore cannot be perceived as a positive effect of future conditions.

As performance and fitness provide insights into the degree of resilience of an animal to a stressor, it is important to broaden the selection of traits studied to better predict the ecological effects of future high CO_2 and temperature on fish. Although measuring the critical swimming speed (U_{crit}), somatic growth and other developmental features gives a good first insight on how OAW might affect sea bass larvae, it is likely that other indicators of fitness need to be evaluated to obtain a truly holistic assessment of the adaptive capacity of the organism (Melzner et al., 2009). Aerobic scope or reproduction output, for example, can be used to assess fitness traits and the energetic budget of the organisms, reflecting either acute

"real time" stress or considering overall energetic variations by incorporating reallocation of energetic stores (Melzner et al., 2009).

6.2.2 Responses to OAW may be minimized by substantial food supply

Temperature and acidification interact with food availability and the feeding level appear to shape the growth response of marine organisms exposed to these stressors (Hettinger et al. 2013; Thomsen et al. 2013; Stiasny et al. 2019). The experiment described in Chapter 4, conducted on juvenile sea bass demonstrated that individuals maintained at cold condition grow faster when fed *ad libitum* ration compared to restricted ration, but no difference in growth was reported between levels of partial pressure of CO_2 (PCO_2). Indeed the specific growth rate (SGR) was similar between fish exposed to ambient and acidified conditions at both *ad libitum* and restricted ration. Juveniles reared at the warm condition exhibited faster growth compared to those reared at the cold condition, but only at ambient PCO_2 level. Individuals from acidified and warm waters had similar growth rates as cold-reared fish at the respective ration, restricted or *ad libitum*. In addition, at both PCO_2 levels growth was increased with ad libitum feeding in comparison to restricted feeding.

While the effect of the potential interaction between stressors has often been described as additive, synergistic or antagonistic (Vinebrooke et al. 2004) the potential interaction between OA and food supply has been characterized by Brown et al. (2018) as non-interactive (the effect of OA is independent of food availability), positive interactive (the response to concurrently elevated levels of food and PCO_2 is positively or negatively greater than the response to a single factor), or negative interactive (the addition of food lead to a stronger negative response to OA). Hence, no interactive effects of OA and food supply in growth were found at cold condition. At warm condition, although an effect of both, ration and PCO_2 levels, was observed, the difference in SGR was the same between the two PCO_2 levels at restricted ration and *ad libitum* feeding. Thus, PCO_2 reduced the growth of juvenile sea bass similarly whether they are fed with *ad libitum* or restricted rations. Therefore, the relation between OA and food supply appeared non-interactive

Although, in this work, a non-interactive relation between OA and food availability was found at the organismal level, looking further into underlying mechanisms that potentially explain differences in growth of European sea bass between treatments lead me to believe that the relation between OA and food supply might be different at a more basal level. Indeed, results of feed conversion efficiency (FCE) measurements suggested that at warm condition, an interaction between PCO_2 levels and food ration exists. The drastic reduction in FCE observed in fish at high PCO_2 level at restricted ration is minimized when fish are fed *ad libitum*. The FCE response in European sea bass exposed to OA is thus positively modified by food. It might be possible that the effectsof the interaction, i.e. somatic growth, emerge only after a longer rearing period, i.e. longer than a one month exposition of different levels of feeding.

Besides reporting an interaction between OA and food levels, Chapter 4 highlighted that the difference in growth between the different OAW conditions could largely be explained by changes in energy allocation at the detriment of digestive efficiency. Indeed, at warm condition, trypsin activity was significantly reduced at elevated PCO_2 revealing an antagonist effect of OA and temperature in trypsin activity response. Thus it appears that to cope with elevated temperature and acidification levels, sea bass juveniles require higher energy input. Furthermore, without sufficient energy, i.e. food supply, energy that would be allocated for trypsin in normal condition might be redirected, likely towards bicarbonate production in the lumen. Therefore, I supposed that OAW act as metabolic stressors impairing the acid-base balance in fish and that it is likely that to counter the decline in homeostasis function, more energy might be allocated to this regulatory process at the cost of digestive efficiency. Although there is no evidence in the literature to support that digestive function might be impacted, the regulation of the acid-base balance was improved in an Antarctic fish (*Notothenia rossii*) exposed to elevated level of PCO_2, at the detriment of several fitness traits including calcification or osmoregulation; likely due to changes in energy allocation (Strobel et al. 2012). Overall, it appears that OAW induces changes in energy pathways in the fish organism and that substantial food supply may provide ample energy allowing the organism to compensate for potential negative impacts of sub-lethal OA and/or OW conditions.

It is not clear what the existence of an interaction between OA and food availability implies. It is true that elevated PCO_2 levels can increase food availability (e.g. for some herbivores; Gaylord et al. (2015)) but the contrary can also be conceivable (Polovina et al. 2008). Thus it will likely differ between species and habitats. This suggests, however, that previous studies, which generally use *ad libitum* feeding when conducting experimentation, might have underestimated OA effects and therefore, there is a need to introduce different and relevant feeding levels in future laboratory work.

6.2.3 Impact of OAW are species specific

The effects of OAW differ not only among traits but also, depending on the trait, among species. Higher skeleton ossification was reported, previously in this section and in Chapter 3, in sea bass larvae exposed to projected future environment. Similar findings have been found in other studies conducted in a number of species exposed to OA alone. Elevated levels of PCO_2 were found to increase the ossification of the vertebrae and gills of Atlantic cod larvae (Stiasny et al. 2019), and the mineralization of the jaw and the crus of an elasmobranch species (*Leucoraja erinaca*) (Di Santo Valentina 2019). While skeletal formation appears similarly impacted by OA among species, the response in other traits differs significantly from one species to another when confronted to the stressor. For example, the absence of effects of OA on sea bass somatic growth (Chapter 2) was consistent with previous findings reviewed by (Cattano et al. 2018), but contrasts with the findings of a number of other studies. In the

anemone clownfish larvae (*Amphiprion percula*) OA treatments induced higher growth rates (Munday, Donelson, et al. 2009), whereas slower growth rates were recorded for the gilthead seabream (*Sparus aurata*). Baumann et al. (2012) and Pimentel et al. (2016) also observed reduced length-at-hatch for inland silversides (*Menidia beryllina*) exposed to high PCO_2 levels.

Similarly, swimming capacity is affected very differently by OAW among species. Swimming performance can be assessed by measuring U_{crit} (Chapter 2; Melzner et al. 2009; Munday et al. 2009; Silva et al. 2016) but also by testing routine swimming (Maneja et al. 2013; Maneja et al. 2015). Swimming performances have been reported to be either unaffected by OA or subject to impairments. For example, OA did not influence U_{crit} in sea bass larvae (Chapter 2), in anemone clownfish (*Amphiprion percula*) larvae (Munday et al. 2009), in larval cobia (*Rachycentron canadum*) and mahi-mahi (*Coryphaena hippurus*) (Bignami et al. 2014), but reduced U_{crit} in the yellowtail kingfish (*Seriola lalandi*) (Watson et al., 2018). Routine swimming appeared to be mostly negatively affected by future OA conditions. Pimentel et al., (2014) reported that OA decreased vertical orientation frequency and swimming duration in dolphinfish (*Coryphaena hippurus*), while Watson et al. (2018) found a reduction of the average speed in response to a stimulus in juveniles yellowtail kingfish (*Seriola lalandi*). It is thus not clear whether predicted PCO_2 levels influence swimming as a physiological performance or if they impact behavior in some species by reducing the willingness to swim.

Subjected to acidified and warming conditions, the response of developmental structures also differs from one species to another. For example, in this study (Chapter 3), future climate projection did not influence the asymmetry of otoliths. These results diverge from a handful of studies reviewed by Holmberg et al. (2019), noticing asymmetry in otoliths size and shape when exposed to acidified waters, yet agree with a couple of experiments conducted in the orange clownfish (*Amphiprion percula*) (Munday et al. 2011), in a shore clingfish (*Lepadogaster lepadogaster*) (Martins 2017) and in the Japanese rice fish (*Oryzias melastigma*) (Mu et al. 2015). Furthermore, while the study (Chapter 3) found that frequency of malformation in sea bass larvae was increased under a OAW combination but not PCO_2 levels alone, Pimentel et al. (2014b) noticed that sole (*Solea solea*) larvae displayed higher percentage of deformities with OAW, but also with elevated PCO_2 levels.

Comparing the results to previous findings, I noticed first the lack in studies assessing the impact of OA together with warming and that although a number of studies reported relatively similar results, a variety of responses among species have also been found in diverse traits. I assume that this difference in responses between species might be due to the ecology of the species and thereby their degree of plasticity.

The difference in sensitivity to projected climate-driven warming, among species might be explained by the thermal tolerance of species. Adult sea bass are believed to support temperatures from 2 to 32°C, and demonstrated maximum growth rates at temperature between 22 and 24°C. Therefore, an increase of water temperature of 5°C, from 15 to 20°C, is

considered beneficial for the species at adult stage as it will likely enhance the somatic growth of the species. The thermal range and temperature for optimal performance, however, is not known in larvae and while growth was increased by elevated temperature, the larvae displayed poorer performance. Pörtner & Farrell (2008) highlighted that the thermal window can shift and is less wide during reproduction or early life stages such as embryos and larvae. It is thus likely that not only the thermal window changes with ontogenic stage but also the temperature optimum for fitness traits, and that 20°C is already a sub-lethal temperature for sea bass larvae. While a life-time exposure to +5°C above ambient temperature, in sea bass is likely to threaten the future of the species, other species might benefit from this temperature implement. Species inhabit environment mainly cooler than the temperature for optimum growth rates (Sandblom et al. 2016) and probably other aerobic performances. Supposedly, they encounter a thermal-safety margin allowing them to endure unexpected change in temperature such as heat waves (Sunday et al. 2014). Therefore, depending on where the individuals are on the scope for aerobic performance and how narrow the thermal window of the species is throughout all ontogenic stages, the same temperature increment will lead to possible increase in growth rates in some species while it will likely reduce fitness in other species via the impairment of important processes due to protein denaturation and enzyme inhibition (Pörtner & Knust 2007). Thus, while elevated temperature predicted for the end of the century can act as a stressor in some species, it can on the contrary stimulate fitness traits in others by getting the species closer to their thermal optimum.

It is unknown, whether a similar tolerance window exists for PCO_2 levels. The potential of resilience to OA of a species, however, is likely tied to the life history of the species and its phenotypic plasticity. A pattern emerging from existing literature is the increased vulnerability to high PCO_2 levels of early life stages compared to later adult stages. In this study, while largely impacted by the combination of temperature and acidification, sea bass larvae appeared relatively unaffected by elevated PCO_2 levels. I assumed that sea bass larvae tolerance to acidified conditions correlates with the life cycle of the species and the gradient of PCO_2 encountered from young stage to adults. Sea bass embryos are spawned offshore in the open sea where environmental conditions are relatively stable (annual variation of <0.1 pH units) (Pickett & Pawson 1994; Perez-Ruzafa and Marcos 2014; Doney et al. 2009). Larvae, however, migrate towards coastal water and early juvenile stages are thus encountered in coastal waters where PCO_2 levels fluctuate strongly over a day or over the seasons (Ringwood & Keppler 2002). Therefore, juvenile and adult experience significant variations in PCO_2 levels (e.g. daily variation up to 1 pH unit) (Hofmann et al. 2011). For example, the stock of sea bass of the Bay of Biscay experienced PCO_2 levels of 452 to 2780 µatm and 612 to 2829 µatm between 1992 and 2004 in the estuary of the Loire and the Gironde Rivers, respectively (Borges et al. 2006). In Atlantic sea bass, exposure of adults to high values and high variability of PCO_2 levels conferred the phenotypic plasticity needed for larvae to sustain consequent OA.

In fish, resilience to OA fluctuations might be evaluated and/or predicted on the basis of their ecology. Vargas et al. (2017) studied species-specific responses to OA by examining the response of individuals from the same species but from remote populations experiencing contrasting oceanographic regimes. All taxa (copepod, gastropods and mussels) displayed the same pattern: organisms from estuarine waters, naturally exposed to high mean PCO_2 levels and high variation in PCO_2 levels, were more tolerant to future OA conditions. These results suggest that chronic exposition to a local environment characterized by high physicochemical variability and high PCO_2 levels might lead to an adaptive response of physiological traits, and that tolerance of species and population to OA might correspond to an environmental gradient in different habitats. In addition, rather than species-specific, responses appeared to be population-specific. In the number of taxa analyzed in the study, fish were not represented. Similarly to our findings, Baumann et al. (2018) reported that despite a decrease in embryos and larvae survival, Atlantic silversides (*Menidia menidia*) were relatively robust to elevated PCO_2 levels up to 2000 µatm, and that this resilience is likely based on the fact that the fish already experienced such conditions in the wild with important diel and seasonal variations. The fact that all taxa were affected in the same way, in combination with results from this study and findings reported by Baumann et al. (2018), lead to conclude that fish species and populations will be affected similarly. Fish and particularly large pelagics, however, are mostly migratory species. Instead of positioning the species along a gradient from coast to open-water, and consequently from a variable to a stable environment, it might be interesting to class fish sensibility to PCO_2 levels according to their migratory life cycle and the duration and/or distance of their stay inshore. This assumption is in accordance with the "Ocean Variability Hypothesis" suggested by Baumann (2019) posing that the resilience of marine species to CO_2 fluctuation depends 1) of the magnitude of the CO_2 fluctuations experienced over a short time period in their habitats and 2) on the duration of their early life stage (Figure 6.1). This generalization emphasizes the need to consider the particularities and fluctuations of the environment of organisms when designing experiments to investigating the effects of OA. Rather than measuring the response of a fitness trait to a fixed level of PCO_2, it would be interesting to assess the plastic limits across organismal traits, populations and species when exposed to OA fluctuations. This might possibly be assessed through experimental tests of critical population-level. Therefore, there is a need to conduct experimentation introducing scenarios that take into account the natural variability of the environment of species. This also suggests that the response of sea bass larvae might differ among populations (e.g. Atlantic and the population from the Mediterranean Sea) and even among stocks making managing decisions even more complex.

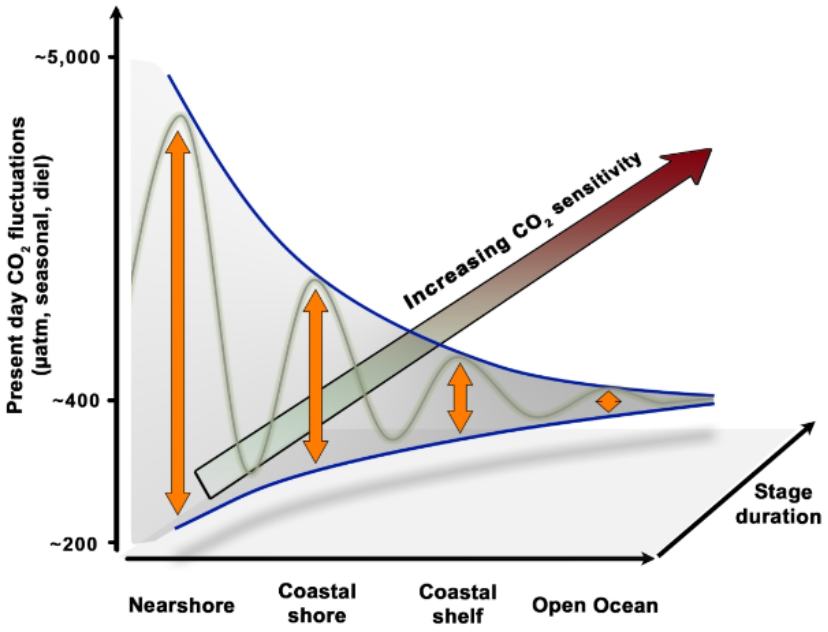


Figure 6.1 Illustration of the Ocean Variability Hypothesis

6.3 The potential for transgenerational acclimation and its implications

Long-term studies are essential to realize an accurate assessment of the effect of projected future climate conditions in marine organisms. While the absence of clear effects of ocean acidification (OA) on the different fitness traits (Chapters 2 and 3) in sea bass larvae might be explained by the phenotypic plasticity of species, but might also be due to transgenerational adaptation. In our studies (Chapter 2 to 4), experiments were conducted on the offspring of wild-caught adults maintained in aquaculture facility for about 5 years. Over the spawning period, water pH of the environment of the parents has been reported to drop to 7.6, which is equivalent to the highest levels of partial pressure of CO_2 (PCO_2) implemented in the different experiments. Therefore, the absence of differences in a physiological trait such as swimming performance might be the result of a previous exposition of the parents to low pH. Similarly, previous studies have highlighted that conditioning the parents to high levels of PCO_2 may decrease the sensitivity of their progeny to OA (Jarrold & Munday 2019; Munday 2014; Murray et al. 2014).

The swimming capacity of early life stages of sea bass appeared to be unaffected by elevated PCO_2 levels and the absence of changes to OA was maintained in the next generation. Warming has been shown to impact negatively on swimming performance in larvae when reaching metamorphosis. When exposing parents to a warm episode during their early development, progeny appeared tolerant to OW and showed similar swimming capacity whether they were maintained in cold or warm conditions. As previously mentioned, changes

in swimming performance is likely to impact foraging, predator escape and dispersion (Leis 2006; Wolter & Arlinghaus 2004). Thus, mitigation of this key fitness trait by transgenerational exposure to OW is highly beneficial for the species and showed that the species might be able to cope with future warming conditions. The potential for transgenerational acclimation, however, still raises a few concerns. First, it is probable that a mechanism supporting one trait might not be as plastic as another mechanism controlling another trait, which would influence the potential for transgenerational acclimation to OA and/or ocean acidification and warming (OAW). For example, processes supporting behavior and olfactory performance (Welch et al. 2014) may be less plastic than metabolic pathways (Miller et al. 2012) or swimming performance. Second, although transgenerational acclimation in sea bass lead to the preservation of the swimming performance of individuals exposed to OAW, it is not clear if at even longer term this plasticity is beneficial for the species. Indeed, ties between plasticity and adaptation represent a significant part of evolutionary research. Opinions diverge, however, on whether plasticity facilitates or slows down evolution (Merilä 2015). While adaptive plasticity might enhance evolution through the selection of specific genes supporting the trait (genetic assimilation) (Pigliucci et al. 2006), it could also delay genetic adaptation by moving the average phenotype closer to the performance height and consequently diminishing the gradient of selection with no changes in allelic frequencies. Finally, in spite of the fact that the majority of the studies show an amelioration of fitness traits through parental conditioning to OA (Parker et al. 2012; Miller et al. 2012), in some bivalves, such as the hard clam (*Mercenaria mercenaria*) or the bay scallop (*Argopecten irradians*), the negative impact of OA was even greater in the second generation (Griffith & Gobler 2017). This indicates that the sensitivity of the offspring is very likely species-specific. Hence, short-term experimental studies do not capture accurately the potential impact of future conditions of acidification and warming in marine species, and effects are either overestimated (the potential for amelioration of negative effects of OW and/or OA through transgenerational acclimation is not considered) or underestimated (the negative effects of OA are actually greater in the offspring despite previous parental exposition).

6.4 Concluding remarks

The results of this thesis (summarized in Figure 6.2) illustrate the need to investigate a range of traits to provide enough insight into the general health of the species and their ability to cope with future ocean acidification and warming (OAW) conditions. For sea bass it appears necessary to examine the impact of predicted acidification and warming on behavior as the stressors affected otolith size and shape and most likely the willingness to swim. Furthermore, it is essential to deeper investigate disturbance in energy partitioning. This could be estimated using individual respiration rates. Measuring animal respiration rates provides an assessment of the sum of energy consuming processes, and thereby can highlight small changes in specific function or if the alteration in one function is compensated for by another. While providing input into energy trade-offs at the individual level, assessing the potential of alteration of an organism can also provide information at the population or even the species level. For example, if organisms are enable to preserve their energy budget, reproductive contribution to the next generation might be jeopardized consequently compromising the abundance and distribution of the species. Although exposition to environmental factors is likely to elevated energy demand, the demand in energy might be compensated by increasing food intake and assimilation. Sensibility of marine organism to climate change is thus modulated by food availability, making predictions for species sustainability even more complex. It is therefore essential to conduct studies integrating different levels of feeding when investigating OAW effects.

Short-term studies allow to have some valuable insights into individual stress tolerance, but do not take into account potential extended fitness in the new environment. Long-term studies, conducted over a number of generations might show contrasting effects that cancel or exacerbate results from short term exposure to the future environment. Knowledge on the potential for acclimation and adaptation to future climate and especially ocean acidification (OA) is growing but remains scarce regarding fish with long life span such as the European sea bass. This dissertation provides a glance into the effects of parental acclimation in the European sea bass; nonetheless, the species would have to be maintained under OAW for several generations to accurately assess transgenerational effects and the potential for evolution. This is very difficult to execute as a practical matter considering that sea bass is a large species with long generation times. In addition, long-term studies should further consider variations of environmental stressors on relevant temporal scales. This study simulated the difference of temperature experience between early stages and juveniles stages but used relatively fixed high levels of PCO_2. In the wild, natural fluctuations occur in all environmental stressors and will probably increase as the mean of stressors rise. Acclimation and adaptation potentials of resilient individuals might benefit from environmental variations, however, further investigations are required to evaluate these effects. To really assess the future marine population in the new environment, it appears necessary to examine the impact of multiple fluctuating stressors on marine organisms over more ecologically relevant scales and exposed to relevant feeding level.

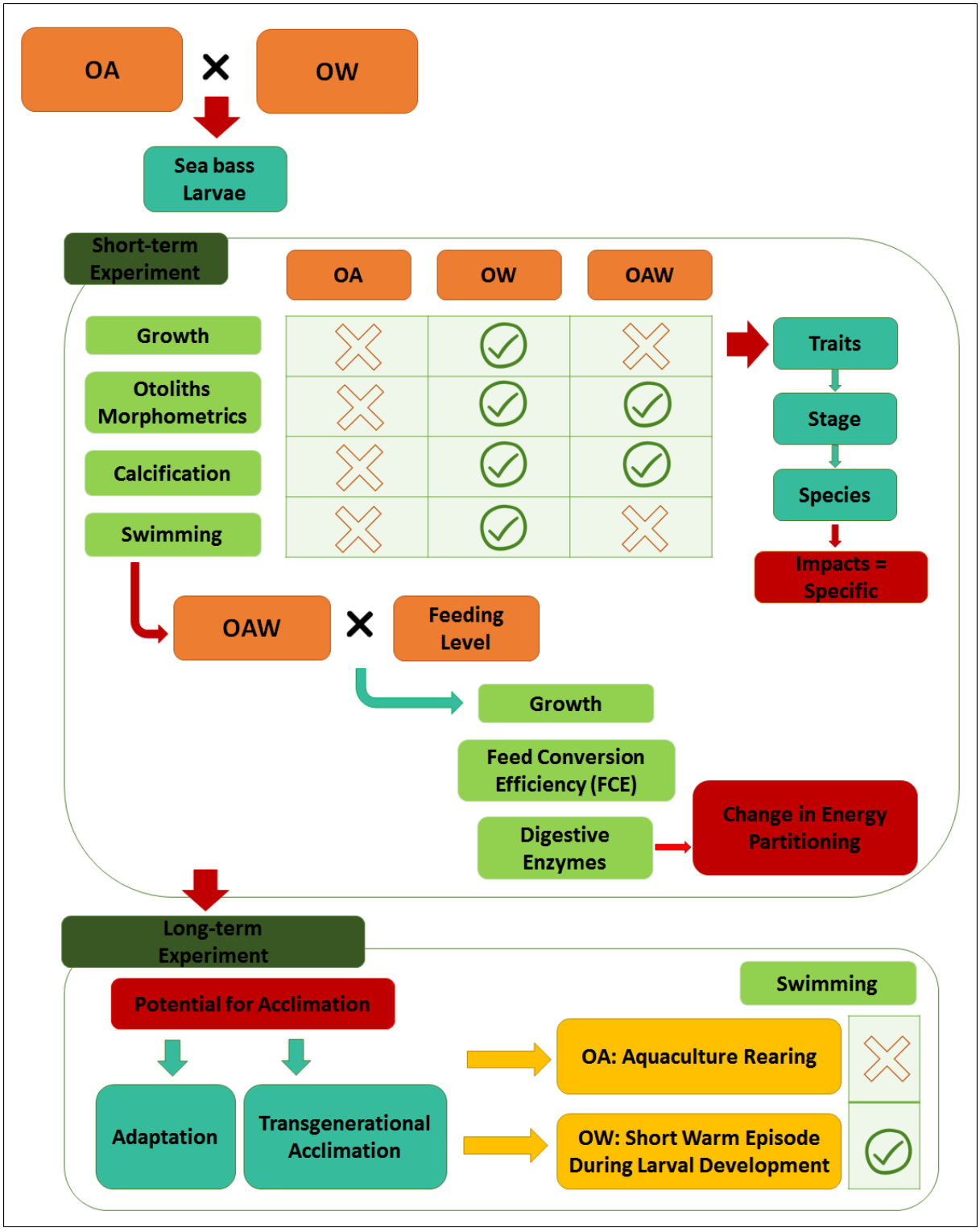


Figure 6.2 Schematic illustration of principal findings of the thesis. Red Coss represent the absence of effects from the corresponding stressor. Abbreviations: OA, ocean acidification; OW, ocean warming; OAW, acean acidification and warming.

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To whom it may concern,

I have briefly reviewed the thesis "Combined effects of ocean acidification and warming on a large pelagic fish, the European sea bass (*Dicentrarchus labrax*)" written by Louise Cominassi. The thesis is written in English. As a native English speaker, I can attest that the writing (grammar and syntax) is acceptable. The English writing is of sufficient quality to move forward with the submission and review of the thesis.

Sincerely,

Prof. Myron A. Peck

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Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg,

Louise Cominassi