Transgenerational effects on the metabolism of the European sea bass (*Dicentrarchus labrax*) in the context of ocean acidification and warming

Sarah Howald

Dissertation with the aim of achieving a doctoral degree at the Faculty of Mathematics, Informatics and Natural Sciences, Department of Biology of the University of Hamburg

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Supervisors of this thesis:	Prof. Dr. Christian Möllmann
	Dr. Felix C. Mark
Examiners of this dissertation:	Prof. Dr. Christian Möllmann
	Dr. Felix C. Mark
Examination comittee of the disputation:	Prof. Christian Möllmann
	Dr. Felix C. Mark
	Prof. Dr. Kai Jensen
	Jun. Prof. Dr. Flemming Dahlke
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Diese Arbeit ist meinem Opa und meinen Kindern gewidmet

Abstract

With the different parts of my thesis, I investigated the capacity of the aerobic metabolism of European sea bass *Dicentrarchus labrax* under current and future ocean acidification and warming (OAW) conditions. In order to get a broad overview of the aerobic metabolism of European sea bass I worked on different developmental stages (larvae and juveniles). While I worked only on the whole organism in larval fish, I added different levels of biological organization (metabolome, mitochondria and whole organism) on the juvenile stage to gain a more profound insight into the aerobic metabolism on this developmental stage. I compared the results of respirometry and growth data of "my" juvenile European sea bass to results from similar experiments conducted on their parental generation.

My thesis was embedded in the FITNESS project (Fish Transgenerational adaptive Strategies to ocean acidification and warming), which allowed me to work with two batches of European sea bass and additionally use the results of a third batch of fish for my analyses:

- Group 1 or F0 was raised and reared at three ocean acidification (OA) conditions prior to the start of my thesis. This group was the parental generation of one of "my" batches of European sea bass and I used the results of experiments on standard metabolic rate (SMR), critical oxygen concentration (PO_{2crit}) and growth of these fish to compare them to the data I obtained on group 2.
- Group 2 or F1 was the offspring of F0. The eggs were raised under the OA conditions of the respective parents. To add ocean warming (OW) to this generation, two temperatures were applied on larval rearing starting at 2 days post hatch. Although this was not a full transgenerational experiment, it allowed me to obtain an estimation of the capacity over successive generations to adapt to a changing ocean.
- Group 3 or Wild was "my" second batch. The fish originated from wild spawners in an aquaculture facility and were raised under six OAW conditions (two OA conditions crossed with 3 OW conditions) in the Ifremer facilities from 2 dph onwards. I used this group for my experiments on mitochondrial as well as on the metabolome level and compared the results to their respective growth data.

The OAW conditions were defined as follows:

Three conditions of OA:

- A: today's ambient situation in coastal waters of Brittany and the Bay of Brest (approx. 650 µatm CO_2)

- Δ500: a scenario according to the IPCC (2021) shared socio-economic pathway SSP3-7.0 (ambient plus 500 µatm CO_2– approx. 1150 µatm CO_2)
- Δ1000: a scenario according to SSP5-8.5 (ambient plus 1000 µatm CO_2 – approx. 1700 µatm CO_2).

The F1 fish were only reared under A and Δ1000.

Temperature condition in F0 fish:

- Larval rearing: 19 °C
- Juvenile rearing: ambient temperatures, without allowing the temperature to drop below 12 °C.

Two conditions of OW in F1 and Wild:

- Cold life condition (C):
  - Larval rearing: 15 °C
  - Juvenile rearing: ambient temperature (up to 18 °C), without allowing the temperature to drop below 15 °C
- Warm life condition (W):
  - Larval rearing: 20 °C
  - Juvenile rearing: Cold life condition + 5 °C

Both OW conditions in F1 and Wild were applied on all OA conditions, resulting in the following conditions: C-A, C-Δ500 (only Wild), C-Δ1000, W-A, W-Δ500 (only Wild) and W-Δ1000.

The aim of my thesis was to answer the following questions:

(1) Will mitochondrial performance be impaired in juvenile European sea bass heart mitochondria after long-term acclimation to OAW or after acute temperature changes?
I hypothesized that:
   a. LEAK respiration would increase after acute warming, due to thermal deterioration of mitochondrial membranes, resulting in impaired mitochondrial performance.
   b. Changes in mitochondrial membrane properties after long-term and developmental warm life conditioning would result in reduced LEAK respiration and therefore restored mitochondrial respiratory control ratios.
   c. Increases in intracellular PCO_2 and bicarbonate concentrations will impair mitochondrial metabolism, e.g. by inhibited citrate synthase or succinate dehydrogenase.

(2) Will OAW lead to synergistic effects on growth rates of larval and juvenile European sea bass?

My hypotheses were:

a. Growth rates of larval and juvenile European sea bass will be increased under OW.
b. Growth rates of larval and juvenile European sea bass will not be affected by OA.
c. Synergistic effects of OAW might impair growth rates of larval and juvenile European sea bass.

(3) Will OAW lead to synergistic effects on metabolic rates of larval and juvenile European sea bass?

I proposed the following hypotheses:

a. Larval RMR and juvenile SMR will be increased under OW
b. Larval and juvenile metabolic rates will not be impaired by OA.
c. Metabolic rates of larval and juvenile European sea bass will be lower under OAW due to synergistic effect.
d. Neither OA nor OW nor OAW will affect juvenile PO_{2crit}.

(4) Will transgenerational plasticity in European sea bass after acclimation of F0 to OA improve growth and metabolic rates of F1 under OAW?

My hypotheses were:

a. F0 and F1 W larvae will display similar growth rates (rearing temperature of 19 and 20 °C, respectively).
b. F0 and F1 C juveniles will be of similar size at 3000 dd (ambient rearing temperature in both generations).
c. F0 and F1 C juveniles will display similar SMR.
d. Growth and metabolic rates of F1 larvae and juveniles will not be impaired by OA.

(5) Will OAW induced changes in growth, metabolic rates and mitochondrial function become visible at the metabolite level?

I hypothesized that:

a. Increased growth rates due to OW will be reflected in increased protein synthesis in muscle and liver tissue.
b. Concentrations of metabolites involved in maintaining homeostasis, such as organic acids and osmolytes, will be altered due to OA, especially in gill tissue.
c. Alterations in metabolic profiles will reflect synergistic effects due to OAW.

Chapter 2 addresses the first question: I determined the capacity of mitochondria in permeabilized heart fibres of Wild juveniles. Acute warming of 5 °C resulted in significantly increased mitochondrial LEAK respiration in mitochondria of Wild C juveniles. This shows that this rather small temperature increase is already challenging for heart mitochondria. While acute temperature increases impaired mitochondrial function, heart mitochondria of warm life-conditioned juvenile European sea bass showed better mitochondrial functionality, for example, through increased respiratory control ratios (RCR). These improved mitochondrial capacities were reflected in higher growth rates of Wild W juveniles. OA did not affect respiration rates of complex I and IV of the electron transport system or RCR and inhibited respiration of complex II under OA was only significant in Wild W juveniles, when heart fibres were facing an acute temperature decrease (of 5 °C).

To answer the second question of my thesis, I analysed growth rates of F0 and F1 larvae and juveniles in chapter 3 and compared them. I also analysed growth rates of Wild larvae and juveniles in chapter 4. OW as a single driver increased growth rates in larval and juvenile F1 and Wild European sea bass. OA as a single driver did not affect larval and juvenile F0, F1 and Wild seabass growth rates. However, if both drivers were combined, F1 W-Δ1000 larval European sea bass were significantly smaller at metamorphosis than in all other treatments. This was not observed in Wild larvae or juveniles and could not be tested in F0 larvae and juveniles, due to the missing OW treatment. F1 W-Δ1000 juveniles displayed higher growth rates than F1 C juveniles, but due to the missing F1 W-A treatment it was not possible to define whether the detrimental effects of OAW persisted into the juvenile phase and were just masked by increased growth due to OW or not. Nevertheless, these data indicate a possible mismatch in the second generation of European sea bass, which can either be due to transgenerational effects or due to developmental plasticity, as the F1 fish were the only group in this thesis, which were reared and raised from fertilization onwards under OA conditions.

To answer question 3: I determined routine metabolic rates (RMR) of F1 larval European sea bass as well as standard metabolic rate (SMR) and critical oxygen concentration ($P_{O_{2crit}}$) of F1 juvenile European sea bass. SMR and $P_{O_{2crit}}$ were determined in F0 juvenile European sea bass prior to my thesis and I could use these data to compare them to F1. OA as a single driver did not have an effect on any of these traits. $P_{O_{2crit}}$ was not affected by OAW. The effect of OW as a single driver could not be tested. SMRs were lowest in F0 juveniles, followed by F1 C and then F1 W-Δ1000 juveniles. The lower SMR in F0 could be due to the different temperature life histories. While F1 C fish were reared continuously at 15 °C, F0 fish were reared at 10 °C during larval development and at 15 °C just after metamorphosis. The higher SMR in F1 W-Δ1000 was observed in larval RMRs, too. F1 W larvae displayed higher RMRs than F1 C larvae. This increase in metabolic rates fits the finding of previous

studies in this and other fish species and was mirrored in higher growth rates in F1 W larvae and F1 W-Δ1000 juveniles compared to F1 C and F0 larvae and juveniles.

Although already compared to answer the questions above, the complete comparison of F0 and F1 larvae and juveniles and the determination of the transgenerational plasticity (TGP) was the purpose of the 4th question of my thesis. However, it was decided within the FITNESS project to apply an additional OW scenario on F1, as it was done on Wild but not on F0 fish. As larval number, space and manpower were restricted, the addition of the OW scenario, came at the cost of a full factorial transgenerational experiment prohibiting the determination of TGP of European sea bass. Nevertheless, the comparison of F0 and F1 fish provides insights into the capacities of two successive generations to survive under the same OA conditions: While OA as a single driver did not impact F0 Δ1000 larvae (raised at 19 °C) and F1 C-Δ1000 larvae (raised at 15 °C) in comparison to F0 A and F1 C-A, respectively, it did decrease size at metamorphosis in F1 W-Δ1000 larvae (raised at 20 °C) in comparison to all other treatments. The detrimental effects of OAW on F1 W- Δ 1000 larvae did not seem to be related to different parental provisioning, as indicated by similar sizes of larvae of all OAW treatments at mouth opening. However, they could be due to effects of OA during embryonal development.

For the last question of my thesis, I used again European sea bass juveniles from the third group (Wild). I determined metabolic profiles of liver, white muscle, heart and gill tissue and compared the findings on metabolic profiles to the findings of other experiments within the FITNESS project: It was possible to explain some of the findings of the experiments in the FITNESS project with the findings on metabolome level. Increased protein and lipid synthesis in liver but not in white muscle tissue might explain why fish grew faster, but showed impaired swimming performance. Increased glycolysis in liver tissue might be the main driver of increased SMR in warm acclimated European sea bass.

As a conclusion of my work, I was able to add relevant information to the body of knowledge of fish physiology in light of climate change. European sea bass might be challenged during larval development in future oceans, but if they arrive as juveniles in nursery areas with abundant food, this species might profit from higher temperatures at its northern distribution range.

Zusammenfassung

In den unterschiedlichen Teilen meiner Doktorarbeit habe ich die Kapazitäten des aeroben Stoffwechsels des Europäischen Wolfsbarsches, *Dicentrarchus labrax*, bei aktuellen und zukünftigen Bedingungen unter Ozeanversauerung und –erwärmung (OAW) untersucht. Ich wollte einen breitgefächerten Überblick über den aeroben Stoffwechsel des Europäischen Wolfsbarschs bekommen und habe daher mit verschiedenen Entwicklungsstadien (Larven und Juvenile) gearbeitet. Während ich bei den Larven am ganzen Tier gearbeitet habe, habe ich bei den Juvenilen an verschiedenen biologischen Organisationsstufen (Metabolom, Mitochondrien und Ganztierebene) gearbeitet um einen tieferen Einblick in den aeroben Stoffwechsel dieses Entwicklungsstadiums zu erhalten. Zudem habe ich die Ergebnisse der Respirometrie- und Wachstumsdaten von „meinen" juvenilen Europäischen Wolfsbarschen mit Daten aus ähnlichen Experimenten an der Elterngeneration verglichen.

Meine Doktorarbeit war Teil des FITNESS-Projektes (Fish Transgenerational adaptive Strategies to ocean acidification and warming), das erlaubte mir an zwei Gruppen des Europäischen Wolfsbarsches zu arbeiten und zudem die Ergebnisse aus einer Dritten für meine Studien zu benutzen:

- Gruppe 1 oder F0 wurde bereits vor meiner Doktorarbeit unter drei Ozeanversauerungsbedingungen (OA) aufgezogen und gehältert. Diese Gruppe ist die Elterngeneration einer „meiner" Gruppen. Ich konnte die Ergebnisse der Respirometrie- und Wachstumsexperimente, also basale Stoffwechselraten (SMR), kritische Sauerstoffkonzentration (PO_{2crit}) und Wachstumsraten, nutzen, um sie mit den Daten zu vergleichen, die ich aus ähnlichen Experimenten an Gruppe 2 erhalten habe.
- Gruppe 2 oder F1 war die Nachwuchsgeneration von F0. Die Eier dieser Generation wurden unter den OA Bedingungen der jeweiligen Elterngeneration gehältert. Um auch Ozeanerwärmung (OW) in die Versuche an dieser Generation mit aufzunehmen, wurden zwei Temperaturen in der Larvenhälterung verwendet, beginnend zwei Tage nach dem Schlupf. Auch wenn das Versuchsdesign kein vollständiges faktorielles Transgenerationsdesign ist, so erlaubte mir dieses Design dennoch einzuschätzen, wie sich die Kapazität sich den ändernden Bedingungen anzupassen ändert, wenn zwei aufeinanderfolgender Generationen diesen Bedingungen ausgesetzt sind.
- Gruppe 3 oder Wild war „meine" zweite Gruppe. Die Fische kamen aus einer Aquakulturanlage und waren der Nachwuchs von wildgefangenen Europäischen Wolfsbarschen. Diese Gruppe wurde unter sechs OAW (zwei OA Bedingungen mit 3 OW Bedingungen gekreuzt) Bedingungen aufgezogen und gehältert, beginnend zwei Tage nach

dem Schlupf. Ich habe diese Gruppe für meine Experimente an Mitochondrien und dem Metabolom genutzt und die gewonnenen Daten mit den Wachstumsdaten der Tiere verglichen.

Die angewendeten OAW Bedingungen waren wie folgt definiert:

Drei OA Bedingungen:

- A: die heutigen Umgebungsbedingungen in küstennahen Gewässern der Bretagne und in der Rade von Brest (ca. 650 µatm CO_2)
- Δ500: ein Szenario, welches den Vorhersagen des SSP3-7.0 des IPCC (2021) folgt (heutige Bedingungen plus 500 µatm CO_2, ca. 1150 µatm CO_2)
- Δ1000: ein Szenario nach SSP5-8.5 (heutige Bedingungen plus 1000 µatm CO_2, ca. 1700 µatm CO_2)

Die F1 Fische wurden nur unter den A und Δ1000 Bedingungen gehältert.

Temperaturbedingungen der F0 Fische:

- Hälterung der Larven: 19 °C
- Hälterung der Juvenilen: aktuelle Umgebungstemperaturen, jedoch immer über 12 °C gehalten

Zwei Temperaturbedingungen von OW in F1 und Wild:

- „Kaltes“ (C) Szenario:
    - Hälterung der Larven: 15 °C
    - Hälterung der Juvenilen: aktuelle Umgebungstemperaturen (bis zu 18 °C), jedoch immer über 15 °C gehalten
- „Warmes“ (W) Szenario:
    - Hälterung der Larven: 20 °C
    - Hälterung der Juvenilen: Kaltes Szenario + 5 °C

Beide OW Bedingungen wurden auf alle OA Bedingungen in F1 und Wild angewendet. Es gab daher die folgenden Gruppen: C-A, C-Δ500 (nur Wild), C-Δ1000, W-A, W-Δ500 (nur Wild) and W-Δ1000.

Das Ziel meiner Arbeit war es die folgenden Fragen zu beantworten:

(1) Ist die Leistungsfähigkeit von Mitochondrien aus den Herzen von juvenilen Wolfsbarschen nach Langzeitakklimation zu OAW oder nach akuten Temperaturänderungen beeinträchtigt? Meine Hypothesen waren:

a. LEAK Respirationsraten sind nach akuter Erwärmung erhöht, da die Mitochondrienmembranen durch die Temperatur beschädigt sind, was zu einer verschlechterten Leistungsfähigkeit von Mitochondrien führt.
b. Veränderungen der Eigenschaften der Mitochondrienmembranen nach Langzeitakklimation führen zu reduzierten LEAK Respirationsraten und damit zu wiederhergestellten mitochondrialen Respiratorischen-Kontrollraten
c. Erhöhte intrazelluläre PCO_2- und Bikarbonatkonzentrationen verschlechtern den mitochondrialen Stoffwechsel, z.B. indem sie die Zitratsynthase oder Succinatdehydrogenase inhibieren.

(2) Führt OAW zu synergistischen Effekten auf die Wachstumsraten von Wolfsbarschlarven und –juvenilen?

Meine Hypothesen waren:

a. Wachstumsraten von Larven und Juvenilen des Europäischen Wolfsbarsches sind unter OW erhöht.
b. Wachstumsraten von Larven und Juvenilen des Europäischen Wolfsbarsches sind unter OA unverändert.
c. Synergistische Effekte von OAW könnten die Wachstumsraten von Larven und Juvenilen des Europäischen Wolfsbarsches verschlechtern.

(3) Führt OAW zu synergistischen Effekten auf die Stoffwechselraten von Larven und Juvenilen des Europäischen Wolfsbarsches?

Meine Hypothesen waren:

a. RMR der Larven und SMR der Juvenilen wird unter OW erhöht sein.
b. Stoffwechselraten von Larven und Juvenilen wird unter OA nicht verschlechtert sein.
c. Weder OA, noch OW, noch OAW werden PO_{2crit} der Juvenilen verändern.

(4) Verbessert transgenerationale Plastizität beim Europäischen Wolfsbarsch die Wachstums- und Stoffwechselraten von F1 bei OAW nach Akklimierung von F0 an OA?

Meine Hypothesen waren:

a. F0 und F1 W Larven werden ähnliche Wachstumsraten zeigen (Hälterungstemperatur 19 °C, bzw. 20 °C).
b. F0 und F1 C Juvenile werden eine ähnliche Größe bei 3000 dd haben (aktuelle Umgebungstemperatur in beiden Generationen).
c. F0 und F1 C Juvenile werden ähnliche SMR haben.
d. Wachstums- und Stoffwechselraten von F1 Larven und Juvenilen werden durch OA nicht verschlechtert.

(5) Kann man Veränderungen, die beim Wachstum, in den Stoffwechselraten und in der mitochondrialen Funktion beobachtet werden, auch im Metabolom wiederfinden? Meine Hypothesen waren:
   a. Durch OW erhöhte Wachstumsraten spiegeln sich in erhöhter Proteinsynthese in Muskel- und Lebergewebe wieder.
   b. Die Konzentrationen von Metaboliten, die in der Erhaltung der Homöostase involviert sind, wie organische Säuren und Osmolyte, werden durch OA verändert, vor allem in der Kieme.
   c. Veränderungen der Metabolitprofile werden die synergistischen Effekte von OAW wiederspiegeln.

Kapitel 2 befasst sich mit der ersten Frage: Ich habe die mitochondrialen Kapazitäten in permeabilisierten Herzfasern von Wild Juvenilen untersucht. Akute Erwärmung um 5 °C führte zu signifikant erhöhten mitochondrialer LEAK Respiration in den Mitochondrien von Wild C Juvenilen. Das zeigt, dass bereits relativ kleine Temperaturansteige eine Herausforderung für die Mitochondrien im Herzen sein können. Während diese akute Temperaturerhöhung die mitochondriale Funktion beeinträchtigt hat, zeigten die Mitochondrien von warmakklimierten Tieren eine verbesserte Funktionalität, z.B. durch erhöhte respiratorische Kontrollverhältnisse (RCR). Diese verbesserten Kapazitäten spiegelten sich in höheren Wachstumsraten der Wild W Juvenilen wieder. OA beeinflusste weder die Respirationsraten von Komplex I und IV des Elektronentransportsystems, noch die RCR. Zudem konnte eine signifikante Hemmung des Komplexes II durch OA nur in den Wild W juvenilen beobachtet werden, wenn die Herzfasern zusätzlich einer akuten Temperaturverminderung (um 5 °C) ausgesetzt waren.

Um die zweite Fragestellung meiner Doktorarbeit zu bearbeiten analysierte ich die Wachstumsraten von F0 und F1 Larven und Juvenilen in Kapitel 3 und verglich sie. Des Weiteren analysierte ich die Wachstumsraten von Wild Larven und Juvenilen in Kapitel 4. OW als alleiniger Stressfaktor erhöhte die Wachstumsraten von Wild und F1 Larven und Juvenilen. OA als alleiniger Stressfaktor veränderte die Wachstumsraten von F0, F1 und Wild Larven und Juvenilen nicht. Die Verbindung von beiden Stressfaktoren führte allerdings zu deutlich kleineren F1 W-Δ1000 Larven, im Vergleich zu allen anderen Larven, zum Zeitpunkt der Metamorphose. Dies konnte so nicht in den Larven und Juvenilen der Wild Gruppe beobachtet werden. In der F0 Gruppe konnte der Effekt von OAW durch die fehlende OW Bedingung nicht untersucht werden. F1 W-Δ1000 Juvenile zeigten ebenfalls erhöhte Wachstumsraten im Vergleich zu F1 C Juvenilen. Da die F1 W-A Bedingung in den Juvenilen fehlte,

lässt sich hier allerdings nicht sagen, ob die schädlichen Einflüsse von OAW, die in F1 Larven beobachtet wurden, in der Juvenilen weiter Bestand hatten und nur durch die Effekte von OW auf das Wachstum maskiert wurden, oder nicht. Dennoch deuten diese Daten an, dass es in den Larven der zweiten Generation unter OA Bedingungen eine Diskrepanz gibt, die entweder durch Transgenerationseffekte ausgelöst sein kann oder durch Plastizität während der Entwicklung, da die F1 Fische die einzige Gruppe in dieser Arbeit sind, welche von der Befruchtung an unter OA Bedingungen gehältert wurde.

Um die dritte Frage zu beantworten habe ich die Stoffwechselraten (RMR) von F1 Larven des Europäischen Wolfbarsches, sowie die basalen SMR und PO_{2crit} von F1 Juvenilen bestimmt. SMR und PO_{2crit} wurden vor meiner Arbeit auch in F0 Juvenilen gemessen und ich konnte diese Daten nutzen, um sie mit den F1 Tieren zu vergleichen. OA als alleiniger Stressfaktor hatte keinen Einfluss auf die untersuchten Stoffwechselprozesse. PO_{2crit} wurde nicht von OAW beeinflusst. Der Einfluss von OW als alleiniger Stressfaktor konnte in diesen Experimenten nicht untersucht werden, da es keine F1 W-A Juvenilen gab. Die SMR waren in den F0 Juvenilen am niedrigsten, gefolgt von den F1 C Juvenilen und dann den F1 W-Δ1000 Juvenilen. Die niedrigen SMR in F0 könnten durch die unterschiedlichen Temperaturen während der Larvenentwicklung hervorgerufen sein. Während die F1 C Tiere durchgängig bei 15 °C gehältert wurden, wurden die F0 Tiere während der Larvenentwicklung bei 19 °C gehältert und erst nach der Metamorphose bei 15 °C. Die höheren SMR von F1 W-Δ1000 Juvenilen wiederum bestätigen die Ergebnisse aus den RMR der Larven: F1 W Larven hatten höhere RMR als F1 C Larven. Dieser Anstieg der Stoffwechselraten wurde auch in anderen Studien an dieser und an anderen Fischarten gefunden und spiegelte sich in den erhöhten Wachstumsraten von F1 W Larven und F1 W-Δ1000 Juvenilen im Vergleich zu den F1 C Larven, bzw. F1 C Juvenilen wider.

Auch wenn die Daten schon in den vorherigen Fragestellungen Verglichen wurden, diente Frage 4 dazu einen möglichst vollständigen Vergleich zwischen F0 und F1 anzustellen und die transgenerationale Plastizität zu untersuchen. Allerdings wurde innerhalb des FITNESS Projektes entschieden, dass zusätzlich ein OW Szenario auf F1 angewendet werden sollte, wie es auch in Wild, aber nicht F0 der Fall war. Da sowohl die Larvenanzahl, als auch Platz und Arbeitskraft begrenzt war, lief diese Entscheidung zu Lasten eines vollständigen Versuchsplan des Transgenerationsexperiments. Damit war es nicht möglich die TGP des Wolfsbarsches zu bestimmen. Dennoch kann der Vergleich von F0 und F1 wichtige Hinweise auf die Kapazität von zwei aufeinanderfolgenden Generationen bei den gleichen OA Bedingungen zu überleben liefern: Während OA als alleiniger Treiber weder F0 Δ1000 Larven (bei 19 °C gehältert) noch F1 C- Δ1000 Larven (bei 15 °C gehältert) beeinflusst hat, waren F1 W- Δ1000 Larven (bei 20 °C gehältert) signifikant kleiner als alle anderen Larven bei der Metamorphose. Diese schädlichen Effekte von

OAW auf F1 W- Δ1000 Larven schienen nicht mit der Versorgung der Larven durch die Eltern zusammenzuhängen, da die Larven aus allen OAW Bedingungen in F1 zum Zeitpunkt der Mundöffnung etwa gleich groß waren. Allerdings könnten sie auf Effekte von OA während der embryonalen Entwicklung hinweisen.

Für die letzte Fragestellung meiner Arbeit habe ich wieder mit Juvenilen aus der dritten Gruppe (Wild) gearbeitet. I habe Metabolitprofile von Gewebe von Leber, weißem Muskel, Herz und Kieme aufgenommen und die Ergebnisse dieser Metabolitprofile mit den Ergebnissen aus den anderen Experimenten innerhalb des FITNESS Projektes verglichen: Es war möglich einige Ergebnisse dieser anderen Experimente im FITNESS Projekt mit den Ergebnissen auf Metabolomebene zur erklären. Erhöhte Protein- und Lipidsynthese im Lebergewebe, aber nicht im Muskelgewebe könnte erklären, warum die Juvenilen zwar schneller gewachsen sind, jedoch schlechtere Schwimmleistungen erbrachten. Erhöhte Glykolyse im Lebergewebe könnte einer der Hauptfaktoren für die erhöhte SMR in den warmakklimieren Wolfsbarschen gewesen sein.

Als Schlussfolgerung meiner Arbeit kann ich sagen, dass ich dem Wissen über Fischphysiologie in Zeiten des Klimawandels einige wichtige Informationen hinzufügen konnte. Der Europäische Wolfbarsch wird während der Larvenentwicklung herausgefordert werden, aber wenn er als Juveniler in Kinderstuben mit ausreichend Nahrung ankommt, kann er von den höheren Temperaturen an dem nördlichen Ende seines Verbreitungsgebietes profitieren.

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Abbreviations

Δ500 – Acidification condition (ambient PCO_2 + 500 µatm)

Δ1000 – Acidification condition (ambient PCO_2 + 1000 µatm)

A – Ambient PCO_2 condition

AA – Amino acids

AAM - Intermediates of amino acid metabolism and amino acid derivates

AP – Atlantic population of European sea bass

BL – Body length

C – Cold life-conditioned group

CC – Intermediates of the citrate cycle

CCO – Cytochrome *c* oxidase

CH - Carbohydrates

CI – Complex I of the electron transport system

CII – Complex II of the electron transport system

CIV – Complex IV of the electron transport system

CT_{max} – Critical thermal maximum

dd – Degree days

DM – Dry mass

dph – Days post hatch

EC – Energy compounds

ETS – electron transport system

FITNESS – Fish Transgenerational adaptive Strategies to ocean acidification and warming

HRMAS – high-resolution magic angle spinning

^{1}H-NMR – proton nuclear magnetic resonance spectroscopy

IPCC – Intergovernmental Panel on Climate Change

LME model – Linear mixed effect model

L_{Omy} – LEAK respiration with oligomycin (cf. 2 Publication I, table 2)

MP – Mediterranean population of European sea bass

MS-222 – Tricaine methane sulfonate

NAP – Northern Atlantic population of European sea bass – Northern Bay of Biscay to British and Irish waters

OA – Ocean acidification

OAW – Ocean acidification and warming

OOA – Osmolytes and organic acids

OW – Ocean warming

P – OXPHOS respiration (cf. 2 Publication I, table 2)

PCA – Principle components analysis

PCO_2 – Partial pressure of CO_2

PLS-DA – Partial-least squares discriminant Analysis

PO_2 – Partial pressure of O_2

PO_{2crit} – Critical oxygen concentration

RCP – Representative concentration pathway

RCR_{Omy} – Respiratory control ratio (cf. 2 Publication IPublication I, table 2)

RMR – Routine metabolic rate

SDA – Specific dynamic action

SMR – Standard metabolic rate

SP – Intermediates of different sugar pathways

SSP – Shared socio-economic pathway

VIP – Variable importance in projection

W – Warm life-conditioned group

WM – Wet mass

1 General Introduction

1.1 Effects of climate change on marine organisms

Since the industrial revolution, anthropogenic activities cause increasing greenhouse gases in our atmosphere. Greenhouse gases include gases such as carbon dioxide (CO_2) and methane and their increase is responsible for the rising temperatures on our planet. This rise in temperature is not only affecting the land, but increasing the sea surface temperature – a problem called ocean warming (OW). Concerning the partial pressure of CO_2 (PCO_2), the atmosphere and the ocean surface layers are in equilibrium. Therefore, rising atmospheric CO_2 concentrations lead to rising CO_2 concentrations in the ocean. The ocean has taken up about 30-50 % of the anthropogenic CO_2. This causes additional problems, as CO_2 dissolves in the ocean and reacts with water to carbonic acid, ultimately increasing the hydrogen ion concentration in the ocean and consequently resulting in a decrease in ocean pH. This is called ocean acidification (OA). Both drivers, OA and OW, together will lead to warmer and more acidic oceans (ocean acidification and warming – OAW). The first studies on the effect of climate change applied only one driver to the species in question, however, it is recognized today that applying at least two drivers seems necessary (Wernberg, Smale, & Thomsen, 2012). The effects of more than one driver can be additive – the effect of both drivers together is the sum of the individual effects, antagonistic – the effect of one driver compensates for the effect of the other, or synergistic – the combined effects of both drivers are greater than their sum.

OW is directly affecting ectothermic organisms, such as fish, as their body temperature is dependent on the surrounding water temperature. Increased body temperature due to OW will alter growth (Peck, 2002; Pörtner et al., 2007), reproduction success (see review Llopiz et al., 2014) and geographic abundance (Pörtner, 2006; Turner et al., 2009) of marine ectotherms. If the conditions are unfavourable, an organism has to acclimatise and in the long term adapt to the new conditions or migrate to more favourable conditions to ensure survival. The body of investigations concerning the effect of the OA on ectothermic organisms in the ocean is growing rapidly, and while most studies from the beginning of this century worked on calcifying organisms and determined negative effects, only a small part was determining the effect of OA or OAW on fish, with contradictory results in the available studies (Kreiss et al., 2015; Heuer and Grosell, 2014; Pope et al., 2014; Bignami et al., 2013; Frommel et al., 2011). Although juvenile and adult fish have well-developed acid-base and ion regulation capacities and are therefore expected to survive in the future ocean, fish larvae are lacking these complex physiological characteristics and might therefore be more vulnerable to the changing

environment (Llopiz et al., 2014). More investigation on a variety of fish species and especially on different life stages appears necessary (Pope et al., 2014).

An organism's capacity to grow, reproduce and generally survive is highly dependent on the relation between oxygen supply and demand within the tissues. Increasing temperature leads to increasing oxygen demand within the body, resulting in increased metabolic rates. At some point, the cardiovascular system is no longer able to supply the different tissues with sufficient oxygen. Due to being a highly aerobic tissue (Driedzic, 1992), heart mitochondria are thought to play a key role in shaping an organism's thermal tolerance, as impaired mitochondrial metabolism would alter ATP supply of cardiomyocytes and consequently alter the performance of the cardiovascular system. It was shown in other studies that increased temperature led to impaired mitochondria, as suggested by decreased oxidative phosphorylation, decreased ATP production efficiency and lost integrity of the complexes of the ETS (Chen & Knowlton, 2010; Chung et al., 2017; Iftikar & Hickey, 2013; Strobel et al., 2013). Oxygen consumption rates of the different complexes of the ETS in mitochondria are a proxy for the metabolic rates of the processes in the mitochondria and can therefore give valuable insights into the capacity of mitochondria to produce ATP.

In addition to the necessity to understand the effects of OAW on subcellular processes, it becomes evident that research on transgenerational effects of OAW is equally important. As summarized by Munday (2014), the performance of fish, whose parents experienced the same environment, has been found improved in several studies, sometimes even completely counterbalancing the negative effects seen within the parental generation. Although transgenerational plasticity might help fish to cope with the changing environment, the offspring generation did not always have a better performance in the tested environment than their parents. Especially behaviour does not seem to have a high plasticity under OA. Studies which investigate transgenerational effects of OA (Schunter et al., 2016), OW (Shama et al., 2014, 2016) or OAW are rare (Munday, 2014) and, as most of the OAW research on fish, are mainly focussing on tropical species, species with a short generation time and/or species, which are economically not important.

1.1.1 Effects of ocean warming on marine organisms

Increasing temperature is leading to increasing oxygen demands and therefore increasing metabolic rates. The standard metabolic rate (SMR) is the basal metabolic rate, maintaining minimum vital functions, without fuelling other energy-demanding processes such as digestion, growth, reproduction and activity. The maximum metabolic rate (MMR) is the maximum rate of oxygen consumption, which the fish can achieve. The difference between SMR and MMR is the aerobic or

metabolic scope and indicates the maximum achievable energy budget for metabolic processes not included in SMR. SMR and MMR and consequently aerobic scope are temperature dependent. While SMR increases only slowly, MMR usually shows a sharp increase until a certain temperature, after which it drops. Consequently, the metabolic scope increases with the increase in MMR and decreases when MMR drops (Figure 1A, Pörtner & Farrell, 2008). When SMR is higher than MMR, the minimum vital functions are no longer supplied with sufficient amounts of oxygen. Therefore, the organism relies on anaerobic metabolism to fuel vital body functions. This state is generally reached at the critical temperature. As anaerobicmetabolism is not possible over long timespans, only short-term survival is possible below the low and above the high critical temperature. The optimal thermal window of an organism, where it thrives best, is the temperature range between lower and upper pejus temperature. As higher temperatures lead to higher metabolic scopes within the thermal window, growth is also faster with increasing temperature in fish within their thermal window (e.g. Chauton et al., 2015). Although the organism can survive temperatures below and above its thermal window, the performance gets worse, as the metabolic scope is lower. The thermal window and the optimal temperature are different between different species, but are also depending on the developmental state, being generally lower for early life stages and reproduction and being highest in juveniles (Figure 1B). The addition of other drivers, such as OA or hypoxia, is generally lowering the metabolic scope and therefore narrowing the thermal window (Figure 1A).

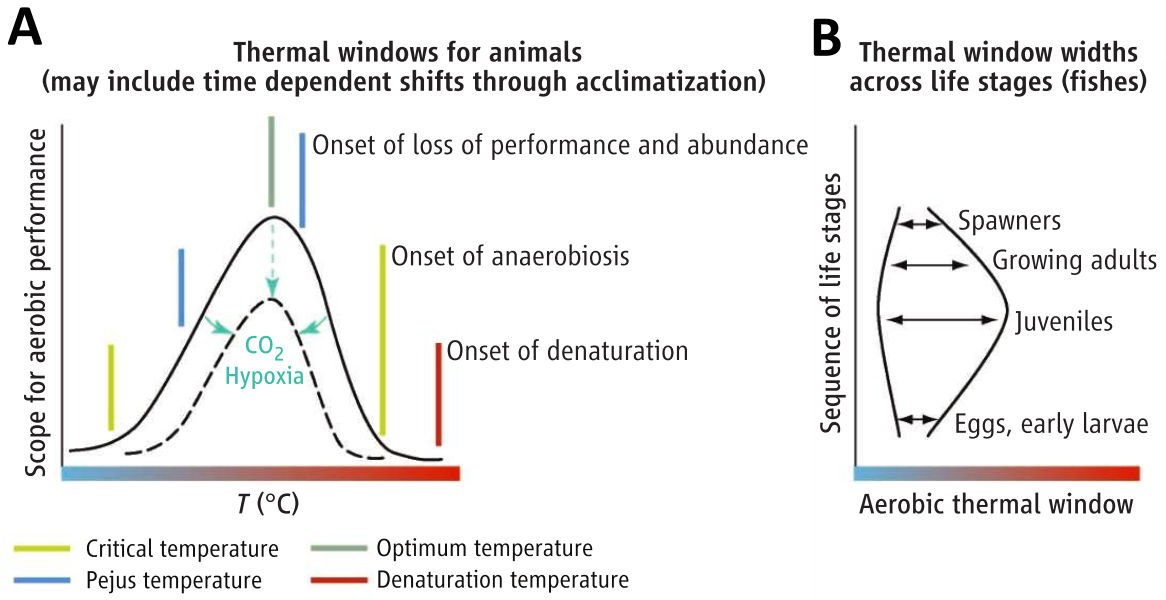

Figure 1 Thermal windows of aerobic performance taken from Pörtner and Farrell (2008). (A) The thermal window of optimal performance of an organism lies within the lower and upper pejus temperature. While survival below the lower and above the upper pejus temperature is possible for long time, it is restricted to short time spans below the lower and above the upper critical temperatures. Additional driver, such as CO_2 and hypoxia lower the metabolic scope and decrease the thermal window. (B) The width of the thermal window is dependent on developmental stage, being smallest in early life stages and spawners and broadest in juveniles.

The onset of the higher pejus temperature is usually linked to the inability of the cardiovascular system to supply the tissues with sufficient oxygen, as the metabolic rates within the tissues are increasing due to temperature and therefore demanding more oxygen. Therefore, it appears crucial to measure oxygen consumption rates and understand the underlying cellular processes to predict an organism's capacity to cope with future ocean conditions. Dahlke et al. (2016) found indications in cod embryos that the organism is not capable to cover the oxygen and energy (ATP) demand under thermal stress. This was shown by limitations to oxygen consumption rates upon warming, which were paralleled by decreased mitochondrial phosphorylation capacities and coupling efficiency, which themselves resulted from defective performance of the electron transport system (ETS). Mitochondrial respiration rates were determined in different tissues after acclimation to OW, OA and OAW (e.g. Chung et al., 2017 and Strobel et al., 2013) with species-specific responses: e.g. in gadoids, Polar cod only responds to OW, but not to OA, while Atlantic cod responds mainly to OAW (Leo et al., 2017). This indicates that the subcellular processes leading to the changes in energy demand and supply within the organisms are not yet fully understood. Especially mitochondrial plasticity might play a key role in determining an organism's capacity to acclimate and in the long term adapt to changed environmental conditions, e.g. in the study of Strobel et al. (2013) warm acclimation led to uncompensated mitochondrial respiration rates in the investigated cold-stenotherm fish species, which reflected uncompensated whole animal metabolic rates from a former study.

In addition to alterations of the energy budget, the ontogeny of fish is also affected by water temperature, resulting in consequences for the later life and/or for the population in general. For example, the development of muscle fibres is directly important for the individual, as the ability to swim determines the foraging as well as escape success and has been found to be affected by temperature in several fish species, including European sea bass (Ayala et al., 2003). The sex ratio on the other hand is not important for the survival of an individual, but it becomes important for the survival of the population if it is biased towards one sex. As the sex is important for aquaculture, the effect of temperature on sex ratio was determined in many cultured fish species, including European sea bass, and was shown to be biased towards populations with more males than females at lower temperature (Blásquez et al., 1998). It was also observed in several fish species, including European sea bass that higher temperatures lead to faster larval development at the cost of swimming capacity as fins, muscle, neural system and behaviour were less developed at the same total length (Koumoundouros et al., 2009; Cominassi et al., 2019). These results suggest that it is not only important to observe larval growth and survival under different OAW scenarios as in many studies on fish larvae, but also traits which might be more important in defining the fitness of the population when not under laboratory or aquaculture conditions.

1.1.2 Effects of ocean acidification on marine organisms

Due to the well-developed acid-base regulation systems, fish were thought to be less vulnerable to OA than calcifying organisms (for review, see Heuer & Grosell, 2014). Yet, indications that this might not be universal led to an increase in OA and OAW research in fish in the last decade. The effects of OA were species and trait specific, e.g. reduced, unimpacted and increased growth due to OA were found: OA impaired growth of juvenile inland silverside (Baumann et al., 2012), while it did not affect growth of Atlantic halibut juveniles (Gräns et al., 2014), cobia (Bignami et al., 2013) and Australasian snapper larvae (McMahon et al., 2020). Australian snapper larvae even benefited from OA with decreased mortality (McMahon et al., 2020). Even increased larval growth was observed in larvae of mahi-mahi and cod, but at the cost of decreased swimming performance in the first (Bignami et al., 2014) and severe to lethal tissue damage in the latter (Frommel et al., 2011). However, the capacity to withstand OA might change with developmental stage: while cod embryos before and during gastrulation displayed low capacities for acid-base regulation, which was paired with decreased survival, their capacities after gastrulation were similar to adult cod (Dahlke et al., 2020). Different results were even found within the same species: Crespel et al. (2017) showed slightly reduced growth in their high acidification condition (ambient + 1000 µatm CO_2) in European sea bass larvae, while in the study of Pope et al. (2014) growth was increased under OA and also under OAW in the same species. However, the increased growth came at costs as well, increasing growth under OAW occurred together with reduced aerobic scope (AS). In both of these studies, the larvae originated from aquaculture facilities where fish usually live at higher than ambient PCO_2 conditions, possibly resulting in transgenerational or developmental acclimation to these conditions. Although the origin of the fish in these studies might have caused a bias, a low sensitivity of European sea bass growth to ocean acidification seems likely.

1.1.3 Combined effects of ocean acidification and warming

While OA as a single driver showed species-specific results, with beneficial as well as harmful effects on fish, OAW led to more unidirectional effects, which were often synergistic. In most species, juvenile and adult fish possess well-developed mechanisms for acid-base regulation (for review, see Heuer & Grosell, 2014). However, these mechanisms need to be fuelled with energy. Consequently, if energy supply is altered additional energy demand will be challenging to satisfy. OA has been found to inhibit complex II (CII) of the ETS in mitochondria of mammals and fish (Simpson, 1967; Wanders et al., 1983; Strobel et al., 2013). It was suggested that mitochondria could use anaplerotic mechanisms, e.g. decarboxylation of aspartate and glutamate, feeding into the Krebs cycle, which

would subsequently stimulate CI to overcome inhibitory effects of OA on CII (Langenbuch & Pörtner, 2002; Strobel et al., 2013). OW, on the other hand, reduced the activity of CI in cod embryos (Dahlke et al., 2016). The reduction of CI resulted in decreased mitochondrial phosphorylation capacity and oxygen consumption rates, while the energy requirements of the tested embryos increased with OA. If this inhibition of CI occurs in combination with the inhibition of CII, the mitochondrial performance would be severely decreased. Especially larvae and embryos might be more vulnerable to such inhibitions, both, are less developed than juveniles, while at the same time investing all available energy into growth without reserving excess capacity for environmental regulation. Therefore, growth rates of larvae might be affected by OAW via mechanisms such as impaired mitochondrial performance much earlier than growth rates of juvenile fish.

1.2 Developmental and transgenerational plasticity

Genetic adaptation of marine organisms is probably not fast enough to keep up with the rapidly changing climate, but might help species with short life spans and generation times to survive in our oceans (Melzner et al., 2009; Hoegh-Guldberg et al., 2007). Species with long generation times will not be able to genetically adapt to the new conditions and might be more vulnerable to OAW (Melzner et al., 2009). These species can survive via transgenerational plasticity (TGP). TGP is one of the nongenetic inheritance mechanisms to shape the response of the F1 generation (offspring) to a certain driver, which was already experienced by the F0 generation (parents). TGP can include the transfer of cell structures, such as mitochondria (Mousseau & Fox, 1998). As described above, although TGP could help the F1 generation to survive under conditions to which they are not yet adapted, this was not always the case (Munday, 2014). Especially behaviour did not seem to have a high plasticity under OA, e.g. the negative effects of OA on escape response in cinnamon anemone fish was reduced in some traits when parents were exposed to OA (Allan et al., 2014), whereas the negative effects of OA on olfactory responses in spiny damselfish were not reduced after parental exposure (Welch et al., 2014). Additionally, Shama et al. (2014; 2016) found different effects depending on the time when the F0 generation was acclimated to OW, during reproductive conditioning or during developmental acclimation. Developmental acclimation is the acclimation to a certain driver during embryonic and/or larval phase. Developmental acclimation of the F0 to OW led to negative effects in F1 on growth and respiratory control ratio (RCR) of mitochondria, while acclimation during reproductive conditioning led to improved growth and RCR in F1 due to beneficial TGP (Shama et al., 2014; 2016).

1.3 European sea bass (*Dicentrarchus labrax*)

The European sea bass, *Dicentrarchus labrax*, is a temperate marine teleost inhabiting a broad range of Europe's coastal waters. It is distributed throughout the Mediterranean and Black Sea and in the eastern Atlantic from Norway to Senegal and can be found in coastal waters until 100m depth (Bjørndal & Guillen, 2018). Most of their life is spent in brackish water, but European sea bass are able to forage in hypo- and hypersalinity lagoons (Chervinski, 1975; Dando & Demir, 1985). Due to this lifestyle, European sea bass have a high tolerance for different temperatures and salinities (Claireaux & Lagardère, 1999; Dalla Via et al., 1998; Chervinski, 1975). European sea bass feed opportunistically on zooplankton during larval stage and crustaceans and fish from juvenile stage onwards (Pickett & Pawson, 1994).

Genetically distinct populations have been proven in the Mediterranean between eastern and western populations (Bahri-Sfar et al., 2000) and while the Atlantic population (AP) is different from the Western Mediterranean population (MP) with a separation at the Gibraltar Strait (Naciri et al., 1999), no distinction within the Atlantic population has been proven yet (Vandeputte et al., 2019). However, as I worked on European sea bass from their northern distribution range, I defined their population as Northern Atlantic population (NAP), which includes the distribution from the northern Bay of Biscay into British and Irish waters.

As a popular edible fish, European sea bass is an important species in aquaculture (160,000 t in 2015), but also important for commercial and especially fishing activities (Bjørndal & Guillen, 2018). Due to their importance in fisheries, several studies on egg and larval distribution were done around the British Isles in the 1980s and 90s (e.g. Russel et al., 1996; Pawson, 1992; 2007; Jennings & Pawson, 1992; Dando & Demir, 1985): Mature European sea bass migrate from the coast to offshore spawning grounds during winter, where temperatures do not decrease below 9 °C. Offshore spawning takes place between March and June and results in offshore egg development. Larvae hatch after about 5 days and are transported by currents towards coastal nursery areas. Metamorphosis is reached after two to three months, usually around the time when they arrive inshore, around this developmental stage the late larvae and early juveniles are able to sense cues of nursery areas and to swim in this direction, even against currents. The juvenile European sea bass spend the following 2-4 years close to the coast and mainly in brackish waters to grow to maturity. Depending on sex, European sea bass mature at two to three years or three to four years, for males and females, respectively (Pérez-Ruzafa & Marcos, 2014). European sea bass life cycle from eggs to spawning adults is pictured in Figure 2.

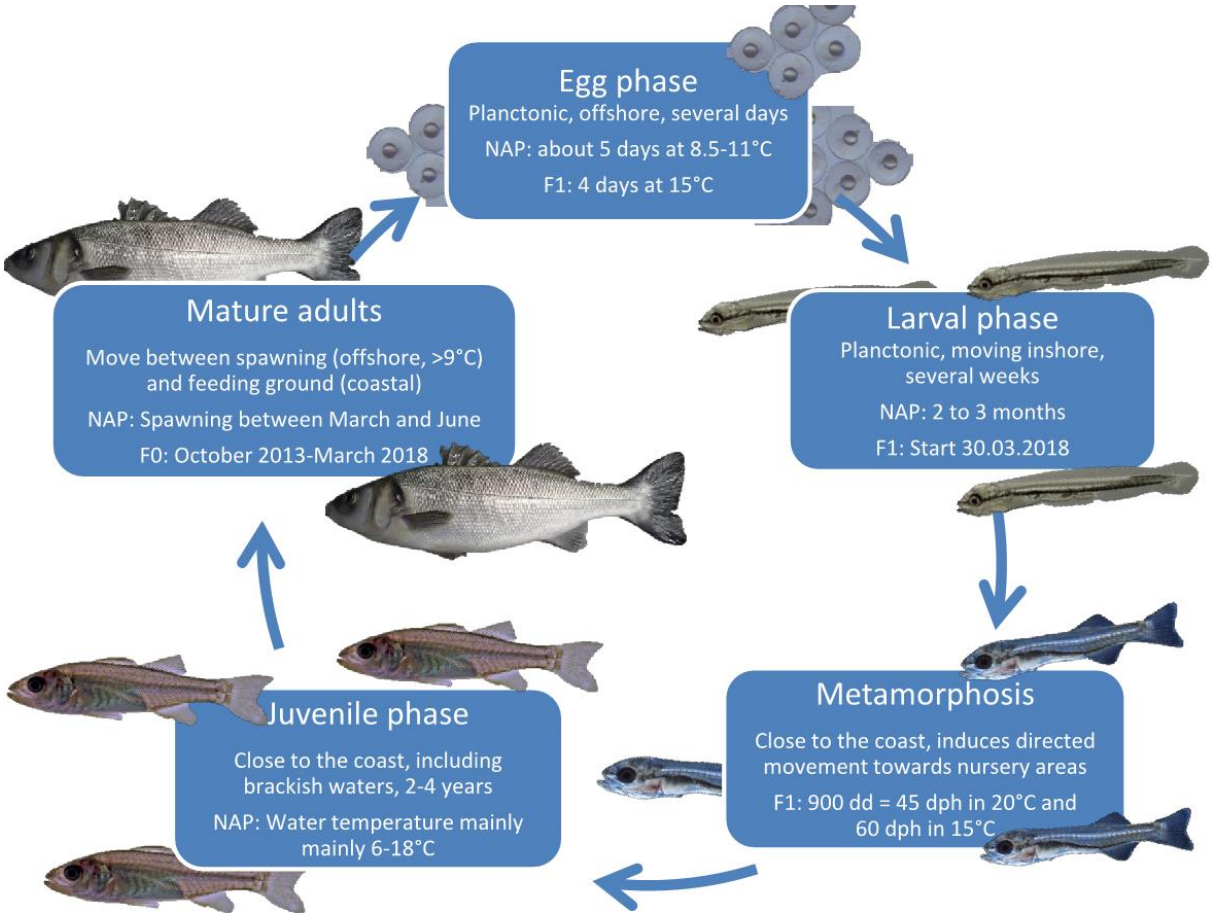

Figure 2 Life cycle of European sea bass from the Northern Atlantic population (NAP), supplemented with dates from F0 and F1 (group 1 and 2, see Figure 3). With dph – days post hatch and dd – degree days (dph · T(°C).

Most of the studies on the thermal biology of European sea bass were done on MP, which live at higher temperatures than NAP and seem to prefer and withstand higher temperatures. Typical habitat temperatures in the Mediterranean are 13-29 °C, depending on season (Person-Le Ruyet et al., 2004), consequently hatching occurred from 8 to 20 °C (Marangos et al., 1986) and optimal temperatures for larval development and juvenile growth were 15 to 17 °C (Koumoundouros et al., 2001; Ayala et al., 2003) and 22 to 28 °C (Lanari et al., 2002; Person-Le Ruyet et al., 2004; Dülger et al., 2012), respectively, in Mediterranean populations. Little experiments were done on AP, but differences in larval development at different temperatures were observed between AP and MP specimen (Ayala et al., 2001), which means that findings on MP are not necessarily transferable to NAP. These differences might be due to the temperature range experienced in nature by the different populations, while the MP experiences relatively high temperatures, NAP experiences temperatures lower than 15 °C for most of the year and mainly within the range of 6-18 °C (Russel et al., 1996). Spawning and larval hatching takes place at 8.5-11 °C in the English Channel (Thompson & Harrop, 1987). Due to the lack of studies on this population, optimal temperatures for larval and

juvenile growth and development were not yet determined. Claireaux and Lagardère (1999) worked on a mixed population (Atlantic and Mediterranean origin, personal communication) and found maximum metabolic rates at 20-24 °C, while the metabolic scope was reduced below 10 °C. While the temperature for maximum metabolic rate is well above the temperature range experienced by the NAP, the temperature of the reduced metabolic scope of 10 °C might shape the northern distribution range of this species and is in accordance with the findings of Pawson et al. (2007) that spawners seek areas where the temperature does not decrease below 9 °C. Although NAP can live and reproduce at these low temperatures, it has been shown that warmer winter temperatures improve growth performance of juvenile European sea bass in the English Channel, but vice versa colder winter temperatures depressed growth, as concluded from year class strength of wild-caught specimens in the 1980s (Pawson, 1992). Another study on NAP juveniles showed that under laboratory conditions growth was higher at 20 °C than at 15 °C (Gourtay et al., 2018), but note that these fish developed at 20 °C during larval rearing, which might have affected the growth performance due to developmental plasticity.

1.4 FITNESS project

My PhD thesis was embedded in the FITNESS project (Fish Transgenerational adaptive Strategies to ocean acidification and warming, funded by the Deutsche Forschungsgemeinschaft (DFG)) which addresses the adaptive capacity of the European sea bass to cope with climate change. The aim of my PhD thesis was to investigate the acclimation capacities of the aerobic metabolism of the European sea bass, *Dicentrarchus labrax,* in the context of OAW. The experiments were done on different developmental stages, namely larvae and juveniles, as well as on different levels of biological organization (metabolome, mitochondria and whole organism). Furthermore, some experiments were compared to previous data gained from the parental generation of “my fish”, to investigate the transgenerational effects on the acclimation capacities.

Within the FITNESS project, three groups of fish were used for experiments (see Figure 3):

- Group 1 (F0): The F0 generation was reared under three different OA conditions (described in detail below) since November 2013, see Crespel et al. (2017; 2019). These fish were produced in an aquaculture facility from a domesticated selected population. At 2 days post hatch (dph) the larvae were transferred to the rearing facilities in the institute and allocated into the different OA conditions. Experiments on these fish started in November 2013 and were partly published in Crespel et al. (2017; 2019). Some results from experiments on these fish are part of this thesis, but the experiments were conducted prior to this thesis and by different persons. F0 fish spawned in March 2018 to produce group 2.

- Group 2 (F1): The F1 generation is the offspring of the ambient and Δ1000 treatment of the F0 group (see details below). The eggs were cultured under the two different OA conditions. After hatching, the larvae were divided into the rearing tanks and two temperatures were applied, resulting in four different OAW conditions (described in detail below). The experiments on these fish started in April 2018 and are part of this thesis.
- Group 3 (Wild): These fish were offspring of wild-caught specimens, reared in the same aquaculture facilities as the parents of group 1. The larvae were obtained from natural spawning and were transferred from the aquaculture facilities to the larval rearing facilities in the institute in January 2016 at 2 dph. They were cultured in six OAW conditions (details below). Experiments on these fish were done in 2016 and 2017 and are part of this thesis

The OAW conditions were defined as follows:

Three conditions of OA:

- A: today's ambient situation in coastal waters of Brittany and the Bay of Brest (approx. 650 µatm CO_2 see Duteil et al., 2016; Pope et al., 2014)
- Δ500: a scenario according to the IPCC (2021) shared socio-economic pathway SSP3-7.0 (ambient plus 500 µatm CO_2– approx. 1150 µatm CO_2)
- Δ1000: a scenario according to SSP5-8.5 (ambient plus 1000 µatm CO_2 – approx. 1700 µatm CO_2).

The F1 fish were only reared under A and Δ1000.

Temperature condition in F0 fish:

- Larval rearing: 19 °C
- Juvenile rearing: ambient temperatures, without allowing the temperature to drop below 12 °C, to avoid temperatures which could inhibit or postpone reproduction.

Two conditions of OW in F1 and Wild:

- Cold life condition (C):
    - Larval rearing: 15 °C
    - Juvenile rearing: ambient temperature (up to 18 °C in 2016 and 19 °C in 2018), without allowing the temperature to drop below 15 °C
- Warm life condition (W):
    - Mirrored cold life condition with an offset of plus 5 °C
    - Larval rearing: 20 °C
    - Juvenile rearing: Cold life condition + 5 °C (up to 23 °C in 2016 and 24 °C in 2018)

Both OW conditions in F1 and Wild were applied on all OA conditions, resulting in the following conditions: C-A, C-Δ500 (only Wild), C-Δ1000, W-A, W-Δ500 (only Wild) and W-Δ1000.

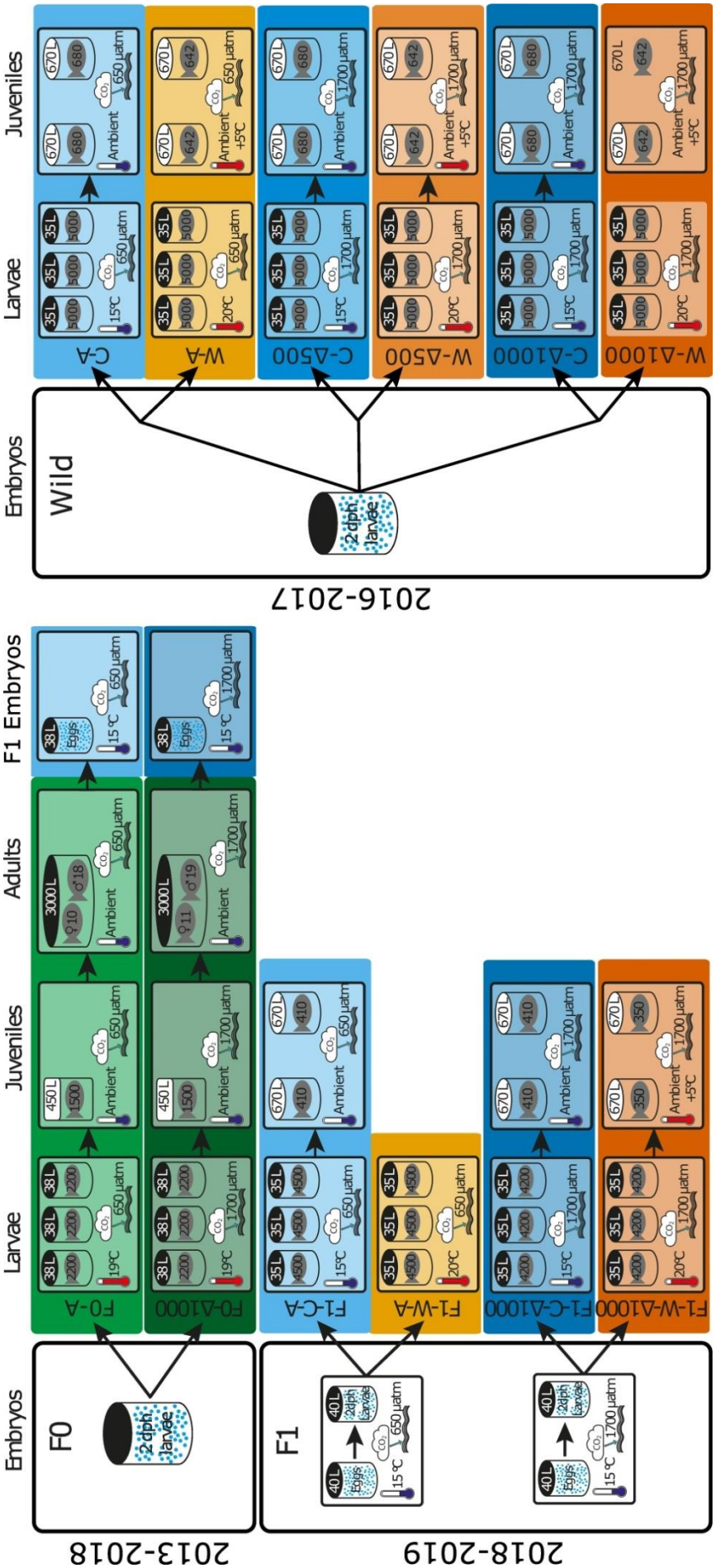

Figure 3 Schematic overview summarizing the rearing conditions under different ocean acidification–warming (OAW) scenarios of the three groups of European sea bass used in this thesis. Parental (F0) and offspring (F1) generations were raised and reared under two acidification conditions: today's ambient levels in the Bay of Brest (650 µatm CO_2, A) and a projected condition following the worst-case scenario prediction of the IPCC (2021) (1700 µatm CO_2, Δ1000). In the third group, originating from wild spawners in an aquaculture facility (Wild), a third acidification condition was applied, reflecting the business as usual condition of the IPCC predictions (1150 µatm CO_2, Δ500; IPCC,2021). F0 was raised under one temperature for all acidification condition (see in text for detail), while in F1 and Wild, two temperatures were applied to each of the acidification conditions: a cold life condition (C) with 15 °C during the larval phase and ambient conditions during the summers (up to 18 °C), and a warm life condition (W) with 20 °C during the larval phase and ambient +5 °C during summers. Duration of the different developmental stages: embryonal phase – several days, larval phase – several weeks, juvenile phase – several years and adult phase – here just until reproduction, but generally several years, too. Age of larvae (days post-hatch, dph), tank volume, shape and inner colour, number of individuals per tank, temperature and PCO_2 are indicated.

1.5 Aim of this thesis

The aim of my PhD thesis was to investigate the acclimation capacities of the aerobic metabolism of the European sea bass (*Dicentrarchus labrax*) in the context of OAW. The experiments were carried out on different developmental stages (larvae and juveniles), as well as on different levels of biological organization (metabolome, mitochondria and whole organism). Furthermore, the effects of the acclimation capacities over two successive generations were investigated. To achieve these goals, I used the three groups of fish under four to six OAW conditions, as described above (see Chapter 1.4 and Figure 3). The applied temperatures of 15-18 and 20-23 °C for C and W life-conditioned fish, respectively, are well within the thermal window of European sea bass from NAP, as described in chapter 1.3. Therefore, W life-conditioned fish should grow and develop faster than C life-conditioned fish when compared at the same age in days post hatch (dph). To be able to compare the fish at the same developmental state, I used the age in degree days (dd), which is calculated by multiplying their age in dph with the temperature of their environment. Doing this should lead to comparable developmental states and sizes.

I aimed to investigate the underlying mechanisms, which shape the capacity of the aerobic metabolism of the European sea bass, to cope with different drivers of climate change. I used different methods to determine the capacity of different parts of the aerobic metabolism, such as measurements of mitochondrial respiration and capacities, standard and routine metabolic rates, growth rates and metabolic profiles. The different groups of fish as well as the different methods were therefore used to address the following questions:

1.5.1 Will mitochondrial performance be impaired in juvenile European sea bass heart mitochondria after long-term acclimation to OAW or after acute temperature changes?

As the performance of heart mitochondria is important for the functioning of the cardiovascular system, it appears crucial to measure oxygen consumption rates of heart mitochondria and understand the underlying cellular processes to predict an organism's capacity to cope with future ocean conditions. To determine the effects of OW, OA, OAW as well as acute temperature changes on mitochondria of European sea bass, I determined mitochondrial respiratory capacities in permeabilized heart fibres of juvenile European sea bass from group 3 (Wild). I proposed the following hypotheses:

(1) Thermal deterioration after acute warming would impair mitochondrial performance in Wild C juveniles due to increased LEAK respiration.
(2) Long-term and developmental warm life conditioning would lead to changes in mitochondrial membrane properties. Therefore, mitochondria of Wild W juveniles would have reduced LEAK respiration in comparison to acutely warmed mitochondria of Wild C juveniles and therefore restored mitochondrial respiratory control ratios.
(3) Mitochondrial metabolism will be impaired by increases in intracellular PCO_2 and bicarbonate concentrations, e.g. by inhibited citrate synthase or succinate dehydrogenase.

1.5.2 Will OAW lead to synergistic effects on growth rates of larval and juvenile European sea bass?

As described above, the use of European sea bass from aquaculture breeding stocks in other studies on the effects of OAW on this species might have caused a bias in the results, therefore I did growth measurements on larvae and juveniles originating from wild-caught breeders (group 3 – Wild) acclimated to four OAW scenarios. The growth data of juvenile Wild fish are presented and discussed in Chapter 4, together with the respective metabolic profiles, explaining possible mechanisms for reduced or unchanged growth. I also followed growth of F1 larvae and juveniles throughout their rearing period and compared their growth rates to growth rates of F0 in publication II. My hypotheses were:

(1) OW will increase growth rates of larval and juvenile European sea bass.
(2) OA will not affect growth rates of larval and juvenile European sea bass.
(3) OAW might lead to lower growth rates in larval and juvenile European sea bass, due to synergistic effects of OA and OW.

1.5.3 Will OAW lead to synergistic effects on metabolic rates of larval and juvenile European sea bass?

Due to the importance of the capacity to supply the tissues with oxygen, I determined metabolic rates of juvenile European sea bass. The data obtained from F0 and F1 juveniles are presented in publication II. Larval metabolic rates were determined continuously during the entire F1-larval period and are also presented in publication II. I proposed the following hypotheses:

(1) OW will lead to increases larval RMR as well as juvenile SMR.
(2) OA will not impair metabolic rates in larvae and juveniles.

(3) OAW will lead to synergistic effects, reflected in lower metabolic rates of larvae and juveniles.

(4) PO_{2crit} in juvenile European sea bass will not be affected by OA, OW or OAW.

1.5.4 Will transgenerational plasticity in European sea bass after acclimation of F0 to OA improve growth and metabolic rates of F1 under OAW?

As TGP can positively affect survival, growth and development of fish larvae, when parents have experiences similar conditions, I compared growth and metabolic rates of F0 and F1 larvae and juveniles in publication II and addressed the question, whether acclimation of two successive generations is positively affecting growth and metabolic rates of juvenile European sea bass under OA and OAW. As F0 was tolerant to OA, I hypothesized that F1 would cope comparably well under OA conditions. My hypotheses were:

(1) Growth rates of F1 W larvae will be similar to growth rates of F0 larvae (rearing temperature of 20 and 19 °C in F1 and F0, respectively).

(2) Size of F1 C juveniles and F0 juveniles will be similar at 3000 dd (rearing temperature equaled ambient temperature in both generations).

(3) Metabolic rates of F1 C juveniles and F0 juveniles will be similar.

(4) OA will not impair growth and metabolic rates of F1 larvae and juveniles.

1.5.5 Will OAW induced changes in growth, metabolic rates and mitochondrial function become visible at the metabolite level?

Metabolomics have been shown to be a valuable tool in assessing the effects of environmental stress in a variety of organisms, including marine fish (as reviewed in Lankadurai et al., 2013). I generated metabolic profiles with ^{1}H-NMR-spectroscopy from heart, liver, gill and white muscle tissue of juvenile European sea bass from group 3 (Wild). Results are presented and discussed together with the respective growth data in publication II. I hypothesized:

(1) Increased growth rates due to OW will be reflected in increased protein synthesis in muscle and liver tissue.

(2) OA will lead to alterations in metabolites involves in maintaining homeostasis, such as organic acids and osmolytes, especially in gill tissue.

(3) OAW might lead to synergistic effects, which will be reflected in alterations of metabolic profiles.

2 Publication I

Future ocean warming may prove beneficial for the northern population of European sea bass, but ocean acidification will not

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RESEARCH ARTICLE

Future ocean warming may prove beneficial for the northern population of European seabass, but ocean acidification will not

Sarah Howald[1,2,*], Louise Cominassi[2], Nicolas LeBayon[3], Guy Claireaux[3,4] and Felix C. Mark[1]

ABSTRACT

The world's oceans are acidifying and warming as a result of increasing atmospheric CO_2 concentrations. The thermal tolerance of fish greatly depends on the cardiovascular ability to supply the tissues with oxygen. The highly oxygen-dependent heart mitochondria thus might play a key role in shaping an organism's tolerance to temperature. The present study aimed to investigate the effects of acute and chronic warming on the respiratory capacity of European sea bass (*Dicentrarchus labrax* L.) heart mitochondria. We hypothesized that acute warming would impair mitochondrial respiratory capacity, but be compensated for by life-time conditioning. Increasing P_{CO_2} may additionally cause shifts in metabolic pathways by inhibiting several enzymes of the cellular energy metabolism. Among other shifts in metabolic pathways, acute warming of heart mitochondria of cold life-conditioned fish increased leak respiration rate, suggesting a lower aerobic capacity to synthesize ATP with acute warming. However, thermal conditioning increased mitochondrial functionality, e.g. higher respiratory control ratios in heart mitochondria of warm life-conditioned compared with cold life-conditioned fish. Exposure to high P_{CO_2} synergistically amplified the effects of acute and long-term warming, but did not result in changes by itself. This high ability to maintain mitochondrial function under ocean acidification can be explained by the fact that seabass are generally able to acclimate to a variety of environmental conditions. Improved mitochondrial energy metabolism after warm conditioning could be due to the origin of this species in the warm waters of the Mediterranean. Our results also indicate that seabass are not yet fully adapted to the colder temperatures in their northern distribution range and might benefit from warmer temperatures in these latitudes.

KEY WORDS: Mitochondrial respiration, Developmental acclimation, Temperate teleost, Heart

INTRODUCTION

The increasing amount of atmospheric CO_2 is working as a greenhouse gas, raising atmospheric temperatures and, as a consequence, also sea surface temperatures (ocean warming). At the same time, about a third of the atmospheric CO_2 is taken up by the oceans, which leads to decreasing seawater pH through the formation of carbonic acid (ocean acidification). These processes together will lead to warmer and more acidic oceans, a trend that has already been observed over the last decades and is predicted to continue. Depending on the representative concentration pathway, the IPCC (2014) predicts that the partial pressure of CO_2 (P_{CO_2}) will increase up to 1000 µatm above current values until the end of this century. Temperature projections for the same time span predict increases of up to 4°C at the coast of Brittany (Sheppard, 2004). Changes in water temperature have direct influence on the metabolic rate of ectothermic organisms, such as fish, with consequences for growth (Pörtner et al., 2007; Peck, 2002), reproductive success (for review, see Llopiz et al., 2014) and biogeography (Turner et al., 2009; Pörtner, 2006). The body of studies looking into the effects of the changing environment on marine ectothermic organisms is growing rapidly. However, only a small number of these studies have investigated the effects of ocean acidification and the combined effects of ocean acidification and warming on fish, with contrasting results between species as well as life stages (Kreiss et al., 2015; Heuer and Grosell, 2014; Pope et al., 2014; Bignami et al., 2013; Frommel et al., 2012). More investigation on a variety of fish species, on different life stages and under ecologically relevant P_{CO_2} concentrations appears necessary (Pope et al., 2014).

The effects of temperature on fish metabolism have been investigated intensively (e.g. Johnson and Katavic, 1986; Mirkovic and Rombough, 1998; Bláquez et al., 1998; Farrell, 2002; Pörtner et al., 2007; Hilton et al., 2010; Strobel et al., 2012). Thermal sensitivity of fish is mainly dependent on the capacity of the cardiovascular system to supply the tissues with oxygen (Pörtner and Lannig, 2009). The heart is a highly aerobic tissue (Driedzic, 1992) and it is therefore believed that the capacity of the heart mitochondria to produce ATP plays a central role in defining thermal tolerance in fish. Although the subcellular processes are not yet fully understood, it has been suggested that the functionality of heart mitochondria determines the temperature of heart failure, and that heart thermal acclimation capacity is relatively limited to safeguard functionality (e.g. Chen and Knowlton, 2010; Chung et al., 2017; Iftikar and Hickey, 2013; Strobel et al., 2013a). Different mitochondrial processes have been shown to be impaired at elevated temperatures, as indicated by decreased oxidative phosphorylation, decreased ATP production efficiency and lost integrity of the protein complexes of the electron transport system (e.g. Fangue et al., 2009; Hilton et al., 2010; Mark et al., 2012; Iftikar and Hickey, 2013). Additionally, high temperatures increase the fluidity of mitochondrial membranes, potentially leading to increased proton leak through the inner mitochondrial membrane and decreased mitochondrial efficiency (Pörtner, 2012). Impaired mitochondrial metabolism thus might lead to alterations in cardiomyocyte ATP supply and consequently affect the performance of the cardiovascular system, which will ultimately determine the thermal sensitivity of the fish. Although juvenile and adult fish generally possess well-developed acid–base regulating mechanisms (for review, see Heuer and Grosell, 2014), increased

[1]Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Integrative Ecophysiology, 27570 Bremerhaven, Germany. [2]Institute of Marine Ecosystem and Fisheries Science, Center for Earth System Research and Sustainability (CEN), University of Hamburg, 22767 Hamburg, Germany. [3]Ifremer, LEMAR (UMR 6539), Laboratory of Adaptation, and Nutrition of Fish, Centre Ifremer de Bretagne, 29280 Plouzané, France. [4]Université de Bretagne Occidentale, LEMAR (UMR 6539), 29280 Plouzané, France.

*Author for correspondence (sarahhowald@gmx.de)

S.H., 0000-0003-4151-3542; L.C., 0000-0002-2606-4753; N.L., 0000-0001-7354-9449; G.C., 0000-0003-1340-6221; F.C.M., 0000-0002-5586-6704

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List of abbreviations

A	ambient P_{CO_2} condition
C	cold life-conditioned group
CCO	cytochrome *c* oxidase
CI–IV	complex I–IV of the electron transport system
CT_{max}	critical thermal maximum
dd	degree days
dph	days post-hatching
L_{Omy}	LEAK respiration with oligomycin
LME model	linear mixed effect model
P	OXPHOS respiration
RCP	representative concentration pathway
RCR_{Omy}	respiratory control ratio with oligomycin
W	warm life-conditioned group
Δ500	ambient P_{CO_2}+500 µatm acidification condition
Δ1000	ambient P_{CO_2}+1000 µatm acidification condition

ocean acidification may act as an additional stressor, e.g. by increasing the ATP demand for acid–base regulation. Consequently, well-functioning mitochondria are even more important if temperature and P_{CO_2} are increased simultaneously. However, while the body of literature on the effects of temperature on mitochondrial function in fish is relatively large (e.g. Fangue et al., 2009; Shama et al., 2014, and references therein), only a handful of studies have investigated the effects of increased P_{CO_2} on fish mitochondria and even fewer have combined ocean acidification and ocean warming (but see Strobel et al., 2013a; Leo et al., 2017). CO_2 can freely diffuse out of the water into the extracellular, intracellular and mitochondrial space. Just as in the blood, decreased intracellular pH due to elevated P_{CO_2} is buffered by actively raising intracellular bicarbonate levels (Strobel et al., 2013a). Mitochondrial membranes are permeable to CO_2 but impermeable to bicarbonate (Arias-Hidalgo et al., 2016), resulting in elevated matrix bicarbonate levels under high CO_2 (Pörtner and Sartoris, 1999; Strobel et al., 2012). Bicarbonate acts as a competitive inhibitor of mitochondrial citrate synthase and succinate dehydrogenase in rodents (Simpson, 1967; Wanders et al., 1983), which can be overcome by a compensatory increase in mitochondrial capacity following hypercapnia acclimation – although this has not yet been documented unequivocally for marine fish (Strobel et al., 2012; 2013a,b). Bicarbonate also acts as a pH-sensing molecule in marine fish and invertebrates (Tresguerres et al., 2014; Barott et al., 2017), with both upregulating and downregulating consequences on mitochondrial metabolism. As over the coming decades ocean temperature and P_{CO_2} will rise hand in hand, it is important to determine their combined effects on mitochondrial metabolism to be able to take ecologically relevant conclusions. Furthermore, there is a relative lack of studies that focus on temperate, large and/or economically relevant species, while at the same time employing realistic P_{CO_2} and exposure scenarios.

In our study, we exposed European seabass, *Dicentrarchus labrax* (L.), from 3 days post-hatching (dph) for 7 months to two temperatures and three P_{CO_2} conditions in a full-factorial design. Temperature and P_{CO_2} conditions reflect the predictions of the IPCC for 2100 (IPCC, 2014). The European seabass is an important aquaculture species (160,000 metric tons in 2015), but also an important target in commercial as well as in recreational fishing activities (Bjørndal and Guillen, 2018). European seabass are distributed throughout the Mediterranean, the Black Sea and the North-Eastern Atlantic from Norway to Senegal in coastal waters from coastal lagoons and estuaries up to 100 m depth (Bjørndal and Guillen, 2018). It has a relatively high tolerance to different temperatures and salinities (Dalla Via et al., 1998; Claireaux and Lagardère, 1999). We studied the effect of ocean acidification and warming on mitochondria of juvenile seabass by determining mitochondrial respiratory capacity in permeabilized heart fibers of seabass juveniles that had been reared under the respective ocean acidification and warming conditions since hatching (7 months). We examined the effects of ocean acidification and warming on mitochondrial ATP-producing processes (OXPHOS respiration) and the counteracting proton leak (LEAK respiration), as well as their resulting ratio (the respiratory control ratio, RCR). An increased LEAK respiration, which is not compensated for by increased OXPHOS respiration, results in a drop in RCR and indicates that mitochondrial functionality and consequently mitochondrial capacity to produce ATP are impaired.

We hypothesized that acute warming would impair mitochondrial performance in juvenile seabass hearts, as LEAK respiration may increase with thermal deterioration of mitochondrial membranes. However, compensational processes after long-term and developmental thermal conditioning could include changes in mitochondrial membrane properties, which would reduce LEAK respiration rates and consequently restore RCR. Additionally, we wanted to fathom the capacity of seabass mitochondria to cope with ocean acidification, especially when combined with ocean warming. We hypothesized that the changes in intracellular P_{CO_2} and bicarbonate concentration elicited by ocean acidification would affect mitochondrial metabolism, e.g. by inhibiting citrate synthase and succinate dehydrogenase, putting further pressure on the cellular energy metabolism.

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 guidelines of 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).

All chemicals were purchased from Sigma-Aldrich, except for tricaine methane sulfonate (MS-222), which was purchased from Pharma Q Limited.

Datasets of mitochondrial respiration and water conditions during rearing, as well as additional information on larval rearing are available online in PANGAEA (www.pangaea.de/).

Animals and experimental conditions

Animals

Larvae were obtained from the aquaculture facility Aquastream (Ploemeur-Lorient, France) at 2 dph (20 January 2016). Brood stock fish were caught in the sea off Morbihan, France. Four females (mean mass 4.5 kg) were crossed with 10 males (mean mass 2.4 kg), which spawned naturally using photothermal manipulation. Conditions in the aquaculture facility during breeding were as follows: 13°C, 35 psu, pH 7.6, 8 h 45 min of light followed by 15 h 15 min darkness. Spawning of eggs took place on 15 January 2016; larvae hatched on 18 January 2016 and were transported to our structures on 20 January 2016.

Larval rearing

Larval rearing was performed in a temperature-controlled room using black 35 l tanks initially stocked with ca. 5000 larvae per tank. Three replicate tanks were used for each temperature–P_{CO_2} combination. Larvae were randomly distributed into the

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experimental tanks at 3 dph (21 January 2016). During the following 3 days, the temperature for the warm life condition was increased stepwise, 1°C during the first day and 2°C during each of the two subsequent days. The P_{CO_2} conditions were applied directly after division into the experimental tanks. Starting at 7 dph (mouth opening), larvae were fed with live artemia, hatched from High HUFA Premium artemia cysts (Catvis, AE's-Hertogenbosch, The Netherlands). Artemia were fed to the larvae 24 h after rearing cysts in sea water up to 33 dph; afterwards, the artemia nauplii themselves were fed with cod liver oil and dry yeast after 24 h and fed to the larvae after 48 h. The artemia were transferred to the larval rearing tanks from two storage tanks (one for each temperature) with peristaltic pumps; their concentration in the tanks was maintained high during the day to allow *ad libitum* feeding; excess artemia left the tank via the waste water outflow. The 15 h photoperiod in the larval rearing room lasted from 07:00 h to 22:00 h; the light intensity increased progressively during the larval rearing period from total darkness to 96 lx (Table S1). Headlamps were used (set to the lowest light intensity) to allow us to work in the larval rearing facility. Larval mortality was 10–80%, regardless of the rearing condition (Table S2). The water surface was kept free of oily films using a protein skimmer. Larval density was reduced regularly during larval rearing as samples for other experiments were taken throughout the entire larval rearing period (at approximately 100, 300, 500, 700, 750 and 900 degree days, dd=dph×temperature in °C). At approximately 980 dd, the early juveniles were transferred from the larval to the juvenile rearing facility, corresponding to 50 dph and 65 dph for 20°C and 15°C rearing, respectively.

Juvenile rearing

As they reached juvenile stage, fish were moved from the larval rearing facilities to juvenile tanks at approximately 1000 dd (50 dph, 8 March 2016, and 65 dph, 23 March 2016, for warm and cold life conditions, respectively). Fish were counted per tank and all fish from one condition were pooled in one tank until the swim bladder test and separation into duplicate tanks at 1541 dd (78 dph, 5 April 2016) and 1301 dd (86 dph, 13 April 2016) for warm and cold life conditions, respectively. The swim bladder test was done to keep only the fish with developed swim bladders. Briefly, the fish were anesthetized and introduced into a test container with seawater with a salinity of 65 psu. Those fish floating at the surface were removed from the test container and placed into the rearing tanks for recovery. The juveniles were reared in round tanks with a volume of 0.67 m^3 and a depth of 0.65 m. Mortality rates of 24.8–43.4% (Table S3) occurred between moving to the juvenile facility and the swim bladder test. During the first 5 days after moving to juvenile rearing facilities, the juveniles were fed artemia nauplii (48 h old and enhanced with cod liver oil and dry yeast) and commercial fish food. Commercial fish food (Neo Start) was fed daily and was adjusted in size (1–3) and amount during the juvenile rearing time, as recommended by the supplier (Le Gouessant, Lamballe, France). More precisely, fish were fed *ad libitum* until 19 August 2016; afterwards, food ratios were calculated every 3–4 weeks for each tank in respect to biomass and temperature of the tank using the formulae provided by Le Gouessant. The daily ration of food was supplied to the tanks by automatic feeders during the daytime; the fish were not fed during the night-time. Photoperiod was adjusted to natural conditions once a week, with slowly increasing light intensity in the juvenile rearing facilities during the first hour each morning. The tanks were cleaned daily after pH measurements. Water flow within the tanks was adjusted once a week, so that oxygen saturation levels were not below 90%, keeping equal flow-through rates in all tanks of one temperature.

Experimental conditions

The larvae and juveniles were reared under six different ocean acidification and warming scenarios, following the predictions of the IPCC (2014) for the next 130 years. The acidification conditions included three different P_{CO_2}: today's ambient situation in coastal waters of Brittany and the Bay of Brest (ambient group – A, approximately 650 µatm; see Pope et al., 2014; Duteil et al., 2016), a scenario according to the IPCC representative concentration pathway RCP 6.0, projecting a ΔP_{CO_2} of 500 µatm to current values (Δ500, approximately 1150 µatm) and a scenario according to RCP8.5, projecting a ΔP_{CO_2} of 1000 µatm (Δ1000, approximately 1700 µatm). All acidification conditions were crossed with two different temperatures to create a 'cold' (C) and a 'warm' (W) life condition scenario. In the cold life condition, larvae were reared at 15°C; juveniles in the cold life condition were reared at 15°C until ambient temperature in the Bay of Brest reached 15°C, and from there on juveniles were reared at ambient temperatures of the Bay of Brest (up to 18°C in 2016). The warm life condition mirrored these thermal profiles, but with an offset of plus 5°C (20–23°C). As larvae and post-larval juveniles would display different growth rates under the two different thermal scenarios, we adopted the concept of degree days (dd, see above) as the basis for comparison between these life conditions.

The sea water used in the aquaria 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, 75 W, Ocene, Louvigné-du-Désert, France), ensuring high water quality. During larval and early juvenile rearing, the water supply for the acidified incubation tanks came from a central header tank, where the water P_{CO_2} conditions were adjusted. The water pH was controlled by an IKS Aquastar system (iks Computer Systeme GmbH, Karlsbad, Germany), which continuously measured pH in one of the replicate tanks and opened a magnetic valve to bubble CO_2 into the header tank when pH in the rearing tank became too high. Water exchange was set to 20 l h^{-1} until 12 dph and 25 l h^{-1} until the end of larval rearing. During juvenile rearing with higher water exchange rates, additional PVC columns were installed to control the pH in the rearing tanks. The water arrived at the top of the column and was pumped from the bottom of the column to the rearing tanks. The CO_2-bubbling apparatus was installed at the bottom of the column and was adjusted by a flow control unit when pH deviated from the desired value. One column supplied both replicate tanks of each condition. Temperature and pH were checked each morning with a handheld WTW 3110 pH meter (with a WTW Sentix 41 electrode, NIST scale; both from Xylem Analytics Germany, Weilheim, Germany) before fish were fed. 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). Total alkalinity was measured once a week following the protocol of Anderson and Robinson (1946) and Strickland and Parsons (1972): 50 ml of filtered tank water (200 µm nylon mesh) was mixed with 15 ml HCl (0.01 mol l^{-1}) and pH was measured immediately. Total alkalinity was then calculated with the following formula:

$$\mathrm{TA} = \frac{V_{\mathrm{HCl}} \cdot c_{\mathrm{HCl}}}{V_{\mathrm{sample}}} - \frac{(V_{\mathrm{HCl}} + V_{\mathrm{sample}})}{V_{\mathrm{sample}}} \cdot \frac{[\mathrm{H}^+]}{\gamma^{\mathrm{H}^+}}, \quad (1)$$

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

The Microsoft Excel macro CO2sys (Lewis and Wallace, 1998) was used to calculate seawater carbonate chemistry, using the constants proposed by Mehrbach and colleagues, refitted by Dickson and Millero (see CO2sys). Oxygen saturation (WTW Oxi 340, Xylem Analytics Germany) and salinity (WTW LF325, Xylem Analytics Germany) were measured once a week together with total alkalinity, from juvenile stage onwards (see Table 1 for all water parameters).

Mitochondrial respirometry

Measurements of mitochondrial respiration rates were performed from approximately 3700 to 4100 dd in all conditions (183–199 dph and 234–249 dph in warm and cold life conditions, respectively). Although the same age in degree days was chosen, the cold life-conditioned fish were significantly smaller than the warm life-conditioned fish [length: 8.72±0.09 cm and 9.59±0.09 mm, respectively, least squares (ls)means±s.e.m., $P<0.001$; and carcass mass: 10.00±0.45 g and 13.38±0.46 g, respectively, lsmeans±s.e.m., $P<0.001$, linear mixed effect models (LME)], resulting in smaller ventricle sizes (0.0105±0.0004 g and 0.0122±0.005 g, respectively, $P<0.05$, lsmeans±s.e.m., LME), as well as lower hepatosomatic indices (1.51±0.05 and 2.45±0.06, respectively, lsmeans±s.e.m., $P<0.0001$, LME). However, condition factor was not different in the two temperature conditions (1.51±0.01 and 1.48±0.01, respectively, lsmeans±s.e.m., $P>0.1$, LME) (see Table S4). P_{CO_2} did not have an effect on fish size. Prior to the experiments, the fish were starved for 2 days. Two batches of eight fish each were processed per day. Juveniles were randomly caught from their tanks and anesthetized with MS-222. The concentration of the anesthetic was adjusted to reach a loss of equilibrium within less than 5 min, typically 0.2 g l^{-1}. Mass, fork length and body length were directly determined with a precision balance (Mettler, Columbus, OH, USA) and a caliper, to the nearest 0.01 g and 0.01 mm, respectively. Afterwards, fish were killed by a cut through the neck, and the heart was completely dissected from the fish, followed by excavation of the ventricle. Excess blood was removed from the ventricle by cleaning it on blotting paper, prior to weighing (Sartorius, Göttingen, Germany). Tissue from a whole ventricle was used for respiration measurements in each respiration chamber of the oxygraphs and respiration rates were normalized to ventricle mass. The ventricle was stored in relaxing and biopsy preservation solution [BIOPS: 10 mmol l^{-1} Ca-EGTA (0.1 µmol l^{-1} free calcium), 20 mmol l^{-1} imidazole, 20 mmol l^{-1} taurine, 50 mmol l^{-1} K-Mes, 0.5 mmol l^{-1} DTT, 6.56 mmol l^{-1} $MgCl_2$, 5.77 mmol l^{-1} ATP, 15 mmol l^{-1} phosphocreatine, pH 7.1; modified after Gnaiger et al., 2000] until all eight ventricles were dissected. The ventricles were manually frayed and were permeabilized on ice with saponin (50 µg ml^{-1}) for 20 min on a shaking table (80 rpm), followed by two cleaning steps for 10 min at 80 rpm in modified mitochondrial respiration medium [MiR05: 160 mmol l^{-1} sucrose, 60 mmol l^{-1} potassium-lactobionate, 20 mmol l^{-1} taurine, 20 mmol l^{-1} Hepes, 10 mmol l^{-1} KH_2PO_4, 3 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} EGTA, 1 g l^{-1} bovine albumin serum (fatty acid free), pH 7.45 at 15°C; modified after Fasching et al., 2014]. During the permeabilization step, the livers and the carcasses of the fish were weighed to calculate the hepatosomatic index (HSI) and condition factor (K) (see Table S4).

Mitochondrial respiration of the permeabilized heart fibers was measured using four Oroboros Oxygraph-2K respirometers with DatLab 6 software (Oroboros Instruments, Innsbruck, Austria). Permeabilized fibers have the advantage of resembling the living state as closely as possible, while still allowing control of the supply of substrates and inhibitors to the mitochondria (Saks et al., 1998; Pesta and Gnaiger, 2012). Measurements were conducted at 15 and 20°C for all treatments to determine the effect of acute temperature changes on mitochondrial metabolism *in vitro*. The oxygen sensors were calibrated in air-saturated buffer prior to the experiments and in oxygen-depleted buffer after each experiment. The measurements were done in MiR06 buffer (MiR05 buffer enriched with 280 U ml^{-1} catalase; Fasching et al., 2014) to allow for reoxygenation with H_2O_2. A standard substrate–uncoupler–inhibitor titration protocol was employed to measure the respiration rates of the different complexes: glutamate (10 mmol l^{-1}), malate (2 mmol l^{-1}) and pyruvate (10 mmol l^{-1}) were used to measure LEAK respiration of complex I [$L(n)_{CI}$; Table 2], followed by addition of ADP

Table 1. Water parameters during larval and juvenile phase of the 2016 batch

Treatment	pH	Temp. (°C)	Salinity (psu)	O_2 (% air sat.)	TA (mol l^{-1})	P_{CO_2} (µatm)
Larvae						
C–A	7.95±0.01	15.3±0.0	33.0±0.1	–	2364±17	656±16
C–Δ500	7.77±0.01	15.3±0.0	33.0±0.1	–	2382±19	1041±26
C–Δ1000	7.58±0.00	15.3±0.0	33.0±0.1	–	2394±26	1682±26
W–A	7.88±0.01	20.0±0.1	33.1±0.1	–	2369±21	832±13
W–Δ500	7.79±0.01	20.0±0.1	33.1±0.1	–	2383±22	1057±30
W–Δ1000	7.60±0.01	20.0±0.1	33.1±0.1	–	2380±23	1672±33
Juveniles						
C–A	7.97±0.01	16.0±0.2	34.2±0.1	90.9±0.5	2396±18	655±18
C–Δ500	7.75±0.01	16.0±0.2	34.2±0.1	92.2±0.6	2404±19	1107±21
C–Δ1000	7.55±0.01	16.1±0.2	34.2±0.1	90.9±0.6	2399±19	1841±40
W–A	7.92±0.01	21.9±0.2	35.0±0.2	90.2±0.9	2418±12	788±22
W–Δ500	7.78±0.01	21.8±0.2	35.0±0.2	90.5±0.7	2420±15	1133±43
W–Δ1000	7.59±0.01	21.9±0.2	35.0±0.2	91.3±0.6	2423±12	1808±65
Ambient seawater						
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

The larval period lasted until 4 March 2016 [46 days post-hatching (dph), ~900 degree days (dd)] and 18 March 2016 (60 dph, ~900 dd) for warm and cold life conditions, respectively; and the juvenile period until 24 October 2016 (280 dph, ~5900 dd) and 8 February 2017 (387 dph, ~6200 dd) for warm and cold life-conditioned fish, respectively. Data are means±s.e.m. over all replicate tanks per condition. pH, temperature (Temp.), salinity, oxygen saturation (during juvenile rearing) and total alkalinity (TA) were measured weekly; P_{CO_2} was calculated with CO2sys. Sea water (SW) measurements were conducted in 2017 and 2018. A, ambient P_{CO_2}; Δ500, ambient+500 µatm CO_2; Δ1000, ambient+1000 µatm CO_2; C, cold life condition; W, warm life condition.

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Table 2. Analyzed mitochondrial metabolic states (after Gnaiger, 2014) during the substrate–uncoupler–inhibitor titration protocol

Description	State	Name
Substrates for CI are available but no ADP	LEAK	$L(n)_{CI}$
Substrates for CI and ADP are available to fuel coupled respiration of CI	OXPHOS	P_{CI}
Substrates for CI and CII as well as saturating ADP are available to fuel coupled respiration of the full electron transport system	OXPHOS	P
Oligomycin inhibits F_1F_O-ATPase resulting in LEAK respiration	LEAK	L_{Omy}
Coupled respiration of CII calculated: $P–P_{CI}$	OXPHOS	P_{CII}
Respiratory control ratio calculated: $P×L_{Omy}^{-1}$	–	RCR_{Omy}

Oxygen was available in all these states. $L(n)_{CI}$, LEAK respiration of complex I; P, OXPHOS respiration; CI, mitochondrial complex I; CII, mitochondrial complex II; L_{Omy}, LEAK respiration with oligomycin.

(2.5 mmol l^{-1}) to measure OXPHOS respiration of complex I (P_{CI}). Succinate (10 mmol l^{-1}) and further ADP (two additions of 2.5 mmol l^{-1}) resulted in OXPHOS respiration of complex I (CI) and complex II (CII) combined (P). Cytochrome c (0.01 mmol l^{-1}) was used as a control for inner mitochondrial membrane integrity; measurements with increases of more than 10% following cytochrome c addition were not used for further analyses. Oligomycin (4 µg ml^{-1}) was used to inhibit F_OF_1-ATPase, resulting in LEAK respiration (L_{Omy}). Stepwise titration of FCCP (0.05 µl of 2 mmol l^{-1} stock solution per step) was used to uncouple the mitochondrial electron transport system and determine its maximum capacity. After uncoupling, CI, CII and complex III (CIII) were successively inhibited with rotenone (0.005 mmol l^{-1}), malonate (5 mmol l^{-1}) and antimycin A (0.0025 mmol l^{-1}), respectively. Residual respiration after antimycin A addition was used to correct all mitochondrial respiration rates. Complex IV (CIV) capacity was then determined by addition of ascorbate (2 mmol l^{-1}) and TMPD (0.5 mmol l^{-1}). Oxygen levels were usually restored by addition of 2 µl H_2O_2 (3% stock solution) after the oligomycin and rotenone steps.

A measure for OXPHOS respiration of CII (P_{CII}) was calculated ($P_{CII}=P–P_{CI}$), although this measure will lead to lower respiration rates than direct determination of P_{CII} respiration, when only substrates for CII are available (Gnaiger, 2009; Mark et al., 2012). For all complexes, the contribution of the respiration of the respective complex to OXPHOS respiration was calculated. This was also done for L_{Omy}, which we tentatively termed the 'L_{Omy} fraction', as a relative indicator of proton leak, despite the fact that membrane potential is potentially higher in L_{Omy} than in natural state 4. Mitochondrial quality and efficiency were evaluated by calculating the respiratory control ratio ($RCR_{Omy}=P×L_{Omy}^{-1}$), which is an indicator of mitochondrial coupling (Gnaiger, 2009; Strobel et al., 2013a).

Statistical analysis

All statistics were performed with R (http://www.R-project.org/). Data were tested for outliers (Nalimov test), normality (Shapiro–Wilk's test, $P>0.05$) and homogeneity (Levene's test, $P>0.05$). Mitochondrial respiratory data were fitted to linear mixed effect models (LME model, 'lme' function of 'nlme' package; https://CRAN.R-project.org/package=nlme). Conditioning and assay temperature, as well as P_{CO_2} and their interactions were included as fixed effects. Because of the significantly different sizes of the fish, fish mass was also included as a fixed effect, whereas the oxygraph chamber and the number of the run on that day were included as random effects. In case of heterogeneity of data, variance structures were included in the random part of the model; the best variance structure was chosen according to the lowest Akaike information criterion (AIC) values. Validity of linearity for P_{CO_2} concentration and mass was cross-tested with generalized additive models ('gam' function of 'mgcv' package; Wood, 2017), as described in Zuur et al. (2009). If linearity was given, the LME model was chosen instead of the generalized additive model. If significant effects were detected in the LME models, *post hoc* Tukey tests were performed with the 'lsmeans' function ('lsmeans' package; Lenth, 2016). Significance for all statistical tests was set at $P<0.05$. All graphs are produced from the lsmeans data with the 'ggplot2' package (Wickham, 2016). All data are shown as lsmeans±s.e.m. Biometrical data were also tested with LME models, with P_{CO_2}, conditioning and assay temperature as fixed effects and origin tank as a random effect. Model validation was carried out in the same way as described above for mitochondrial respiratory data.

RESULTS

Effects of acute *in vitro* warming on mitochondrial function in cold life-conditioned fish

P_{CII} (Fig. 3) respiration rates increased significantly with acute warming in the Δ1000 group (LME, $P<0.05$; Table 3). All other analyzed parameters were not affected by acute warming: P (Fig. 1), P_{CI} (Fig. 2) and the relative contributions of CI and CII to P (Table 3; CI fraction: 53.4±3.3–61.1±3.3%, Fig. S1; and CII fraction: 38.8±3.3–46.5±3.3%, Fig. S2).

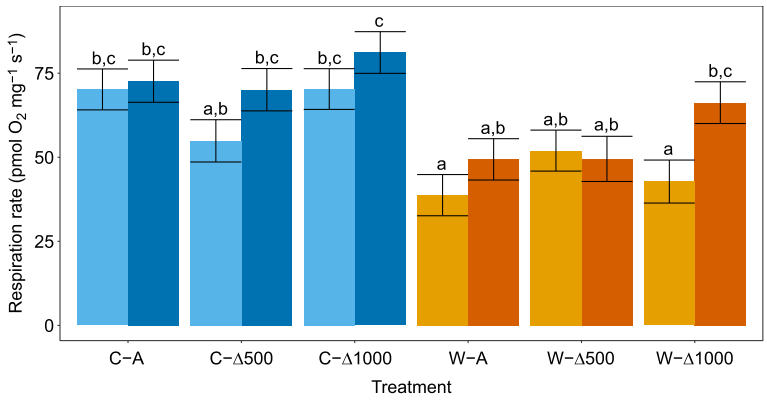

Fig. 1. Respiration rates of permeabilized heart ventricle of European seabass. Respiration rate (oxidative phosphorylation capacity, P) data are least squares (ls)means±s.e.m. Different letters indicate significant differences [linear mixed effects (LME), $P<0.05$]; blue: cold life-conditioned fish (C), orange: warm life-conditioned fish (W), light shading: cold assay temperature, dark shading: warm assay temperature. A, ambient P_{CO_2}; Δ500, ambient+500 µatm CO_2; Δ1000, ambient+1000 µatm CO_2. $n_{C–A}$=16/15, $n_{C–Δ500}$=15/16, $n_{C–Δ1000}$=16/16, $n_{W–A}$=16/15, $n_{W–Δ500}$=17/11, $n_{W–Δ1000}$=13/15, for cold/warm assay temperature, respectively.

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Table 3. *F*-values of fixed effects from the linear mixed models on mitochondrial respiration of permeabilized heart fibers of juvenile European seabass

	Den. d.f.	CT (1 d.f.)	AT (1 d.f.)	P_{CO_2} (2 d.f.)	Mass (1 d.f.)	CT:AT (1 d.f.)	CT:P_{CO_2} (2 d.f.)	AT:P_{CO_2} (2 d.f.)	CT:AT:P_{CO_2} (2 d.f.)
RCR_{Omy}	126	12.50***	$1.66^{n.s.}$	$0.19^{n.s.}$	4.73*	$1.31^{n.s.}$	$2.18^{n.s.}$	$0.05^{n.s.}$	$0.72^{n.s.}$
Oxygen consumption rate									
P	147	40.83***	20.76***	$2.46^{‡}$	$0.65^{n.s.}$	$0.09^{n.s.}$	$2.16^{n.s.}$	$2.04^{n.s.}$	4.20*
P_{CI}	146	29.08***	10.10**	$1.17^{n.s.}$	$1.09^{n.s.}$	$0.00^{n.s.}$	3.33*	$0.20^{n.s.}$	3.46*
P_{CII}	144	12.30***	26.30***	$2.05^{n.s.}$	$0.22^{n.s.}$	$0.00^{n.s.}$	$0.15^{n.s.}$	4.14*	3.52*
L_{Omy}	141	122.51***	39.75***	$1.78^{n.s.}$	$2.87^{‡}$	5.43*	4.03*	$0.93^{n.s.}$	$0.18^{n.s.}$
CIV	129	38.64***	42.90***	$0.47^{n.s.}$	10.83**	$2.82^{‡}$	$0.57^{n.s.}$	$0.08^{n.s.}$	$0.15^{n.s.}$
OXPHOS respiration fraction									
P_{CI} fraction	144	$2.93^{‡}$	$0.56^{n.s.}$	$0.14^{n.s.}$	$2.91^{‡}$	$0.00^{n.s.}$	$2.49^{‡}$	$2.23^{n.s.}$	$0.46^{n.s.}$
P_{CII} fraction	142	$2.37^{n.s.}$	$1.36^{n.s.}$	$0.07^{n.s.}$	$2.41^{n.s.}$	$0.02^{n.s.}$	3.16*	$1.90^{n.s.}$	$0.45^{n.s.}$
L_{Omy} fraction	144	9.82**	4.59*	$0.65^{n.s.}$	$1.76^{n.s.}$	$3.28^{‡}$	$2.57^{‡}$	$0.70^{n.s.}$	$1.27^{n.s.}$
CIV capacity	130	$2.85^{‡}$	$1.48^{n.s.}$	$1.00^{n.s.}$	$2.75^{‡}$	$0.19^{n.s.}$	$1.80^{n.s.}$	$1.11^{n.s.}$	$1.14^{n.s.}$

Denominator degrees of freedom (Den. d.f.) and the number of degrees of freedom (1 or 2) are indicated. CT, conditioning temperature; AT, assay temperature; RCR_{Omy}, respiratory control ratio with oligomycin; *P*, oxidative phosphorylation capacity; CIV, complex IV; L_{Omy}, LEAK respiration with oligomycin. $^{‡}P<0.1$; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

CIV capacity increased with acute warming in both hypercapnia groups (LME, Δ500: $P<0.05$ and Δ1000: $P<0.05$; Table 3) but not in fish at ambient P_{CO_2} (Fig. 4). However, this increase was not strong enough to change the relationship between CIV capacity and *P* (Fig. S3).

L_{Omy} respiration rate increased significantly with acute warming in the A and Δ1000 treatments (LME, $P<0.05$ and $P<0.01$, respectively; Table 3 and Fig. 5). Assay temperature had a significant effect on the relative contribution of L_{Omy} to *P* (L_{Omy} fraction; Table 3). However, no specific differences were detected with the *post hoc* tests (Fig. S4). As P_{CO_2} did not affect the L_{Omy} fraction, we pooled data over P_{CO_2} treatments, which emphasized that acute warming led to impaired mitochondria in the cold life-conditioned fish, as indicated by a significantly higher L_{Omy} fraction under warm assay temperatures compared with cold assay temperatures (Table 4; LME, $P<0.05$).

Effects of acute *in vitro* cooling on mitochondrial function in warm life-conditioned fish

Acute cooling led to significantly decreased *P* and P_{CII} respiration rates in the Δ1000 group (LME, $P<0.01$, Fig. 1 and $P<0.01$, Fig. 3, respectively). All other analyzed parameters were not affected by acute cooling: P_{CI} (Fig. 2), CIV (Fig. 4), L_{Omy} (Fig. 5), and the relative contributions of CI, CII, CIV and L_{Omy} to *P* (Table 3; CI fraction: 50.2±3.2–57.5±3.3%, Fig. S1; CII fraction: 41.7±3.2–49.7±3.1%; Figs S2, S3 and S4).

Effects of long-term thermal conditioning on mitochondrial function

Warm life conditioning led to decreased *P* and P_{CI} respiration rates in the A and Δ1000 group, either at both assay temperatures (P_{CI} in the A group, Fig. 2, LME, $P<0.01$) or only at the cold assay temperature (*P* in the A and Δ1000 group, Fig. 1, LME, $P<0.001$ and $P<0.01$, respectively; and P_{CI} in the Δ1000 group, LME, cold assay temperature, $P<0.05$).

No significant effects of warm life conditioning were observed for P_{CII} (Fig. 3) and CIV capacity (Fig. 4) or the relative contributions of CI and CII to *P* respiration (Table 3). CIV capacity was 1.5–2 times higher than *P* respiration (Fig. S3) and did not differ between thermal life conditions.

L_{Omy} respiration rate was significantly decreased in warm life-conditioned fish compared with cold life-conditioned fish in the A group (Fig. 5; LME, $P<0.01$ and $P<0.0001$, both assay temperatures, respectively) and in the Δ500 group (LME, $P<0.0001$, warm assay temperature).

Thermal life condition and assay temperature had significant effects on the relative contribution of L_{Omy} to *P* (L_{Omy} fraction; Table 3). As P_{CO_2} did not affect the L_{Omy} fraction, P_{CO_2} treatment data

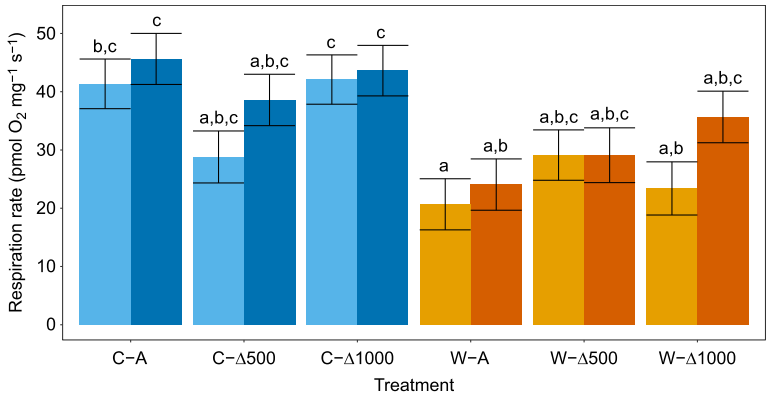

Fig. 2. OXPHOS respiration rate of complex I (P_{CI}) in permeabilized heart ventricle fibers of European seabass. Data are lsmeans±s.e.m. Different letters indicate significant differences (LME, $P<0.05$); blue: cold life-conditioned fish (C), orange: warm life-conditioned fish (W), light shading: cold assay temperature, dark shading: warm assay temperature. A, ambient P_{CO_2}; Δ500, ambient+500 µatm CO_2; Δ1000, ambient+1000 µatm CO_2. n_{C-A}=16/15, $n_{C-\Delta500}$=14/16, $n_{C-\Delta1000}$=16/16, n_{W-A}=16/14, $n_{W-\Delta500}$=17/11, $n_{W-\Delta1000}$=13/15, for cold/warm assay temperature, respectively.

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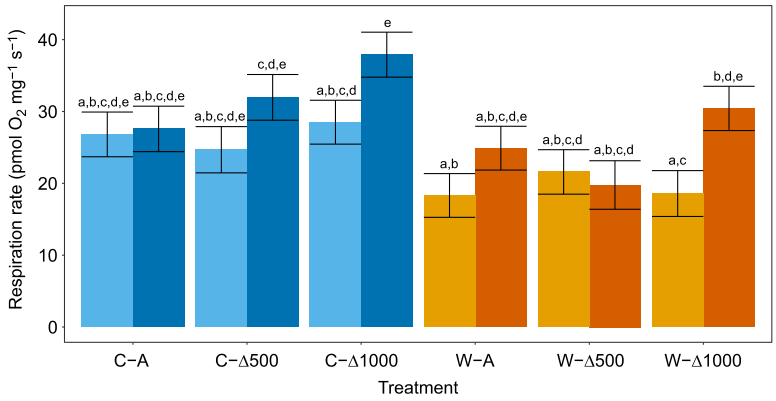

Fig. 3. OXPHOS respiration rate of complex II (P_{CII}) in permeabilized heart ventricle fibers of European seabass. Data are lsmeans±s.e.m. Different letters indicate significant differences (LME, P<0.05); blue: cold life-conditioned fish (C), orange: warm life-conditioned fish (W), light shading: cold assay temperature, dark shading: warm assay temperature. A, ambient P_{CO_2}, Δ500: ambient+500 µatm CO_2, Δ1000: ambient+1000 µatm CO_2. $n_{C–A}$=15/15, $n_{C–Δ500}$=14/16, $n_{C–Δ1000}$=16/16, $n_{W–A}$=15/14, $n_{W–Δ500}$=16/11, $n_{W–Δ1000}$=13/15, for cold/warm assay temperature, respectively.

were pooled. These data indicated by a significantly higher L_{Omy} fraction in the warm compared with the cold assay temperature that acute warming led to impaired mitochondria in the cold life-conditioned fish, whereas the L_{Omy} fraction did not differ between the thermal life history groups when compared at the respective conditioning temperature (Table 4; LME, P<0.05). Conditioning to warmer temperatures led to a significantly decreased L_{Omy} fraction (Table 3).

Mitochondria were well coupled in all treatments (RCR_{Omy}>4), with significantly higher RCR_{Omy} in warm life-conditioned fish than in cold life-conditioned fish (LME, 9.43±1.38 and 6.46±1.33, respectively, P<0.05). There were no significant effects of assay temperature, P_{CO_2} or any interaction terms on RCR_{Omy} (Table 3).

Effects of acclimation to different P_{CO_2} on mitochondrial function

Elevated P_{CO_2} alone did not have significant effects on any of the studied complexes and processes of the electron transport chain (Table 3). However, we found synergistic effects with temperature which became visible as interaction effects with life condition or assay temperature only in the Δ500 or Δ1000 fish, as specified above.

DISCUSSION

Mitochondrial functional capacities were examined in seabass juveniles raised in six combinations of three P_{CO_2} and two temperature treatments. The data provide evidence that heart mitochondria of juvenile seabass can be impaired by acute warming, as observed in increased L_{Omy} respiration rates, for example. In contrast, warm life conditioning increased mitochondrial efficiency in comparison to that of cold life-conditioned fish, as seen through increased RCR_{Omy}.

Ocean acidification did not affect mitochondrial functioning in juvenile seabass as a single factor, as indicated by no significant effects of P_{CO_2} alone on mitochondrial capacities. However, ocean acidification intensified the effects of acute or long-term warming. This was most prominent in the high acidification warm life condition treatment, e.g. P respiration rates were only significantly affected by acute temperature change in the W–Δ1000 fish, due to the decrease of P_{CII} in this group. CI, CIV and RCR_{Omy} were not affected by P_{CO_2}. This observation reflects the findings of previous studies in polar fish, where thermal effects on mitochondrial capacity were much more prominent than those of ocean acidification (e.g. Leo et al., 2017; Strobel et al., 2013b). However, the reduced ability of CII to cope with acute temperature changes in the W–Δ1000 fish is in agreement with other studies which found that CII was inhibited by elevated P_{CO_2} in mammals and fish (Simpson, 1967; Wanders et al., 1983; Strobel et al., 2013a). In juvenile seabass, CII was only inhibited by high P_{CO_2} in warm life-conditioned fish facing an acute temperature decrease. Therefore, juvenile seabass mitochondria appear generally able to cope with the inhibiting effect of high P_{CO_2} on CII. Other studies suggested that mitochondria could employ anaplerotic

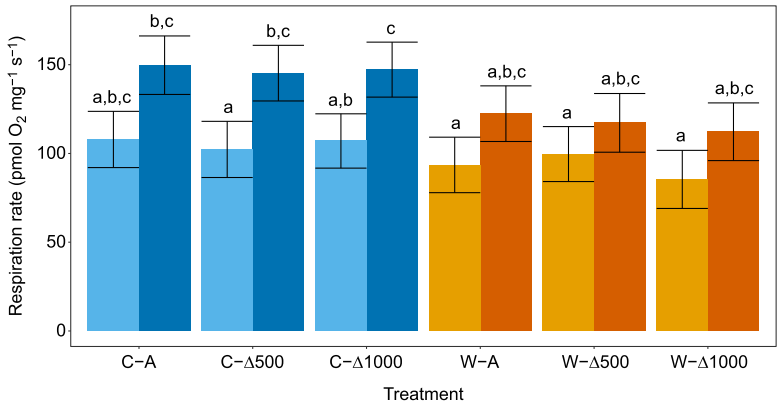

Fig. 4. Complex IV (CIV) respiration rates of permeabilized heart ventricle fibers of European seabass. Data are lsmeans±s.e.m. Different letters indicate significant differences (LME, P<0.05); blue: cold life-conditioned fish (C), orange: warm life-conditioned fish (W), light shading: cold assay temperature, dark shading: warm assay temperature. A, ambient P_{CO_2}; Δ500: ambient+500 µatm CO_2; Δ1000: ambient+1000 µatm CO_2. $n_{C–A}$=12/12, $n_{C–Δ500}$=14/16, $n_{C–Δ1000}$=15/16, $n_{W–A}$=15/14, $n_{W–Δ500}$=17/11, $n_{W–Δ1000}$=11/13, for cold/warm assay temperature, respectively.

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Table 4. L_{Omy} fraction of *P* respiration in permeabilized heart fibers of European seabass

AT	C (%)	W (%)
Cold	16.58±1.60[a]	14.58±1.65[a]
Warm	20.79±1.65[b]	14.41±1.70[a]

Data are least squares (ls)means±s.e.m. pooled over all P_{CO_2} conditions; different letters indicate significant differences (linear mixed effects, LME, P<0.05). C, cold life-conditioned fish; W, warm life-conditioned fish; AT, assay temperature. $n_{C–Cold}$=46, $n_{C–Warm}$=45, $n_{W–Cold}$=46, $n_{W–Warm}$=40.

mechanisms, such as decarboxylation of aspartate and glutamate, feeding into the Krebs cycle via oxaloacetate and oxoglutarate and stimulating CI with additional NADH-related substrates to overcome inhibitory effects of high P_{CO_2} of CII (Langenbuch and Pörtner, 2002; Strobel et al., 2013a). However, if environmental temperature or P_{CO_2} concentration further increase, decreased *P* respiration rates due to reduced CII respiration could occur at the respective habitat (conditioning) temperature and not only under acute temperature change.

Acute warming impairs heart mitochondria of cold life-conditioned juvenile seabass by increasing L_{Omy} respiration, significantly so in the C–A and C–Δ1000 groups, as seen in the increased L_{Omy} fraction. Increased leak respiration rates (L_{Omy} as well as L_{Omy} fraction) indicate decreasing mitochondrial membrane integrity, translating into less ATP produced for the same amount of oxygen consumed. Increases in mitochondrial enzyme activity and respiration rates of mitochondrial complexes, as well as *P* respiration rates with acute temperature increase have been shown in other fish species, e.g. Antarctic nototheniids, European perch and Atlantic cod (Strobel et al., 2013a; Ekström et al., 2017; Leo et al., 2017). Iftikar and Hickey (2013) showed in hearts of New Zealand triple fin fishes that compromised mitochondria at acutely elevated temperature will ultimately lead to heart failure. Consequently, as acute warming of only 5°C impaired mitochondria of the cold life-conditioned fish, it appears likely that cold life-conditioned juvenile seabass can suffer from heart metabolic deficiencies, if acute temperature changes exceed 5°C. This reduced tolerance to acute temperature increase in the cold life-conditioned fish seems to contradict the fact that European seabass are generally highly tolerant to a wide range of temperatures (Dalla Via et al., 1998; Claireaux and Lagardère, 1999). It also seems to contradict the high critical thermal maximum (CT_{max}) of European seabass (28.12±0.09 to 32.50±0.04°C in Mauduit et al., 2016; and 31.3±0.3°C in Anttila et al., 2017). However, in seabass acclimated to 17°C, Anttila et al. (2017) found arrhythmia occurred at around 22°C, although CT_{max} was above 30°C. Furthermore, the Arrhenius breakpoint temperature for maximum heart rate, which is connected to the thermal optimum of growth and aerobic scope, was 19.3±0.3°C and the temperature with the highest maximal heart rate, a measure of the thermal limits of cardiac function, was 21.8±0.4°C in these fish (Anttila et al., 2017). These findings support our conclusion that cold life-conditioned juvenile sea bass might be less able to cope with large acute temperature changes than their warm life-conditioned siblings.

While acute warming impairs the performance of juvenile seabass heart mitochondria, the warm life-conditioned fish showed higher mitochondrial functionality, indicating that the chosen cold life-conditioning temperature is not the optimal temperature for Atlantic juvenile seabass. The thermal biology of *D. labrax* has been the topic of several studies, although mainly on Mediterranean populations (e.g. Marangos et al., 1986; Koumoundouros et al., 2001; Lanari et al., 2002; Person-Le Ruyet et al., 2004; Dülger et al., 2012; but see Russel et al., 1996; Ayala et al., 2003; Gourtay et al., 2018). However, in contrast to the Mediterranean populations, which are exposed to higher habitat temperatures (typical annual range 13–29°C; Person-Le Ruyet et al., 2004), the Atlantic population experiences temperatures lower than 15°C for most of the year and mainly within the range 6–18°C along the coast of France up to the North Sea (Russel et al., 1996). Our fish are the offspring of fish caught in the Bay of Biscay. In these latitudes, spawning, egg development and larval hatching take place at temperatures of 8–13°C (Jennings and Pawson, 1992) and later life stages experience mainly temperatures between 6 and 18°C (Russel et al., 1996). Therefore, the temperature range we used for incubating the larvae in the cold life condition was slightly above the natural temperature range of seabass larvae from the chosen distribution area. However, for juvenile incubation the cold life condition temperature range of 15–18°C was well within the natural temperature range during summer. The temperature of the warm life-conditioned juveniles was consequently above the temperature range of their natural habitat in the Bay of Brest. Our study thus provides evidence that the seabass from the chosen population are not yet fully adapted to lower temperatures, as the warm life-conditioned juveniles displayed much better mitochondrial functionality than the cold life-conditioned animals, reflecting their evolutionary origin in warmer waters.

As a consequence of warm life conditioning, L_{Omy} and *P* respiration rates were both significantly decreased, while RCR_{Omy}

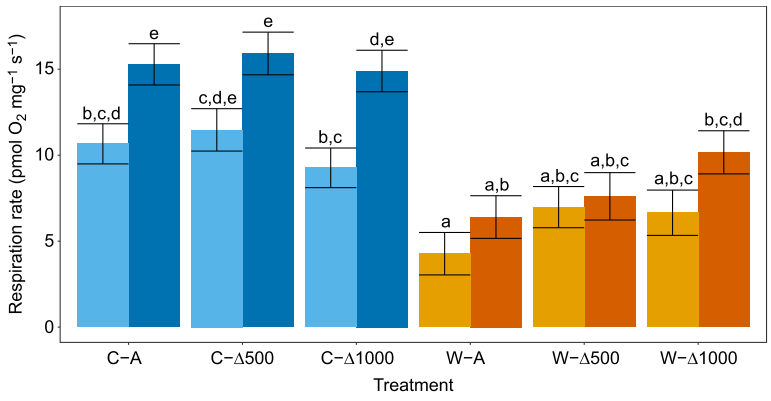

Fig. 5. LEAK respiration with oligomycin (L_{Omy}) of permeabilized heart ventricle fibers of European seabass. Data are lsmeans±s.e.m. Different letters indicate significant differences (LME, P<0.05); blue: cold life-conditioned fish (C), orange: warm life-conditioned fish (W), light shading: cold assay temperature, dark shading: warm assay temperature. A, ambient P_{CO_2}; Δ500, ambient+500 µatm CO_2; Δ1000, ambient+1000 µatm CO_2. $n_{C–A}$=16/15, $n_{C–Δ500}$=15/16, $n_{C–Δ1000}$=16/16, $n_{W–A}$=16/15, $n_{W–Δ500}$=17/11, $n_{W–Δ1000}$=13/15, for cold/warm assay temperature, respectively.

was significantly increased, which was the case in all treatments. The increased RCR_{Omy} in warm life-conditioned fish in comparison to cold life-conditioned fish indicates that although mitochondrial capacity was decreased in warm life-conditioned fish (decreased P), mitochondrial efficiency was increased (decreased L_{Omy} and increased RCR_{Omy}). This could translate into higher growth rates: Shama et al. (2014) found that lower P capacity resulted in optimized metabolic rate that could generate higher scope for growth in sticklebacks acclimated to warmer temperatures. Additionally, in brown trout, lower food intake and failure to grow were correlated to high L_{Omy} respiration rate and lower mitochondrial coupling in liver and muscle mitochondria (Salin et al., 2016a). In other words, individuals with more efficient oxidative phosphorylation tend to grow better than those with less efficient mitochondria. Our study reflects these findings: the warm life-conditioned juveniles showed higher RCR_{Omy} and lower P respiration rates, while being significantly larger than the cold life-conditioned fish, even when compared at equal age in degree days.

In our study, CIV or cytochrome *c* oxidase (CCO) activity was not affected by thermal life condition. As terminal electron acceptor of the electron transport system, CCO is important in aerobic respiration and was found to be the controlling site of mitochondrial respiration and ATP synthesis (Villani and Attardi, 2001; Gnaiger, 2009, 2012; Kadenbach et al., 2010). CCO generally displays excess capacity, especially in heart tissue (Gnaiger et al., 1998). In our study, CIV had excess capacity 1.5- to 2-fold higher than P respiration rate in all treatments, which is within the scope typically found in fish (1.5–3.2, Hilton et al., 2010; 1.8–2.7, Iftikar et al., 2015; 1.9–2.6, Salin et al., 2016b). Therefore CIV is not limiting the capacity of juvenile seabass mitochondria.

Conclusion

Although we used specimens originating from a northern population of seabass for this study, the results altogether indicate that the mitochondrial metabolism still supports (and favors) temperatures as found in Mediterranean specimens. Consequently, juvenile seabass in the North Atlantic might benefit from increased temperatures. Within the limits of this study, we also observed a high capacity to cope with ocean acidification, although this was less pronounced under ocean acidification and warming. The results of this study indicate that juvenile European seabass will be able to survive in an acidifying and warming ocean; however, there are further bottlenecks that may constrict their survival in a future climate. Firstly, other life stages, especially egg and larval stages, might be more vulnerable to temperature changes and increased P_{CO_2}; and secondly, other important traits, such as behavior or reproductive capacity and phenology might be affected differently by ocean acidification and warming. Consequently, other traits and life stages shall be analyzed in further studies.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.H., G.C., F.C.M.; Methodology: S.H., N.L., G.C., F.C.M.; Validation: S.H., F.C.M.; Formal analysis: S.H., G.C., F.C.M.; Investigation: S.H., L.C., N.L., G.C., F.C.M.; Resources: G.C., F.C.M.; Data curation: F.C.M.; Writing - original draft: S.H., F.C.M.; Writing - review & editing: S.H., L.C., G.C., F.C.M.; Visualization: S.H.; Supervision: G.C., F.C.M.; Project administration: F.C.M.; Funding acquisition: G.C., F.C.M.

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Data availability

Datasets of mitochondrial respiration and water conditions during rearing, as well as additional information on larval rearing are available online from PANGAEA (www.pangaea.de).

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.213017.supplemental

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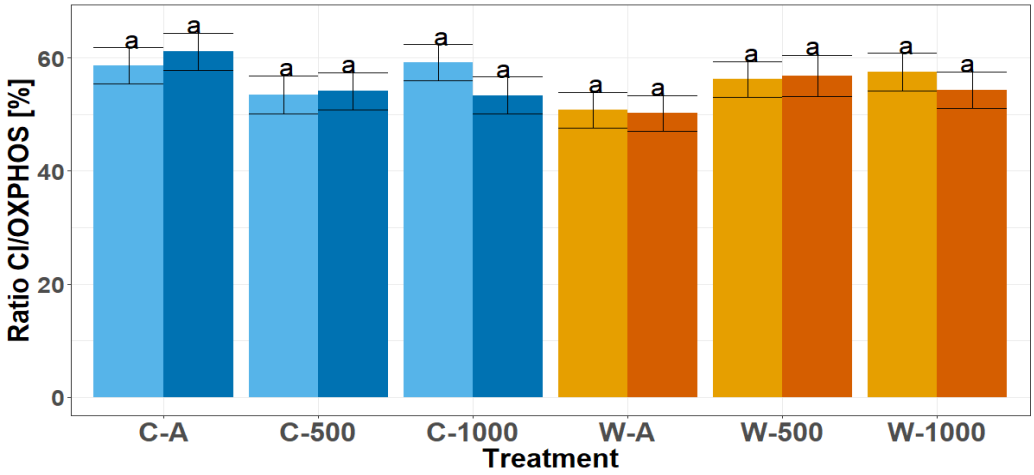

Figure S1 Ratio of CI respiration over OXPHOS respiration of permeabilized heart ventricle fibers of European seabass (expressed as percentage). Shown are lsmeans ± s.e.m. Different letters indicate significant differences (LME, $p<0.05$); blue: cold life conditioned fish (C), orange: warm life conditioned fish (W), light color: cold assay temperature, dark color: warm assay temperature, A: Ambient PCO_2, 500: ambient + 500 µatm CO_2, 1000: ambient + 1000 µatm CO_2; $n_{C\text{-}A}$=16/14, $n_{C\text{-}500}$=14/16, $n_{C\text{-}1000}$=16/15, $n_{W\text{-}A}$=16/14, $n_{W\text{-}500}$=17/10, $n_{W\text{-}1000}$=13/14, for cold/warm assay temperature respectively.

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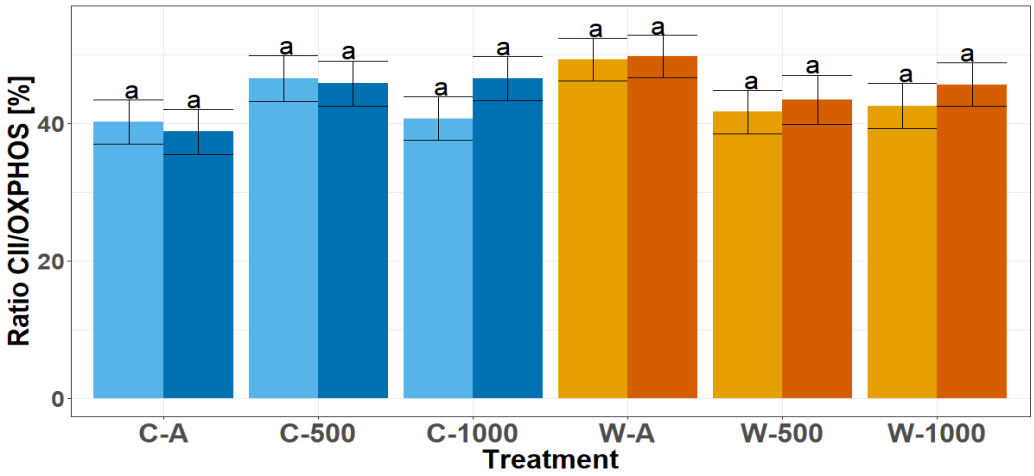

Figure S2 Ratio of CII respiration over OXPHOS respiration of permeabilized heart ventricle fibers of European seabass (expressed as percentage). Shown are lsmeans ± s.e.m. Different letters indicate significant differences (LME, $p<0.05$); blue: cold life conditioned fish (C), orange: warm life conditioned fish (W), light color: cold assay temperature, dark color: warm assay temperature, A: Ambient PCO_2, 500: ambient + 500 µatm CO_2, 1000: ambient + 1000 µatm CO_2; n_{C-A}=15/14, n_{C-500}=14/16, n_{C-1000}=16/15, n_{W-A}=15/14, n_{W-500}=16/10, n_{W-1000}=13/14, for cold/warm assay temperature respectively.

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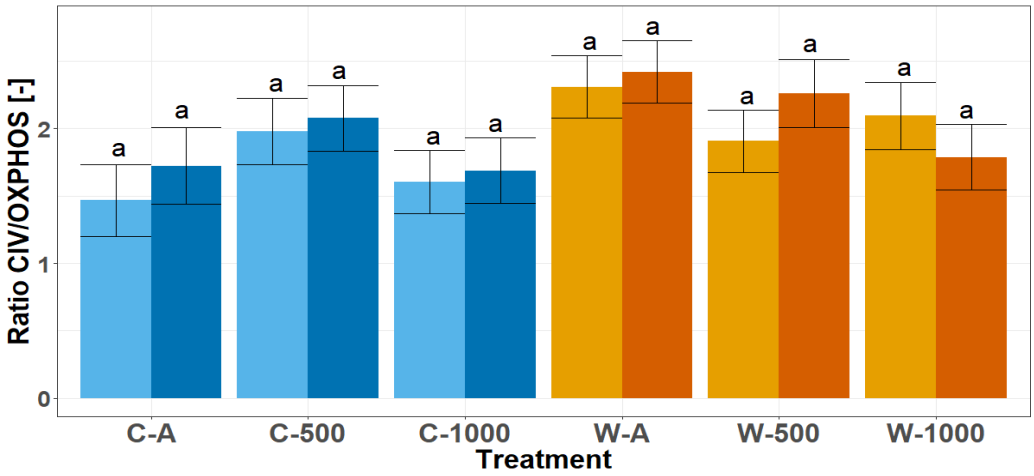

Figure S3 Ratio of CIV capacities over OXPHOS respiration of permeabilized heart ventricle fibers of European seabass (expressed as percentage). Shown are lsmeans ± s.e.m. Different letters indicate significant differences (LME, $p<0.05$); blue: cold life conditioned fish (C), orange: warm life conditioned fish (W), light color: cold assay temperature, dark color: warm assay temperature, A: Ambient PCO_2, 500: ambient + 500 µatm CO_2, 1000: ambient + 1000 µatm CO_2; $n_{C\text{-}A}$=11/10, $n_{C\text{-}500}$=14/16, $n_{C\text{-}1000}$=15/15, $n_{W\text{-}A}$=15/13, $n_{W\text{-}500}$=17/11, $n_{W\text{-}1000}$=11/13, for cold/warm assay temperature respectively.

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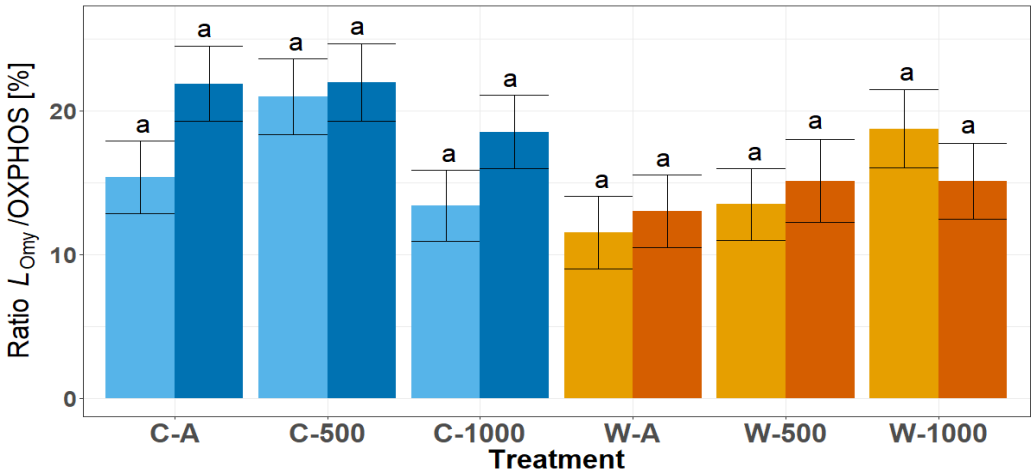

Figure S4 Ratio of L_{Omy} respiration over OXPHOS respiration (L_{Omy} -fraction) of permeabilized heart ventricle fibers of European seabass (expressed as percentage). Shown are lsmeans ± s.e.m. Different letters indicate significant differences (LME, p<0.05); blue: cold life conditioned fish (C), orange: warm life conditioned fish (W), light color: cold assay temperature, dark color: warm assay temperature, A: Ambient PCO_2, 500: ambient + 500 µatm CO_2, 1000: ambient + 1000 µatm CO_2; $n_{C\text{-}A}$=15/14, $n_{C\text{-}500}$=15/15, $n_{C\text{-}1000}$=16/16, $n_{W\text{-}A}$=16/15, $n_{W\text{-}500}$=17/11, $n_{W\text{-}1000}$=13/14, for cold/warm assay temperature respectively.

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Table S1 Light intensity during larval phase

Age [d]	2	7	9	12	16	20	27	31	36	44
Light intensity [lux]	0	0-1	1	2	5	7	10	31	59	96

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Table S2 Larval mortality in % in the different larval rearing tanks (n=3); A – Ambient PCO_2, Δ500 – ambient + 500 µatm CO_2, Δ1000 – ambient + 1000 µatm CO_2, T – temperature, Rep 1-3 – replicate tank 1-3.

	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

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Table S3 Juvenile mortality in % in the different tanks (n=1); A: Ambient *P* CO_2, Δ500: ambient + 500 µatm CO2, 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

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Table S4 Biometrical data of fish used for mitochondrial respiration: AT: assay temperature, Treatments: C: cold life condition (up to 18°C), W: warm life condition (up to 23°C), A: ambient PCO_2, Δ500: ambient PCO_2 + 500 µatm, Δ1000: ambient PCO_2 + 1000 µatm, HSI: hepatosomatic index, K: condition factor, values are means ± standard error.

Treatment	AT [°C]	n	Ventricle weight [g]	Carcass weight [g]	Body length [mm]	HSI [-]	K [-]
C – A	15	16	0.0114±0.0008	10.04±0.78	87.21±1.96	1.44±0.10	1.46±0.05
C – Δ500	15	15	0.0101±0.0006	10.59±0.67	88.83±2.14	1.37±0.09	1.50±0.05
C – Δ1000	15	16	0.0102±0.0007	9.92±0.62	86.12±1.88	1.45±0.12	1.53±0.03
C – A	20	16	0.0108±0.0009	9.97±0.84	85.22±2.46	1.41±0.06	1.56±0.03
C – Δ500	20	16	0.0104±0.0009	10.32±0.85	88.28±2.39	2.00±0.42	1.46±0.04
C – Δ1000	20	16	0.0102±0.0007	10.28±0.81	87.69±2.01	1.82±0.17	1.49±0.04
W – A	15	17	0.0122±0.0007	13.75±0.86	97.58±1.81	2.39±0.13	1.46±0.02
W – Δ500	15	18	0.0147±0.0011	14.99±1.12	98.37±2.31	2.35±0.12	1.53±0.02
W – Δ1000	15	13	0.0123±0.0007	13.46±0.88	96.14±2.25	2.53±0.11	1.49±0.02
W – A	20	16	0.0104±0.0007	11.32±0.71	91.88±1.85	2.28±0.13	1.43±0.02
W – Δ500	20	11	0.0125±0.0009	13.23±0.82	95.13±1.80	2.49±0.10	1.52±0.03
W – Δ1000	20	16	0.0129±0.0013	13.83±1.17	97.00±2.59	2.44±0.09	1.46±0.02

3 Publication II

Effects of ocean acidification over successive generations decrease resilience of larval European sea bass to ocean acidification and warming, but juveniles could benefit from higher temperatures in the NE Atlantic

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RESEARCH ARTICLE

Effects of ocean acidification over successive generations decrease resilience of larval European sea bass to ocean acidification and warming but juveniles could benefit from higher temperatures in the NE Atlantic

Sarah Howald[1,2], Marta Moyano[2,3], Amélie Crespel[4], Luis L. Kuchenmüller[1], Louise Cominassi[2,5], Guy Claireaux[4,6], Myron A. Peck[2,7] and Felix C. Mark[1,*]

ABSTRACT

European sea bass (*Dicentrarchus labrax*) is a large, economically important fish species with a long generation time whose long-term resilience to ocean acidification (OA) and warming (OW) is not clear. We incubated sea bass from Brittany (France) for two generations (>5 years in total) under ambient and predicted OA conditions (P_{CO_2}: 650 and 1700 µatm) crossed with ambient and predicted OW conditions in F1 (temperature: 15–18°C and 20–23°C) to investigate the effects of climate change on larval and juvenile growth and metabolic rate. We found that in F1, OA as a single stressor at ambient temperature did not affect larval or juvenile growth and OW increased developmental time and growth rate, but OAW decreased larval size at metamorphosis. Larval routine and juvenile standard metabolic rate were significantly lower in cold compared with warm conditioned fish and also lower in F0 compared with F1 fish. We did not find any effect of OA as a single stressor on metabolic rate. Juvenile $P_{O_2,crit}$ was not affected by OA or OAW in both generations. We discuss the potential underlying mechanisms resulting in the resilience of F0 and F1 larvae and juveniles to OA and in the beneficial effects of OW on F1 larval growth and metabolic rate, but contrastingly in the vulnerability of F1, but not F0 larvae to OAW. With regard to the ecological perspective, we conclude that recruitment of larvae and early juveniles to nursery areas might decrease under OAW conditions but individuals reaching juvenile phase might benefit from increased performance at higher temperatures.

KEY WORDS: *Dicentrarchus labrax*, Performance, Multi-stressor effects, Metabolic rate, Larval growth, Juvenile growth, Teleost

[1]Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Integrative Ecophysiology, 27570 Bremerhaven, Bremen, Germany. [2]Institute of Marine Ecosystem and Fisheries Science, Center for Earth System Research and Sustainability (CEN), University of Hamburg, 20146 Hamburg, Hamburg, Germany. [3]Center for Coastal Research, University of Agder, Postbox 422, 4604 Kristiansand, Norway. [4]Ifremer, LEMAR (UMR 6539), Laboratory of Adaptation and Nutrition of Fish, Centre Ifremer de Bretagne, 29280 Plouzane, Bretagne, France. [5]Institute of Arctic Biology, University of Alaska, Fairbanks, PO Box 757000, Fairbanks, AK 99775, USA. [6]Universite de Bretagne Occidentale, LEMAR (UMR 6539), 29280 Plouzane, Bretagne, France. [7]Coastal Systems (COS), Royal Netherlands Institute for Sea Research (NIOZ), 1797 SZ 't AQ4 Horntje, Texel, The Netherlands.

*Author for correspondence (fmark@awi.de)

S.H., 0000-0003-4151-3542; M.M., 0000-0002-3026-9225; A.C., 0000-0002-6351-9008; L.C., 0000-0002-2606-4753; G.C., 0000-0003-1340-6221; M.A.P., 0000-0001-7423-1854; F.C.M., 0000-0002-5586-6704

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INTRODUCTION

Climate change is increasing ocean surface temperatures (ocean warming, OW), as well as decreasing ocean pH (ocean acidification, OA). OW as a single stressor on fish metabolism has been investigated intensively since the 1980s in a variety of fish species and life stages and directly influences their metabolism and therefore their growth (Johnson and Katavic, 1986; Peck, 2002; Pörtner et al., 2007) and reproduction success (for review, see Llopiz et al., 2014), as well as their distribution range and abundance (Turner et al., 2009; Pörtner, 2006). OW can increase growth rates of larval and juvenile fish (McMahon et al., 2020a; Baumann, 2019; Chauton et al., 2015), within their thermal window. Although studies on larvae are less numerous than those on adults and juveniles, it has become obvious that larvae are less resilient to OW than adults and juveniles (Dahlke et al., 2020a).

Initially, fish had been thought to be less vulnerable to OA because of their well-developed acid–base regulation systems (Heuer and Grosell, 2014), yet their capacity to cope with OA and ocean acidification and warming (OAW) as co-occurring stressors has been investigated intensively during the last decade with species- and life stage-specific results (Cattano et al., 2017): OA levels between 700 and 1600 µatm CO_2 can lead to increased larval growth (mahi-mahi: Bignami et al., 2014; clownfish: Munday et al., 2009), but decreased larval swimming performance (mahi-mahi: Bignami et al., 2014; dolphinfish: Pimentel et al., 2014) and larval metabolic rate (dolphinfish: Pimentel et al., 2014). OA also induced severe to lethal tissue damage (cod larvae: Frommel et al., 2011), decreased swimming performance, maximum metabolic rate and aerobic scope (Australasian snapper juveniles: McMahon et al., 2020b), and increased larval otolith size, with possible implications for hearing sensitivity (cobia and mahi-mahi: Bignami et al., 2013, 2014). In other species, growth was decreased by OA (inland silverside juveniles: Baumann et al., 2012), or not affected (Atlantic halibut juveniles: Gräns et al., 2014; cobia larvae: Bignami et al., 2013; Australasian snapper larvae: McMahon et al., 2020a). In some species, OA even improved performance (e.g. increased survival of Australasian snapper larvae: McMahon et al., 2020a). Dahlke et al. (2020b) showed that Atlantic cod embryos demonstrated poor acid–base regulation capacity before and during gastrulation, connected to increased mortality under OA and OAW. In contrast, acid–base regulation capacity after gastrulation was similar to that of adult cod. If the two stressors were combined, the effects became more unidirectional and were synergistic in most fish species, e.g. OAW increased growth and survival in larval and juvenile sea bass in their Atlantic populations, but decreased physiological performance (Pope

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et al., 2014). The cumulative consequences of these changes are yet to be determined.

An important factor for projecting whether a species will be able to keep their distribution range under changing conditions is their potential and capacity to acclimate and adapt over generations. Few studies have so far reared fish for more than one generation or examined transgenerational effects for fish in the context of OAW, with trait- and species-specific capacities to adapt to future conditions. For example, in cinnamon anemone fish (*Amphiprion melanopus*), the negative effect of OA on escape responses was reduced in some traits if the parents were exposed to OA (Allan et al., 2014), whereas in spiny damselfish (*Acanthochromis polyacanthus*), negative effects on olfactory responses were not reduced after parental exposure to OA (Welch et al., 2014). In addition to the low number of studies on transgenerational effects, they usually used small fish, with short generation times, and applied only one stressor, either OW or OA. Little is known about the combined effect of several stressors on economically important larger-sized fish with longer generation times and thus multi-stressor, transgenerational studies on such fish are necessary to project future distribution of fish.

Consequently, in our study, we used European sea bass *Dicentrarchus labrax* as a larger, long-lived model species. Sea bass is an economically important species in industrial and recreational fishing as well as in aquaculture (160,000 tons in 2015; Bjørndal and Guillen, 2018). Sea bass can reach an age of 24 years in the Atlantic population (Irish waters; Kennedy and Fitzmaurice, 1972). Although they are generally rather resilient towards environmental fluctuations, effects of OW and OA have been reported for several seabass life stages: OW increased growth rate in larval sea bass, although at the expense of decreased swimming performance (Atlantic population, 15–20°C; Cominassi et al., 2019). Exposure to OA throughout larval development increased mineralization and reduced skeletal deformities (Atlantic population, 19°C and 15 and 20°C, respectively; Crespel et al., 2017; Cominassi et al., 2019). In combination, OAW did not have additional effects on larval growth, swimming ability and development from those already observed separately (Atlantic population; Cominassi et al., 2019). Juvenile sea bass are highly tolerant to temperature (Dalla Via et al., 1998; Claireaux and Lagardère, 1999) and show some degree of tolerance to OA as a single stressor at the mitochondrial level (Atlantic population; Howald et al., 2019). The effect sizes of OA and OW are different: OW as a single stressor increased growth and digestive efficiency, while OA did not affect these traits. The two stressors combined caused reduced growth and digestive efficiency compared with the impact of OW alone. Low food ratios enhanced this effect, resulting in an even more pronounced growth and digestive efficiency reduction than under OAW alone (Atlantic population; Cominassi et al., 2020).

This study aimed to investigate the effect of OAW as well as the effect of OA over two successive generations (F0 and F1) on larval and juvenile growth and metabolism. Therefore, we incubated sea bass from an Atlantic population for two generations (>5 years in total) under current and predicted OA conditions (P_{CO_2}: 650 and 1700 µatm) and applied a warming condition on larvae and juveniles of the F1 generation (ambient, 15–18°C; and Δ5°C, 20–23°C). To study the effect of OA (F0, F1), OW (F1) and OAW (F1) on sea bass, we investigated growth (F0, F1) through ontogeny as a proxy for whole-organism fitness. In addition, we measured routine metabolic rate (RMR; F1) of larvae, as well as standard metabolic rate (SMR; F0, F1) and critical oxygen concentration ($P_{O_2,crit}$; F0, F1) of juvenile sea bass, to unravel the underlying mechanisms resulting in possible growth differences. In F0, no effect of OA on larval and juvenile growth or juvenile SMR and $P_{O_2,crit}$ was found (Crespel et al., 2017, 2019). Those traits were compared in F0 and F1 fish to determine the effects due to parental acclimation to different OA levels. Our hypotheses were: (1) OW will lead to increased growth and metabolic rate in F1 larvae and juveniles; (2) OA alone will not have significant effects on larval and juvenile growth and metabolism in F1, as sea bass seem to be quite tolerant to OA and no detrimental effects were found in F0; and (3) in combination, OA will lead to synergistic OAW effects, reflected in lower growth in larvae and juveniles.

MATERIALS AND METHODS

The present work was performed within the facilities of the Ifremer-Centre de Bretagne (agreement number: B29-212-05). Experiments were conducted according to the ethics and guidelines of French law and legislated by the local ethics committee (Comité d'Ethique Finistérien en Experimentation Animal, CEFEA, registering code C2EA-74) (authorizations APAFIS 4341.03, #201620211505680.V3 and APAFIS 14203-2018032209421223 for F0 and F1, respectively).

Animals and experimental conditions

Sea bass, *Dicentrarchus labrax* (Linnaeus 1758), were reared from early larval stage onwards in two OA treatments in F0 and four OAW treatments in F1. A flow chart summarizing temperature and P_{CO_2} conditions as well as replicate tank number, tank volume and number of individuals per tank is shown in Fig. 1; the timeline for fish rearing is shown in Fig. S1. F0 fish were reared in two OA scenarios, following the predictions of the Intergovernmental Panel on Climate Change (IPCC, 2021) for the next 130 years: today's ambient situation in coastal waters of Brittany and the Bay of Brest (A, ∼650 µatm; see Pope et al., 2014; Duteil et al., 2016) and a scenario according to SSP5-8.5, projecting a ΔP_{CO_2} of 1000 µatm (Δ1000, ∼1700 µatm). Adults from these two treatments were used in the reproduction experiments to generate F1. Sea bass of F1 were reared under the same OA conditions as their respective parents. Additionally, two different temperatures were applied on each OA condition in F1 to create a cold (C) and a warm (W) life condition scenario or four OAW conditions (C-A, C-Δ1000, W-A and W-Δ1000), respectively. As larvae and post-larval juveniles would display different growth rates under the different life condition scenarios, we adopted the concept of degree days [dd=dph×temperature (T in °C)] as a basis for comparison between them. This concept allows comparison of fish at their physiological age rather than their chronological age and has been shown to be an effective way of normalizing growth at different temperatures (Peck et al., 2012).

Larval rearing was performed in a temperature-controlled room and water temperature was fixed to 19°C in F0, and 15 and 20°C in F1 C and W, respectively. In juveniles and adults, water temperature of F0 and F1 C sea bass was adjusted to ambient temperature in the Bay of Brest during summer (up to 19°C), but was kept constant at 15 and 12°C for juveniles and adults, respectively, when ambient temperature decreased below these values. The F1 W was always 5°C warmer than the F1 C treatment.

During larval rearing, the photoperiod was set to 24 h darkness during the first week and 16 h light and 8 h darkness (12 h each in F1) every day afterwards. Light intensity increased progressively during the larval rearing period from total darkness to about 100 lx (Table S1). To work in the larval rearing facilities, headlamps were used (set to the lowest light intensity). In the juvenile and adult

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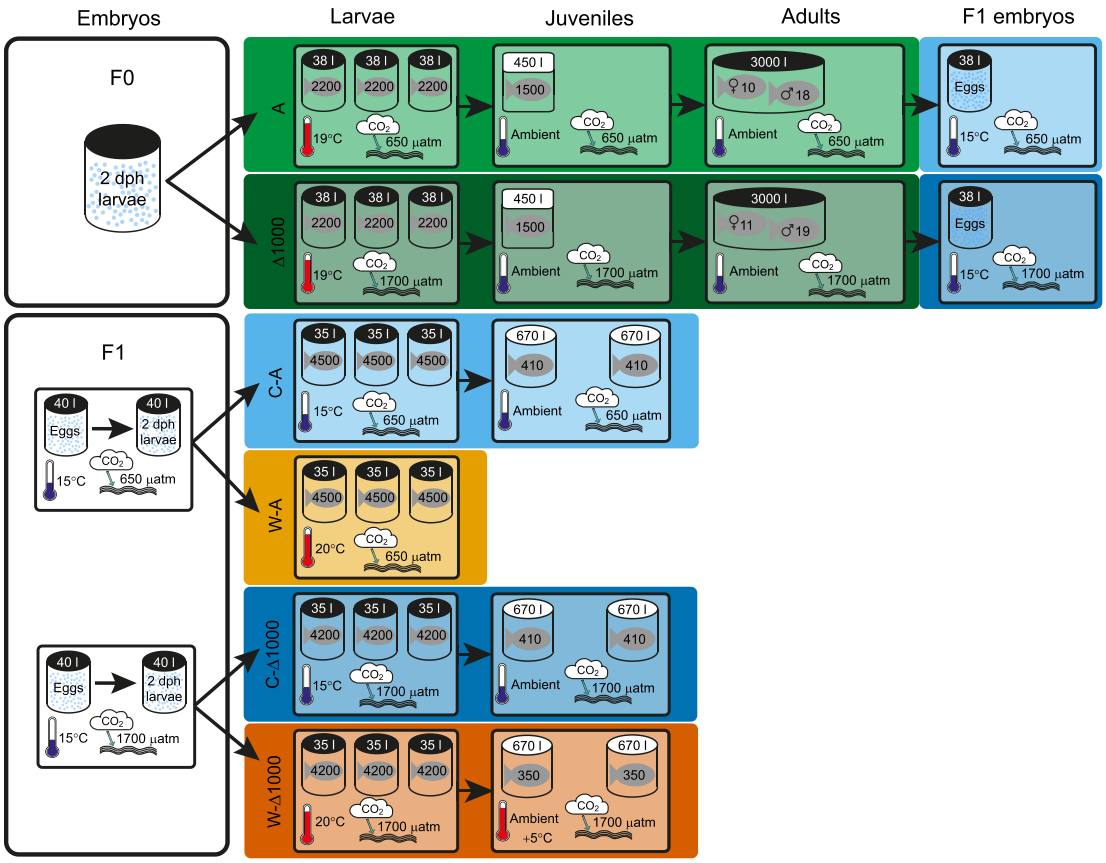

Fig. 1. Schematic overview summarizing the rearing conditions of two generations of sea bass under different ocean acidification–warming (OAW) scenarios. Rearing conditions in the experiments on two successive generations of European sea bass. Parental (F0) and offspring (F1) generations were raised and reared under two acidification conditions: today's ambient levels in the Bay of Brest (650 µatm; A) and a projected condition following the worst-case scenario prediction of the IPCC (2021) (1700 µatm; Δ1000). In F1, two temperatures were applied to each of the two acidification conditions: a cold life condition (C) with 15°C during the larval phase and ambient conditions during the summers (up to 18°C), and a warm life condition (W) with 20°C during the larval phase and ambient +5°C during summers. Age of larvae (days post-hatching, dph), tank volume, number of larvae/adults per tank, temperature and P_{CO_2} are indicated.

rearing facilities, photoperiod followed natural conditions (adjustment once a week).

F0 generation

Larval rearing

F0 larval rearing and origin is described in detail in Crespel et al. (2017, 2019), briefly, larvae were obtained from the aquaculture facility Aquastream (Ploemeur-Lorient, France) at 2 dph (October 2013). F0 larvae were randomly distributed among the two OA conditions described above. Larvae were reared in nine black 38 l tanks initially stocked with *ca*. 2200 larvae per tank in triplicate for all conditions. Larvae were fed *ad libitum* via continuous delivery of *Artemia* nauplii until 28 dph. Afterwards, commercial dry pellets (Neo Start, LeGouessant, France) were fed for the rest of the larval period.

Juvenile rearing

Juvenile rearing is described in detail in Crespel et al. (2019). Briefly, the early juveniles were counted per tank and transferred from larval to juvenile rearing facilities at ∼820 dd (45 dph). Juveniles of one condition were combined and kept in square 450 l tanks (n=1500 fish per condition). At 8 months (about 250 dph), juveniles were PIT tagged (i.e. marked with passive integrated transponders). Juveniles were fed daily with commercial fish food (Neo Start), which was adjusted in size and amount, as recommended by the supplier (Le Gouessant, Lamballe, France). Food ratios were adjusted after each sampling for growth, approximately every 30 days or 3–4 weeks in F0 and F1, respectively (see below), using the formulae provided by Le Gouessant. Daily food ratios were supplied to the tanks by automatic feeders during the daytime.

Adult rearing

During the reproductive season in 2017 (fish were 3.5 years old), sex steroid plasma concentration was measured regularly in all adult F0 fish. The individuals with the highest concentrations were kept in round black tanks with a volume of 3 m^3 and a depth of 1.3 m. Each of the two tanks (one for each condition) was stocked with 22 males and 11 females, resulting in fish density of 11.6 kg m^{-3} and 11.0 kg m^{-3} in A and Δ1000, respectively. Mass and length were regularly measured and commercial fish food was adjusted

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accordingly. Fish were fed Vitalis CAL (Skretting, Norway) during the reproduction season and Vitalis REPRO (Skretting, Norway) during the rest of the year. Vitalis REPRO was supplied to the tanks with automatic feeders during the daytime. Vitalis CAL was supplied to the tank manually in three to four rations on week days.

F1 generation

Embryos were obtained by artificial reproduction of F0 fish. Briefly, once the water temperature reached 13°C and the first naturally spawned eggs were observed in the egg collectors, females were injected with gonadotropin-releasing hormone (GnRH, 10 µg kg^{-1}) to accelerate oocyte maturation (23 March 2018). After 3 days (26 March 2018), eggs and milt were stripped from ripe females and males, respectively, and artificial fertilization was performed following the protocol of Parazo et al. (1998). Briefly, eggs (10 ml l^{-1}) were mixed with seawater and milt (0.05 ml milt l^{-1} seawater). Ten females (1.56±0.24 kg) were crossed with 18 males (1.07±0.16 kg) and 11 females (1.28±0.30 kg) were crossed with 19 males (0.99±0.19 kg) in the A and Δ1000 groups, respectively. Fertilized eggs were incubated in 40 l tanks (without replicates) at 15°C and under the same P_{CO_2} conditions as the respective F0. Hatching occurred after 4 days (30 March 2018).

Larval rearing

Two days after hatching (2 April 2018), larvae were distributed into 12 black 35 l tanks. Triplicate tanks were allocated to each of the four OAW treatments with *ca*. 4500 and 4200 larvae per tank in A and Δ1000 tanks, resulting in a total of *ca.* 13,500 and 12,800 larvae per condition in A and Δ1000, respectively. The temperature of the tanks allocated to the warm life condition was increased stepwise by 1°C day^{-1} during the following 5 days. Starting at 7 dph (mouth opening), larvae were fed with live artemia, hatched from High HUFA Premium artemia cysts (Catvis, AE 's-Hertogenbosch, The Netherlands). Artemia were fed to the larvae 24 h after rearing cysts in seawater. Larvae were fed *ad libitum* with artemia during the day; excess artemia left the tank via the waste water outflow. Larval mortality was 26–96%, without any pattern for the OAW condition (Table S2). High mortality of sea bass larvae, especially during early larval rearing, is common in science and aquaculture (e.g. Nolting et al., 1999; Suzer et al., 2007; Villamizar et al., 2009). We could not find any signs of infection either in the tanks with high mortality or in the tanks with lower mortality rates. However, as larval mortality was unreasonably high (96%) within the first week in one of the replicate tanks of the W-A treatment, remaining larvae in this tank were euthanized (sedation with eugenol followed by an overdose of MS-222) and not used for further analysis. The water surface was kept free of oily films using a protein skimmer. Water exchange was set to 25 l h^{-1} and increased stepwise to 40 l h^{-1} at the end of larval rearing.

Juvenile rearing

At ∼950 dd, the early juveniles were counted per tank and transferred from larval to juvenile rearing conditions (48 dph, 17 May 2018 and 63 dph, 01 June 2018 for W and C, respectively). For F1 W, only the Δ1000 fish were transferred to juvenile rearing facilities. Juveniles were randomly allocated to duplicate tanks per condition. A swim bladder test was done at 1680 dd (83 dph, 21 June 2018) and 1661 dd (104 dph, 12 July 2018) for F1 W and F1 C, respectively. Briefly, the fish were anaesthetized and introduced into a test container with a salinity of 65 psu (Marine SeaSalt, Tetra, Melle, Germany). In F1 W, all floating fish with a developed swim bladder were counted and kept in the rearing tanks, resulting in 355 fish per tank (710 fish in total). In F1 C, 410 fish per tank were randomly selected (820 fish per condition), to have similar stocking densities in W and C. Non-floating fish as well as excess F1 C fish were counted and euthanized (sedation followed by an anaesthetic overdose). The juveniles were reared in round tanks with a volume of 0.67 m^3 and a depth of 0.65 m. During the first 5 days after moving to juvenile rearing, the juveniles were fed *Artemia* nauplii and commercial fish food. Afterwards, commercial fish food was fed as described above.

Experimental conditions

Seawater preparation

The seawater used in the aquaria 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), degassed using a column packed with plastic rings, filtered using a 2 µm membrane and finally UV sterilized (PZ50, 75 W, Ocene) assuring high water quality.

Water conditions for the rearing tanks were preadjusted to the desired OAW condition in header tanks. Seawater arrived in a reservoir next to the rearing facilities, after passing the tungsten heater; in F1, two different reservoirs were used to create the different temperature conditions. The temperature-controlled water supplied the header tanks within the rearing facilities to adjust the water to the desired OA condition. Each header tank supplied water to all replicate tanks of the respective condition.

In F0 larvae and juveniles, the water pH in the header tank was controlled by an automatic injection system connected to a pH electrode (pH Control, JBL), which injected either air (A) or CO_2 (Δ1000), to control water pH. For the Δ1000 F1 larvae, the CO_2 bubbler was installed in the middle of the header tank and the water was mixed continuously with a pump. CO_2 bubbling was adjusted by a flow control unit, when pH deviated from the desired value.

Older F0 A juveniles (>2 years) and adults, as well as F1 A larvae and juveniles received water directly from the respective reservoir, without header tank. Additionally, as water exchange rates became too high for the automatic injection system and the header tank, PVC columns were installed to control the pH in the rearing tanks. The temperature-controlled water arrived at the top of the column and was pumped from the bottom of the column to the rearing tanks. The CO_2 bubbler was installed at the bottom of the column and was adjusted by a flow control unit, when pH deviated from the desired value.

Calculation of water chemistry

The Microsoft Excel macro CO2sys (Lewis and Wallace, 1998) was used to calculate seawater carbonate chemistry; the constants after Mehrbach et al. (1973; as cited in CO2sys) refitted by Dickson and Millero (1987; as cited in CO2sys) were employed.

From October 2015 onwards (late juveniles of F0), total alkalinity was measured following the protocol of Anderson and Robinson (1946) and Strickland and Parsons (1972): 50 ml of filtered tank water (200 µm nylon mesh) was mixed with 15 ml HCl (0.01 mol l^{-1}) and pH was measured immediately. Total alkalinity (mol l^{-1}) was then calculated with the following formula:

$$\mathrm{TA} = \frac{V_{\mathrm{HCl}}\, c_{\mathrm{HCl}}}{V_{\mathrm{sample}}} - \frac{(V_{\mathrm{HCl}} + V_{\mathrm{sample}})}{V_{\mathrm{sample}}} \frac{\{\mathrm{H}^+\}}{\gamma^{\mathrm{H}^+}}, \quad (1)$$

where TA is total alkalinity (mol l^{-1}), V_{HCl} is the volume of HCl (l), c_{HCl} is the concentration of HCl (mol l^{-1}), V_{sample} is the volume of

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Table 1. Water parameters during the F0 larval and early juvenile phase of European sea bass

Phase	Treatment	pH_{NBS}	pH_{total}	Temperature (°C)	Salinity (psu)	TA (µmol l^{-1})	P_{CO_2} (µatm)	PO_4^{3-} (µmol l^{-1})	SiO_4 (µmol l^{-1})
Larvae	A	7.96±0.01	7.89±0.01	19.2±0.3	33.8±0.2	2294±3	589±10	0.57±0.01	8.94±0.06
	Δ1000	7.59±0.00	7.54±0.03	19.2±0.3	33.8±0.2	2306±9	1521±97	0.57±0.01	8.94±0.06
Juveniles	A	8.05±0.01	7.94±0.03	15.3±0.1	34.3±0.2	2294±10	516±31	0.71±0.08	8.35±0.26
	Δ1000	7.61±0.01	7.53±0.02	15.3±0.1	34.3±0.2	2280±16	1489±42	0.71±0.08	8.35±0.26

The larval period lasted until 45 days post-hatching (dph; ~900 degree days, dd); the early juvenile period lasted until 1.5 years. Data are means±s.e.m. over all measurements per condition (triplicate tanks for larvae, single tanks for juveniles). Temperature and pH (NBS scale) were measured daily. pH (total scale), salinity, phosphate, silicate and total alkalinity (TA) were measured once at the beginning and once at the end of the larval phase and 9 times during the juvenile phase; P_{CO_2} was calculated with CO2sys. A, ambient P_{CO_2}; Δ1000, ambient+1000 µatm CO_2 (see Crespel et al., 2017; Crespel et al., 2019).

the sample (l), H^+ is hydrogen activity (10^{-pH}) and γ^{H+} is the hydrogen activity coefficient (here γ^{H+}=0.758).

Water quality control

Temperature and pH were checked each morning with a handheld WTW 330i or 3110 pH meter (Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW Sentix 41, NIST scale) before feeding the fish. The pH meter and the automatic injection system were calibrated weekly with fresh buffers (Merck, Germany) until F0 juveniles reached 2 years. Measured values never differed more than 2% from the target values. Afterwards, the pH meter was calibrated daily with NIST certified WTW technical buffers pH 4.01 and pH 7.00 (Xylem Analytics Germany).

Total pH was determined twice during F0 larval rearing (start and end) and 9 times during F0 juvenile rearing following Dickson et al. (2007) using *m*-cresol purple as an indicator. Additionally, water samples were sent to LABOCEA (France) to measure total alkalinity by titration, as well as phosphate and silicate concentration by segmented flow analysis following Aminot et al. (2009).

In later F0 juveniles (>2 years) and adults as well as F1 larvae and juveniles, total alkalinity was measured monthly or weekly in F0 and F1, respectively, following the protocol described above. Oxygen saturation (WTW Oxi 340, Xylem Analytics Germany) and salinity (WTW LF325, Xylem Analytics Germany) were measured together with total alkalinity (monthly in F0 and weekly in F1). The tanks were cleaned daily after pH measurements. Water flow within the tanks was adjusted once a week, so that oxygen saturation levels were kept >85%, with equal flow rates in all tanks of one temperature. All water parameters are summarized in Table 1 for F0 larvae and juveniles and Table 2 for F0 adults (2 years before spawning) and F1 larvae and juveniles.

Growth

Larval growth

F0 larvae

Larval growth was measured as described in Crespel et al. (2017). Briefly, 10 larvae per tank were sampled each week, starting at 15 dph and ending at 45 dph, when 30 larvae per tank were sampled. For growth measurements, larvae were anaesthetized with phenoxyethanol (200 ppm) and their wet mass (WM), as well as body length (BL) were measured. BL in F0 larvae was measured with a calliper from the tip of the snout to the end of the notochord until flexion; afterwards, fork length was considered as BL (see Fig. S2).

F1 larvae

In F1 larvae, individuals were sampled every 200 dd from 100 to 900 dd to follow growth throughout the larval phase. At each sampling point, 20 larvae per tank were anaesthetized with MS-222 (50 mg l^{-1}, Pharma Q) prior to feeding and directly photographed individually with a microscope (Leica M165C). The larvae were then frozen in liquid nitrogen and stored at −80°C until dry mass (DM) measurements. The software ImageJ (Schneider et al., 2012) was used to determine BL of larvae (see Fig. S2 for the definition of BL).

Juvenile growth

BL and WM were measured approximately every 30 days in F0 and every 3–4 weeks in F1 juveniles. Early juveniles were starved for 1 day prior to growth sampling. Later on, this was increased to 2 days, to make sure that digestive tracts were empty. Juveniles were caught from their tanks and anaesthetized with MS-222 (Pharma Q). The concentration of anaesthetic was adjusted to reach a loss of equilibrium within <5 min, typically 0.2 g l^{-1}. WM and BL were

Table 2. Water parameters in the 2 years before spawning of F0 (2016–2018) and during the F1 larval and juvenile phase of European sea bass

Phase	Treatment	pH_{free}	Temperature (°C)	Salinity (psu)	O_2 (% air saturation)	TA	P_{CO_2} (µatm)
F0	A	7.95±0.02	14.1±0.6	33.6±0.3	92.4±1.7	2406±49	670±40
	Δ1000	7.59±0.02	14.1±0.6	33.6±0.3	92.4±1.9	2411±46	1616±74
F1 larvae	C-A	8.06±0.01	15.3±0.1	31.8±0.1	94.3±1.0	2360±23	504±19
	C-Δ1000	7.53±0.01	15.5±0.1	31.8±0.1	94.3±0.8	2330±22	1872±74
	W-A	7.96±0.01	20.2±0.2	31.7±0.0	84.9±3.4	2311±32	656±22
	W-Δ1000	7.61±0.01	20.2±0.2	31.8±0.0	88.1±1.7	2321±32	1624±59
F1 juveniles	C-A	7.94±0.01	16.1±0.2	33.0±0.1	92.4±0.5	2376±15	696±19
	C-Δ1000	7.60±0.01	16.3±0.2	33.0±0.1	94.3±0.5	2380±14	1603±32
	W-A	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	W-Δ1000	7.57±0.02	22.7±0.2	33.0±0.2	86.3±1.3	2323±16	1866±83
	SW	8.07±0.01	15.0±0.5	34.6±0.3	101.0±0.8	2441±23	609±37

The larval period lasted until 48 dph (~900 dd) and 63 dph (~900 dd) for the warm and cold life condition, respectively; the juvenile period lasted until 180 dph (~4000 dd) and 319 dph (~5100 dd) for warm and cold conditioned fish, respectively. Data are means±s.e.m. over all replicate tanks per condition. Temperature, pH, salinity, oxygen and total alkalinity (TA) were measured weekly in F1 and monthly in F0; P_{CO_2} was calculated with CO2sys; seawater (SW) measurements were conducted in 2017 and 2018. A, ambient P_{CO_2}; Δ1000, ambient+1000 µatm CO_2; C, cold life condition; W, warm life condition.

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directly determined with a precision balance (Sartorius MC1 AC210P) and callipers. For all sampling, only the morning hours were used, to avoid diurnal artefacts in data.

Data handling

For F1 larvae and juveniles, mean specific growth rate (SGR, % day^{-1}) of each tank was calculated after Sutcliffe (1970) with the following formula:

$$\mathrm{SGR} = 100\ (e^{g} - 1). \quad (2)$$

The instantaneous growth coefficient (g) was calculated as:

$$g = \frac{\ln S_1 - \ln S_0}{\Delta t}, \quad (3)$$

where S_0 and S_1 are initial and final size (BL, WM or DM) and Δt is the time between the two measurements (days). Initial and final sizes were calculated for three quantiles (0.05, 0.5 and 0.95) for each tank ('ecdf' function in R).

Q_{10} was calculated with the following formula:

$$Q_{10} = \left(\frac{\mathrm{SGR_W}}{\mathrm{SGR_C}}\right)^{\left(\frac{10}{T_W - T_C}\right)}, \quad (4)$$

where SGR is specific growth rate, T is temperature, and subscripts W and C represent the W and C condition.

Respirometry

F1 larvae

Larval respiration measurements were conducted from approximately 350 to 950 dd in all conditions (18–47 dph and 25–63 dph in W and C, respectively).

Larval respiration was measured in an intermittent flow system. The setup consisted of up to eight 4 ml micro-respiration chambers with a glass ring (Unisense A/S, Aarhus, Denmark), equipped with a glass-coated magnetic stirrer (Loligo® Systems, Viborg, Denmark) and a stainless steel mesh (Loligo® Systems), to separate the stirrer from the larva. The magnetic stirrers were connected to one stirrer controller (Rank Brothers Ltd, Cambridge, UK). The chamber was closed with a custom-made glass lid with three metal ports: two with a diameter of 0.8 mm for water inflow and outflow during flushing, and one with a diameter of 1.2 mm to insert the oxygen sensor into the chamber. Oxygen concentration within the chamber was measured with oxygen microsensors connected to a FireSting oxygen meter (PyroScience GmbH, Aachen, Germany). The respiration chambers were placed within a rack without shielding between the individual chambers. The rack holding the respiration chambers was fully submerged in a water reservoir, which received flow-through water from the respective header tanks of the larval rearing. Water conditions within the water reservoir were kept at 15.5±1.5 and 21.2±1.0°C for W and C larvae, combined with the OA condition of the origin tank of the respective larvae. The reservoir was a black container, which shielded the respiration setup from external disturbance. During the flushing periods, water from the reservoir was pumped into the respiration chambers using computer-controlled flush pumps (Miniature DC pump, Loligo® Systems), relays and software (AquaResp, Copenhagen, Denmark). Four chambers were connected to one flush pump and controlled by one computer. Oxygen microsensors were calibrated to 0% saturation (nitrogen purged seawater) and 100% saturation (fully aerated seawater) prior to each measurement.

Respiration measurements were done in the larval rearing facilities with the same light conditions as for larval rearing. Larvae were fasted at least 3 h prior to respiration measurements to minimize the effect of specific dynamic action (SDA) on metabolic rate. Preliminary tests with measurements overnight showed that oxygen consumption during the 12 h after the 3 h fasting period was similar, suggesting no contribution of SDA and thus that the 3 h fasting period was sufficient for our setup. Larvae were individually placed in the respiration chambers. Oxygen partial pressure was measured every second for ∼4 h. Cycles were composed of 420 s flush, followed by 60 s wait time (time after flush pump stopped to wait for a stable drop in oxygen concentration) and 600 to 180 s measurement time (13–20 cycles per larvae). Measurement time was decreased with increasing larval size. Oxygen concentration was restored to normoxia during the flush time of each cycle and was usually kept above 75% air saturation. Background respiration was measured for 30 min (one slope) after 11 and 18 measurements in F1 C and F1 W larvae, respectively. The mean bacterial respiration was calculated for each temperature treatment and subtracted from total respiration of all larvae of this temperature to obtain oxygen consumption of the larva. Background respiration was typically 0.5–6% of total respiration. Only declines in oxygen concentration displaying R^2>0.80 were used for analysis. After the measurement, larvae were checked to make sure they were alive, anaesthetized with MS-222 (50 mg l^{-1} Pharma Q), photographed individually and frozen in liquid nitrogen. Length and DM of the larvae were obtained as described above (see Table S3). After each experiment, the respiration system was rinsed with fresh water and allowed to dry. For disinfection, the respiration chambers, the tubing of the flush pump and the oxygen sensors were additionally rinsed with ethanol, which was allowed to sit in the chambers and the tubing for at least 30 min followed by rinsing with distilled water.

Juveniles

Setup for F0 juveniles

Measurements on 15 month old F0 juveniles (F0 old) were as described in Crespel et al. (2019); measurements on 5 month old F0 juveniles (F0 young) were done similarly – and differences are indicated in parentheses. Briefly, F0 juvenile respiration was measured individually in one of four (eight) intermittent flow respirometry chambers with a volume of 2.1 l (60 ml), which were submerged in a tank that received flow-through seawater at 15±0.25°C and the respective acidification condition. The water was recirculated within the chamber with a peristaltic pump with gas-tight tubing. The oxygen probe (FireSting oxygen meter, PyroScience GmbH or multichannel oxygen meter, PreSens Precision Sensing GmbH) was placed within the recirculation loop. Oxygen sensors were calibrated to 0% saturation (sodium sulphite, saturated) and 100% saturation (fully aerated seawater) prior to each experiment. The flush pumps were controlled by relays and software (AquaResp, Copenhagen, Denmark). The setup was placed behind a curtain to avoid disturbance. Background respiration was measured after each experiment and estimated for the whole experiment by linear regression assuming zero background respiration at the beginning of the run as the entire system was disinfected with household bleach between each trial.

Setup for F1 juveniles

F1 juvenile respiration was measured in an intermittent flow system. The setup consisted of up to eight 450 ml custom-made respiration chambers. The chambers were made from Lock&Lock glass containers with plastic lid. Four rubber ports were placed into the

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lid: two for water inflow and outflow during flushing cycles and two to connect the chamber to a mixing pump (Miniature DC pump, Loligo® Systems). Oxygen concentration was measured with robust oxygen probes placed within the circulation loop and connected to a FireSting oxygen meter (PyroScience GmbH) or to a multichannel oxygen meter (PreSens Precision Sensing GmbH). The respiration chambers were fully submerged in a flow-through water reservoir. Water conditions within the water reservoir were kept at 14.9±1.0 and 22.3±1.8°C for C and W larvae, combined with the OA condition of the origin tank of the respective juvenile. During the flushing periods, water from the reservoir was pumped into the respiration chambers using computer-controlled flush pumps (EHEIM GmbH & Co. KG, Deizisau, Germany), relays and software (AquaResp). Four chambers were connected to one flush pump and controlled by one computer, running either the FireSting or the PreSens oxygen meter. The setup was covered with black foil to avoid disturbance. Oxygen sensors were calibrated to 0% saturation (nitrogen purged seawater) and 100% saturation (fully aerated seawater) prior to each experiment. Background respiration was measured for 30 min (one slope) after each measurement and the run was discarded, if background respiration was >10%. After each experiment, the whole system excluding the oxygen sensors was disinfected with household bleach or Virkon® (Antec International Limited, Suffolk, UK) and rinsed with freshwater afterwards.

Measurement protocol

Respiration measurements of F0 juveniles were done on approximately 5 month old (119–165 dph) and 15 month old (454–495 dph) juveniles. F1 juvenile respiration measurements were conducted from 2900 to 3900 dd (137–178 dph, 5 months) and 4700 to 5100 dd (291–318 dph, 10 months) for F1 W and F1 C, respectively. F1 C fish were older than F1 W fish at the measurement time in order to have comparable fish sizes (see Table S4).

Juvenile sea bass were fasted for 48–72 h prior to respiration measurements to minimize the effect of residual SDA (Dupont-Prinet et al., 2010). Juveniles were randomly taken from their tank and placed individually in the respiration chambers. The whole setup was shielded from external disturbance with curtains or black foil, but the individual respiration chambers were not shielded from each other. F0 juveniles were chased until exhaustion prior to introduction into the chambers (maximum metabolic rate data partly given in Crespel et al., 2019). Each experiment lasted for about 70 h in F0 and 65 h in F1. Oxygen partial pressure was measured every second and was usually kept above 80%, until the start of the $P_{O_2,crit}$ trial (see below). Each cycle was composed of 360 s (F0) and 540 s (F1) flush time, during which oxygen concentration was restored to normoxia (until $P_{O_2,crit}$ trial), followed by 30 s wait and 210 s (F0) and 180 s (F1) measurement time. In F0, only the measurements taken after the fish fully recovered from chasing stress were used to calculate SMR, usually after 10 h. In F1, the first 5 h of each experiment were not used for analysis of SMR, to account for acclimation of the fish to the respirometer and recovery from handling stress, resulting in approximately 390 and 310 cycles in F0 and F1 juveniles, respectively. Analyses were performed only on declines in oxygen concentration displaying R^2>0.85 and R^2>0.90 in F0 and F1, respectively. On the third morning, a $P_{O_2,crit}$ trial was done on F0 old and F1 juveniles, see below. After finishing the trial or the respiration measurement for F0 young, fish were removed from the chamber. Juveniles were weighed and BL was measured prior to the experiment for F0 and after the experiment for F1. F0 old juveniles were identified by their PIT tag and returned to their origin tank after the experiment. F0 young juveniles and F1 juveniles were killed by a cut through the spine after the experiment.

$P_{O_2,crit}$ trial

On the third morning, oxygen concentration in the tank surrounding the chambers was continuously decreased, in F0 old by passing the water through a gas equilibration column supplied with nitrogen gas before pumping it into the tank. In F1, the decrease in oxygen concentration was done by bubbling nitrogen directly into the surrounding water bath. The decrease lasted over a period of 4–6 h hours to determine $P_{O_2,crit}$. When the fish lost equilibrium in the oxygen-depleted chambers, they were removed from their chamber and treated as described above.

Data handling

In F0 juveniles, the metabolic rate (MR, in mg O_2 h^{-1} kg^{-1} wet mass in F0) was calculated using AquaResp software. In F1, oxygen concentration was converted from % air saturation to nmol l^{-1} and mmol l^{-1} in larvae and juveniles, respectively ('conv_O2' function of the 'respirometry' package; https://CRAN.R-project.org/package=respirometry). MR was calculated from the raw data with the following formula:

$$\mathrm{MR} = \mathrm{Slope}\ V_{resp}, \tag{5}$$

where slope is the oxygen decline in the respiration chamber during one measurement cycle (nmol O_2 l^{-1} h^{-1} and mmol O_2 l^{-1} h^{-1} for larvae and juveniles, respectively), and V_{resp} is the volume of the respirometer (l).

RMR of F1 larvae was calculated as the mean MR throughout the measuring period (~4 h). SMR of F0 juveniles was calculated following the protocol of Chabot et al. (2016) as described in Crespel et al. (2019). SMR of F1 juveniles was calculated in R with the 'calcSMR' function of the 'fishMO2' package (http://github.com/denis-chabot/fishMO2), derived from this protocol. Briefly, the best SMR was chosen as described in Chabot et al. (2016) as either the SMR derived from the mean of the lowest normal distribution (MLND) method (SMR_{MLND}) or the SMR derived from the quantile method with P=0.2 (SMR_{quant}). SMR_{MLND} was used when the coefficient of variation (CV) was <7% or <5.4%, in F0 and F1, respectively; otherwise, SMR_{quant} was applied. Both RMR and SMR were divided by fish mass (resulting in RMR_{Raw} and SMR_{Raw}) and then corrected for allometric scaling with the following formulas:

$$\mathrm{RMR} = \mathrm{RMR}_{\mathrm{Raw}} \left(\frac{\mathrm{DM}}{\mathrm{DM}_{\mathrm{mean}}}\right)^{1-\mathrm{coeff}_{\mathrm{Larvae}}}, \tag{6}$$

$$\mathrm{SMR} = \mathrm{SMR}_{\mathrm{Raw}} \left(\frac{\mathrm{WM}}{\mathrm{WM}_{\mathrm{mean}}}\right)^{1-\mathrm{coeff}_{\mathrm{Juv}}}, \tag{7}$$

where RMR_{Raw} and SMR_{Raw} are RMR (nmol O_2 µg DM^{-1} h^{-1}) and SMR (mmol O_2 kg WW^{-1} h^{-1}) calculated as described in the text, DM is larval dry mass (µg), WM is juvenile wet mass (kg), DM_{mean} and WM_{mean} are mean DM and WM of all larvae and juveniles, respectively, and $coeff_{Larvae}$ and $coeff_{Juv}$ is the allometric scaling coefficient for larvae (0.89) and juveniles (0.99), respectively. The allometric scaling coefficients used were the slopes of linear regressions of MR over mass in the whole larval (F1) and juvenile (F0 and F1 together) dataset. Q_{10} was calculated with the same formula as used for SGR (see Eqn 4).

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$P_{O_2,crit}$ was calculated with the 'calcO2crit' function of the 'fishMO2' package (http://github.com/denis-chabot/fishMO2), or according to Claireaux and Chabot (2016).

Statistical analysis

All statistics were performed with R (http://www.R-project.org/). All data were tested for outliers (Nalimov test), normality (Shapiro–Wilk's test) and homogeneity (Levene's test). None of the datasets met the assumptions for ANOVA; therefore, all data were fitted to linear mixed effects models (LME models, 'lme' function of the 'nlme' package; https://CRAN.R-project.org/package=nlme). Rearing tank was included as a random effect in all models. For the respirometry experiments, respirometer was also included as random effect. In case of heterogeneity of data, variance structures were included in the random part of the model. The best variance structure was chosen according to lowest Akaike information criteria (AIC) values. After fitting fixed and random effects, a backwards model selection process was applied to determine the significant and fixed variables and interactions. If significant effects were detected in the LME models, *post hoc* Tukey tests were performed with the 'lsmeans' function ('lsmeans' package, Lenth, 2016). Significance for all statistical tests was set at $P<0.05$. All graphs were produced from the lsmeans-data with the 'ggplot2' package (Wickham, 2016). All data are shown as lsmeans±s.e.m.

Growth data

Larval BL (F0 and F1 larvae)

Larval BL at mouth opening was only measured in F1 larvae. As these were reared in a full-factorial design, temperature condition, P_{CO_2} concentration and their interactions were included as fixed effects in the model. Across generations, the dataset for larval BL at metamorphosis and over time was imbalanced; therefore, it was not possible to test the effect of temperature, P_{CO_2} condition, generation and their interaction separately. Instead, treatment was used as fixed variable in the model for larval BL at metamorphosis, resulting in six groups: F0 A, F0 Δ1000, F1 C-A, F1 C-Δ1000, F1 W-A and F1 W-Δ1000. For larval BL over time, treatment, age and the interaction between group and age were included as fixed effects in the model.

Larval DM (F1 larvae)

Larval DM was only measured in F1 larvae; therefore, temperature condition, P_{CO_2} concentration, age and their interactions were included as fixed effects in the model for log-transformed larval DM over time. Larval DM at mouth opening and metamorphosis was analysed with temperature condition, P_{CO_2} concentration and their interactions as fixed effects.

Juvenile BL and WM over time (F1 juveniles)

As F0 and F1 juveniles had different temperature life histories as well as rearing conditions, their growth rates over time were not directly compared. Because of an imbalanced dataset in F1 juveniles, it was not possible to test the effect of temperature, P_{CO_2} condition and their interaction separately. Instead, as for larval BL, treatment was used as fixed variable, resulting in three groups: F1 C-A, F1 C-Δ1000 and F1 W-Δ1000. Treatment, age and the interaction between treatment and age were included as fixed effects in the models for juvenile BL and log-transformed juvenile WM over time.

Juvenile BL and WM at 3000 dd (F0 and F1 juveniles)

Juvenile BL and WM were compared at 3000 dd across generations. Because of the imbalanced dataset, treatment was again used as fixed effect. For juvenile BL and WM, treatment included the following five groups: F0 A, F0 Δ1000, F1 C-A, F1 C-Δ1000 and F1 W-Δ1000.

Respirometry

Larval RMR (F1 larvae)

As larvae were reared in a full-factorial design, temperature condition, P_{CO_2} concentration and their interactions were included as fixed effects in the model.

Juvenile SMR and $P_{O_2,crit}$ (F0 and F1 juveniles)

Because of an imbalanced dataset for juvenile respirometry, it was not possible to test the effect of temperature, P_{CO_2} condition, generation, age and their interactions separately; instead, treatment was used as fixed variable, resulting in seven groups for SMR: F0 A-young, F0 Δ1000-young, F0 A-old, F0 Δ1000-old, F1 C-A, F0 C-Δ1000 and F1 W-Δ1000; and five groups for $P_{O_2,crit}$: F0 A-old, F0 Δ1000-old, F1 C-A, F0 C-Δ1000 and F1 W-Δ1000.

RESULTS

Growth

Neither temperature nor P_{CO_2} treatment had a significant effect on larval size at mouth opening stage in F1 larvae (Fig. 2A,D, Table 4). During the following larval development, higher temperatures significantly increased growth if larvae were compared at the same age (dph): F1 C larvae were smaller than F0 and F1 W larvae at higher temperature (Fig. 3A,B, Table 4). SGR ranged from 7.85 to 9.75% day^{-1} for larval DM and 11.67 to 14.76% day^{-1} for larval BL (Table 3). The higher growth rates in F1 W larvae resulted in a Q_{10} of 1.67–2.12 and 1.81–2.35 for DM and BL (Table 3). P_{CO_2} had no effect on the growth of F0 and F1 C larvae, but reduced growth significantly in F1 W larvae (Table 4). Because of the longer larval duration in colder temperatures (900 dd equals 45 dph at 20°C and 60 dph at 15°C), F1 C larvae were of comparable size to F1 W-A and F0 larvae at metamorphosis. In contrast, F1 W-Δ1000 larvae were significantly smaller at metamorphosis than any other group of larvae (Fig. 2B,E, Table 4).

In juveniles, the overall positive effect of temperature on growth persisted, with F1 W juveniles displaying significantly higher growth rates than F1 C juveniles (Fig. 3C,D, Table 4). SGR ranged from 2.88 to 5.16% day^{-1} for juvenile WM and 0.84 to 1.55% day^{-1} for juvenile BL; the higher growth rates resulted in a Q_{10} of 2.41–2.72 and 2.31–2.52 for WM and BL, respectively, in F1 Δ1000 juveniles. If compared at the age of 3000 dd (165, 140 and 181 dph for F0, F1 W and F1 C juveniles, respectively), the difference in size was inverted compared with metamorphosis: F1 W-Δ1000 juveniles were now significantly larger than any other group (Fig. 2C,F, Table 4). P_{CO_2} did not have any significant effect on the growth of F0 or F1 C juveniles. The effect of P_{CO_2} on F1 W juveniles was not determined because of the missing F1 W-A treatment.

Metabolic rate

Metabolic rate estimations were done on larvae with a mean size ranging from approximately 1.5 to 3.0 mg DM and 11.5 to 14 mm BL with no significant differences in size between treatments (BL and DM; Table S3). For juveniles, mean size ranged from approximately 3 to 62 g WM and 9 to 20 cm BL (Table S4), with no significant differences in size (BL and WM) or condition factor between acidification treatments of the same age and generation (ANOVA, $P>0.05$ for F0 old; LME, $P>0.05$ for F0 young and F1 C) or between F1 C and F1 W (LME, $P>0.05$). The positive effect of temperature on growth was mirrored in larval RMR in F1: RMR was significantly lower in F1 C than in F1 W. But in contrast to growth, no effect of

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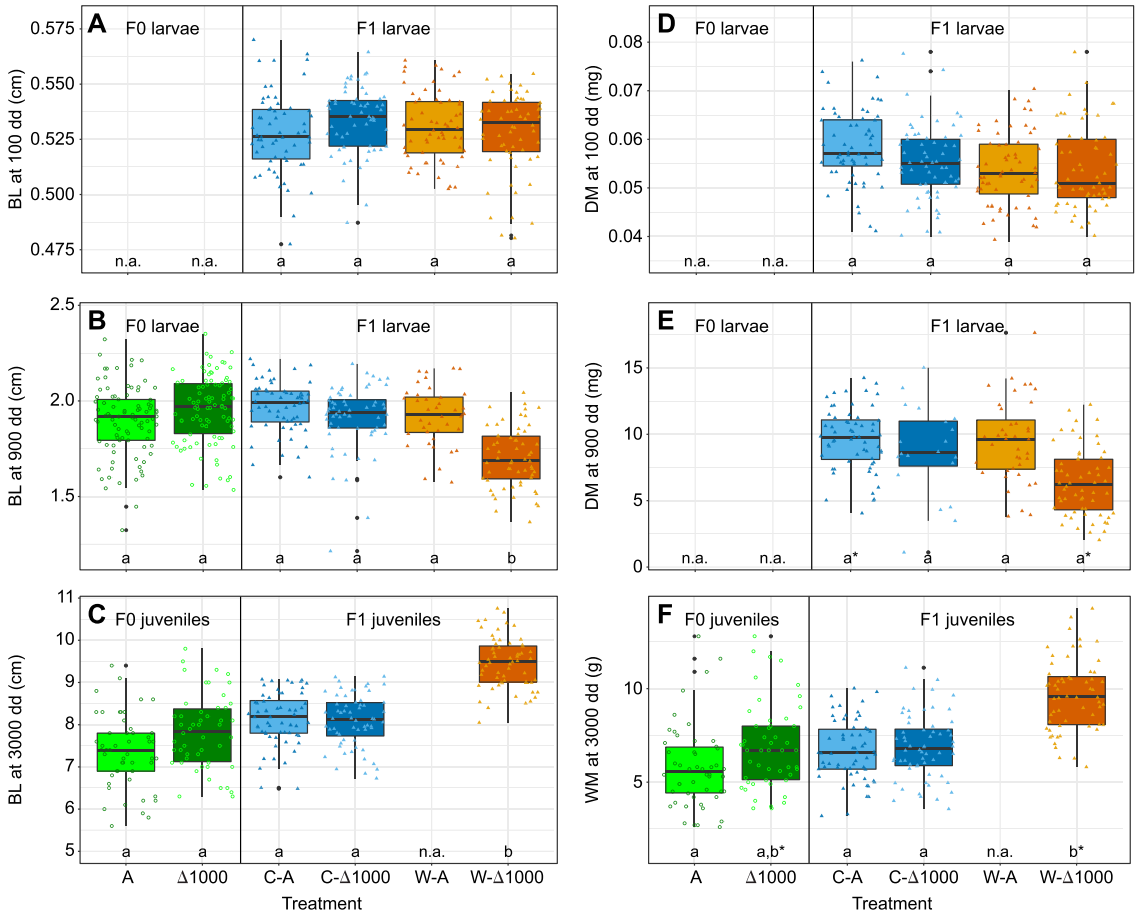

Fig. 2. Body length and mass of European sea bass. Body length (BL) and mass (DM, dry mass; WM, wet mass) data are given for F0 and F1 fish at approximately 100 degree days (dd; mouth opening, 7 dph; A,D), 900 dd (metamorphosis; B,E) and 3000 dd (C,F). Box plots show median, 25th and 75th percentile, 1.5x interquartile range and outliers. Overlying symbols are the individual data points for each treatment; different letters indicate significant differences [linear mixed effect (LME) models, $P<0.05$]; asterisks indicate statistical trends (LME, $P<0.1$). A, ambient P_{CO_2}; Δ1000, ambient+1000 µatm CO_2; C, cold life condition; W, warm life condition; n.a., treatment was not available or not measured in this state. n=40–90.

P_{CO_2} treatment or an interaction of temperature and P_{CO_2} treatment on larval RMR was observed (Fig. 4A, Table 4). A Q_{10} of 2.24 and 2.51 was calculated for larval RMR for F1 A and F1 Δ1000 larvae, respectively. Similarly, juvenile SMR was significantly lower in F1 C than in F1 W juveniles (Fig. 4B, Table 4), with a Q_{10} of 1.61 for F1 Δ1000 juveniles. The comparison between the two generations showed that the SMR in the F0 juveniles did not change significantly between 5 and 15 month old juveniles, but F0 SMR estimates were significantly lower than those in F1 juveniles (Table 4). Comparable to larval RMR, there was no significant effect of P_{CO_2} in juvenile SMR at each thermal treatment. Although the LME model states a significant effect of treatment on $P_{O_2,crit}$ (Fig. 4C, Table 4), *post hoc* tests revealed only a significant difference between F0 Δ1000 and F1 C-A ($P<0.04$); all other groups were not significantly different from each other.

DISCUSSION

Long-term experiments exploring the potential of fish to adapt to OAW are still scarce, especially in larger, temperate species with long generation times. In this long-term experiment, we observed that OW as single driver increased growth rate and RMR in the warm F1 larval sea bass, but as a result of the decreased larval phase duration at warmer temperatures, F1 C-A and F1 W-A larvae had a similar size at metamorphosis. OA as single driver had no effect on F1 larval and juvenile growth or on metabolism at ambient (cold) temperature. Under OAW, F1 W-Δ1000 larvae were significantly smaller at metamorphosis than any other group, while maintaining similar RMR to F1 W-A larvae. As they grew into juveniles, F1 W-Δ1000 fish were bigger than F1 C fish at 3000 dd and had the highest SMR. Unfortunately, the F1 W-A group could not be kept until juvenile phase. Although F0 and F1 W larvae were both raised at increased temperatures, we observed that the detrimental effects of OAW occurred only in F1 W-Δ1000 and not in F0 Δ1000. We also observed that juvenile SMR was lower in F0 than in F1 C and F1 W, with no effect of OA in F0 and F1 C. Juvenile $P_{O_2,crit}$ was not affected by OA or OAW in both generations.

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Table 3. Specific growth rate and the respective Q_{10} of F1 larval and juvenile mass and body length of European sea bass

Parameter	Treatment	*n*	0.05 quantile	0.5 quantile	0.95 quantile
Larval dry mass					
SGR (% day^{-1})	C-A	3	9.19±0.37	9.57±0.23	9.63±0.25
	C-Δ1000	1	7.85	9.26	9.75
	W-A	2	12.92±0.38	14.25±0.11	14.76±0.08
	W-Δ1000	3	11.67±0.29	12.92±0.26	13.73±0.20
Q_{10}	A		1.84	1.96	2.12
	Δ1000		1.81	1.67	1.80
Larval body length					
SGR (% day^{-1})	C-A	3	2.27±0.08	2.41±0.04	2.40±0.06
	C-Δ1000	3	2.14±0.07	2.33±0.02	2.43±0.03
	W-A	2	3.09±0.15	3.37±0.06	3.50±0.02
	W-Δ1000	3	2.88±0.01	3.01±0.06	3.26±0.04
Q_{10}	A		1.98	2.22	2.35
	Δ1000		1.81	1.95	1.98
Juvenile wet mass					
SGR (% day^{-1})	C-A	2	3.07±0.09	2.94±0.03	3.04±0.04
	C-Δ1000	2	2.96±0.05	2.88±0.01	2.92±0.04
	W-A		n.a.	n.a.	n.a.
	W-Δ1000	2	5.16±0.02	4.93±0.11	4.82±0.13
Q_{10}	A		n.a.	n.a.	n.a.
	Δ1000		2.72	2.63	2.41
Juvenile body length					
SGR (% day^{-1})	C-A	2	0.91±0.02	0.87±0.00	0.87±0.00
	C-Δ1000	2	0.85±0.02	0.84±0.00	0.86±0.01
	W-A		n.a.	n.a.	n.a.
	W-Δ1000	2	1.55±0.01	1.49±0.02	1.46±0.06
Q_{10}	A		n.a.	n.a.	n.a.
	Δ1000		2.52	2.45	2.31

Specific growth rate (SGR; means±s.e.m. over all replicate tanks per condition) and Q_{10} are given for the 0.05, 0.5 and 0.95 quantile of the cohort. A, ambient P_{CO_2}; Δ1000, ambient+1000 µatm CO_2; C, cold life condition; W, warm life condition; n.a., treatment was not available or not measured at this state.

Effects of OW on European sea bass growth and metabolism

F1 C larvae were reared at 15°C, reflecting ambient temperature towards the middle to the end of the spawning season in the Bay of Brest. We applied a warming scenario of +5°C on F1 W larvae, which reflects typical rearing temperatures in aquaculture, as well as natural temperatures towards the middle to end of the spawning season in the Mediterranean (Ayala et al., 2003). This thermal treatment (20°C) is well below the upper thermal limits for seabass larvae from the Bay of Brest (27°C; Moyano et al., 2017). OW as a single driver at ambient P_{CO_2} significantly increased growth rate and decreased the time to reach metamorphosis in F1 W-A larvae in comparison to F1 C-A larvae. As a result of the longer larval phase duration, size at metamorphosis was comparable between F1 C-A larvae and F1 W-A larvae. Faster growth at higher temperatures and similar size at metamorphosis despite different temperatures has been shown in other studies of sea bass from Mediterranean and Atlantic populations (Ayala et al., 2001, 2003). OW also increased RMR in F1 W-A larvae compared with F1 C-A larvae. The increase in RMR was similar to the increase in SGR, reflected by a similar Q_{10} [1.96, 2.22 and 2.24 for SGR of DM and BL (0.5 quantile), and RMR, respectively]. This reflects the expected Q_{10} increase of 2–3 for biological processes and confirmed our hypothesis that OW will lead to increased growth and RMR in larval sea bass of this particular population. We did not determine the effects of OW as a single driver on growth and metabolism in F1 juveniles because of the absence of F1 W-A.

Effects of OA on European sea bass growth and metabolism

OA as single driver within the cold temperature condition did not affect either growth and metabolism (RMR, SMR) or $P_{O_2,crit}$ in F1 European sea bass larvae or juveniles. In the wild, sea bass eggs are spawned in stable open ocean conditions and larvae develop during drifting towards the coast; therefore, larvae were thought to be less resilient to OA than juveniles and adults. This has already been shown not to be the case for sea bass in scenarios up to SSP5-8.5 and similar (Pope et al., 2014; F0 in Crespel et al., 2017) and was further confirmed by this study, as larval growth and RMR were not affected by OA within the cold temperature group. As juvenile sea bass inhabit coastal areas and have been shown to be tolerant to a broad range of environmental factors, including temperature and salinity (Dalla Via et al., 1998; Claireaux and Lagardère, 1999), their tolerance to OA was expected and could be confirmed in this study – no effects of OA within the cold temperature group on growth, SMR and $P_{O_2,crit}$ were observed. Our study also supports the hypothesis of Montgomery et al. (2019) that an observed 20% decrease in $P_{O_2,crit}$ under an acute increase of P_{CO_2} (3- to 5-fold increase in P_{CO_2} within ∼6 h) in European sea bass will vanish after long-term acclimation to OA.

Combined effects of OA and OW on European sea bass growth and metabolism

However, the combined effects of OA and OW (OAW) changed the picture for larval resilience. While growth rates increased sufficiently in F1 W-A to reach the same size at metamorphosis as F1 C-A, F1 W-Δ1000 larvae were significantly smaller at metamorphosis than larvae from any other treatment, but maintained RMR as high as that of F1 W-A larvae. Q_{10} values revealed that temperature had a stronger effect on metabolic rate than on growth under OA: 1.67 and 1.95 for SGR of DM and BL (0.5 quantile) and 2.51 for RMR, respectively. This suggests that F1 W-Δ1000 larvae either allocated the energy differently, such as using more energy for movement or different regulatory processes,

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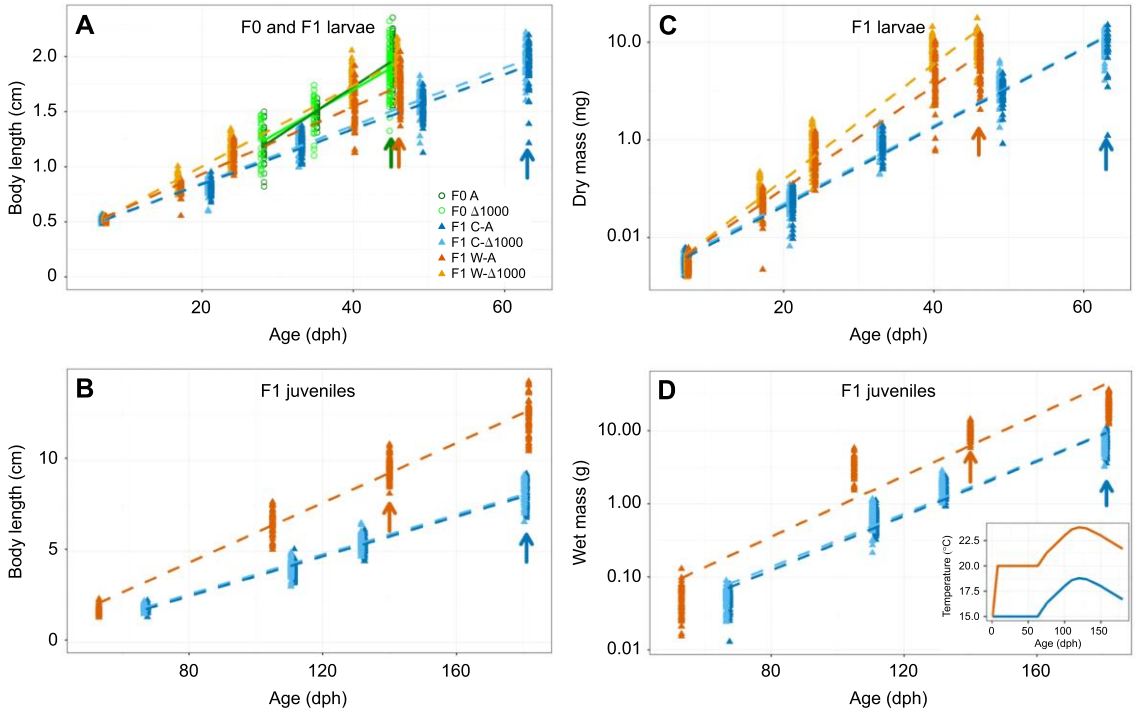

Fig. 3. Growth of F0 and F1 larvae and F1 juveniles with linear regression lines. Shown are individual data points of body length (A,B), larval dry mass (C) and juvenile wet mass (D). F1 C larvae grew significantly slower than F1 W (A,C) and F0 larvae (A). F1 W-Δ1000 larvae grew significantly slower than F1 W-A (A,C) and F0 A larvae (A). F1 W-Δ1000 juveniles grew significantly faster than F1 C juveniles (B,D). No differences were observed between P_{CO_2} treatments in F0 larvae (A), F1 C larvae (A,C) and F1 C juveniles (B,D). All data were tested with LME models; *F*- and *P*-values are summarized in Table 4. Arrows indicate the data points at metamorphosis (900 dd, A,C) and at 3000 dd (B,D); data for different P_{CO_2} conditions of the same age are slightly offset for better visibility. Inset in D shows the temperature history of F1 larvae and juveniles. A, ambient P_{CO_2}; Δ1000, ambient+1000 µatm CO_2; C, cold life condition; W, warm life condition.

or that their energy production and oxygen usage was not as efficient as in the other groups. Although it is possible that the higher RMRs are due to a higher activity of the F1 W-Δ1000 larvae during the measurements, larvae were regularly observed during the trials and the inter-individual variability in movement did not seem related to treatment. Therefore it seems more plausible that larvae under OAW needed energy for different regulatory processes, probably combined with decreased energy production efficiency. In this sense, we previously found that OAW decreased the efficiency of complex II (CII) of the electron transport system (ETS) in cardiac mitochondria of juvenile sea bass in the W-Δ1000 treatment under acute temperature change (Howald et al., 2019). Inhibition of CII by OA was also found in other studies on mammals and fish (Simpson, 1967; Wanders et al., 1983; Strobel et al., 2013). In Atlantic cod embryos, reduced activity of complex I (CI) of the ETS resulted in reduced mitochondrial phosphorylation capacity and subsequently in reduced oxygen consumption rates, while energy requirements were simultaneously increased (Dahlke et al., 2017). Although CII was only affected in juvenile sea bass under acute temperature change, it is probable that larvae are more vulnerable than juveniles (Dahlke et al., 2020a): similar to embryos (Leo et al., 2018), they are less developed while at the same time investing all available energy into growth without reserving excess capacity for environmental regulation and are therefore already affected at their acclimation temperature if OA and OW are combined. This inability to cope with OAW has not been observed in European sea bass larvae before; contrastingly, in former studies, growth of larval European sea bass has been shown to be resilient to OA even at a rearing temperature of 19°C (Pope et al., 2014; F0 larvae in Crespel et al., 2017). Potential explanations why these differences first occurred in F1 are probably related to their parents being reared under OA conditions, as well as effects of different rearing protocols, which are both addressed below (see ‘Effects of OA on European sea bass growth and metabolism over two successive generations’).

In contrast to larvae, F1 W-Δ1000 juveniles displayed a greater thermal plasticity and grew significantly faster than F1 C juveniles, resulting in larger fish at 3000 dd in the F1 W-Δ1000 than in F1 C-A and F1 C-Δ1000 treatments. High growth rates were supported by high SMR, which was also highest in F1 W-Δ1000 juveniles in comparison to F1 C-A and F1 C-Δ1000. As we did not incubate the F1 W-A treatment to juvenile phase, it is unclear whether the detrimental effects of OAW on growth and metabolism in larval European sea bass would have persisted into the juvenile phase. The increased growth rate and bigger size at 3000 dd in F1 W-Δ1000 juveniles in comparison to F1 C-A and F1 C-Δ1000 juveniles might either indicate that OA did not affect growth in juveniles or that growth under OW was so accelerated in juveniles that F1 W-Δ1000 fish were able to catch up and grow to bigger sizes than F1 C fish, masking the negative effects of OAW. The latter suggestion is supported by the findings for SMR and by the Q_{10} of SMR and SGR: in F1 Δ1000 juveniles, SMR was less affected by temperature (Q_{10} 1.61) than SGR [Q_{10} 2.63 and 2.45 for SGR of WM and BL

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Table 4. *F*- and *P*-values of fixed effects from the linear mixed effect models on growth and metabolic rate of F0 and F1 larval and juvenile European sea bass

	OAW treatment		P_{CO_2} treatment		Temperature		P_{CO_2}×temperature	
Parameter	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Larval dry mass								
Mouth opening	n.a.	n.a.	1.18	0.3	4.49	0.06	2.13	0.18
Metamorphosis	n.a.	n.a.	11.69	0.01	6.37	0.05	2.73	0.16
Over time	n.a.	n.a.	17.27	0.0032	2.61	0.1447	8.01	0.0221
Larval body length								
Mouth opening	n.a.	n.a.	0.23	0.66	0.21	0.64	1.72	0.23
Metamorphosis	10.04	0.0008	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Over time	275.09	<0.0001	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Juvenile wet mass								
3000 dd	16.41	0.0222	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Over time	240.515	0.0005	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Juvenile body length								
3000 dd	46.93	0.0049	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Over time	1111.59	<0.0001	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
RMR	n.a.	n.a.	0.01	0.94	29.62	<0.0001	0.06	0.82
SMR	95.44	<0.0001	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
$P_{O_2,crit}$	3.79	0.0064	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

OAW, ocean acidification–warming; dd, degree days; RMR, routine metabolic rate; SMR, standard metabolic rate; $P_{O_2,crit}$, critical O_2 concentration; n.a., treatment was not available or not measured in this state.

(0.5 quantile)]. Q_{10} for SGR and SMR is well in the range found in other studies on European sea bass from the Atlantic [Q_{10} for SGR of WM ~2.4 (15–20°C), calculated from Gourtay et al., 2018; and Q_{10} for SMR 2.09 (14–22°C), calculated from Montgomery et al., 2021 preprint] and from the Western Mediterranean populations [Q_{10} for SGR of WM and RMR of 2.40 and 1.70, respectively (13–25°C), calculated from Person-Le Ruyet et al., 2004]. Person-Le Ruyet et al. (2004) explained the different Q_{10} of RMR and SGR with increased growth rates as being due to increased feed intake. As the fish in our study were fed *ad libitum*, they were able to increase food intake to support high growth rates too. The better capacity of juveniles to cope with and even profit from higher temperatures even under OAW in comparison to larvae is probably a result of the reproduction biology of European sea bass, as well as the generally higher capacity for acid–base regulation in juveniles in comparison to larvae. Larvae develop during spring in the open ocean, where temperatures are stable and relatively cold (8–13°C for Atlantic specimen; Jennings and Pawson, 1992), with optimal larval growth temperatures of 15–17°C (Mediterranean specimens; Koumoundouros et al., 2001; Ayala et al., 2003). In contrast, juveniles live in shallow coastal areas, and so encounter higher temperatures during summer but also higher daily and seasonal variation (6–18°C for Atlantic specimens; Russel et al., 1996) with optimal growth temperatures of 22–28°C (Mediterranean specimens; Lanari et al., 2002; Person-Le Ruyet et al., 2004). Consequently, in terms of growth and metabolism, juvenile sea bass at the northern distribution range might benefit from higher temperatures, as already found in other studies (Howald et al., 2019; Montgomery et al., 2021 preprint), and do not seem to be severely affected by OA.

Effects of OA on European sea bass growth and metabolism over two successive generations

In addition to the effects of the single and combined stressors OA, OW and OAW on individual groups of fish, we also studied the effects of OA in two successive generations on the ability of sea bass larvae and juveniles to cope with upcoming conditions. This study is to our knowledge the first to examine the effects of OA on European sea bass or other long-lived teleosts in more than one generation. Interestingly, the detrimental effect of OAW on larval growth was only observed in F1 and not in F0 larvae of European sea bass, despite their respective parental generation's identical thermal history, and thus appears to be an OA effect. There may be several reasons for this. First, the provisioning of necessary resources when parents have already encountered the same conditions as the future offspring, e.g. via egg size and composition (Munday, 2014), could explain the observed trend in F1 W-Δ1000 larvae. Parental effects can lead in different directions and can last throughout the larval phase: for example, parental effects influence growth in stickleback under OW and OA (Shama et al., 2014; Schade et al., 2014) and explained differences in embryo mortality and hatching success in Atlantic cod under OW (Dahlke et al., 2017). In our study, we did not measure egg size and quality, nor did we incubate offspring of F0 A in cross-factorial Δ1000 scenarios, so we cannot directly quantify parental or transgenerational effects. However, the size of F1 larvae at mouth opening, up to which point the larvae depend on yolk sac reserves, did not differ across treatments. Thus, using this landmark as an indirect indicator, parental provisioning does not seem to explain differences in larval growth rates. Second, the incubation protocol differed between F0 and F1. While F0 larvae were first incubated under OA conditions at 2 dph, F1 sea bass were constantly reared under OA conditions from fertilization onwards, although warming was also applied from 2 dph onwards. It is possible that the effects of OA during embryogenesis shaped the reaction of F1 larvae to OAW, e.g. via epigenetic signalling. As reviewed by Dahlke et al. (2020a), it seems that spawning adults and embryos are the most vulnerable life stages in fish, possessing the lowest tolerance to OW, e.g. Atlantic cod embryos exposed to OAW showed reduced hatching success and oxygen consumption rates (Dahlke et al., 2016) and OA decreased the Q_{10} of RMR in Atlantic silverside embryos (Schwemmer et al., 2020). To summarize, the different reaction of F0 and F1 larvae to OAW could be due to parental effects or effects during embryogenesis and more research is necessary to determine the underlying mechanisms.

As the different temperature life histories and replication schemes (no replicate tanks in F0 juveniles) did not allow a direct comparison of growth rates between F0 and F1 juveniles, we compared size at

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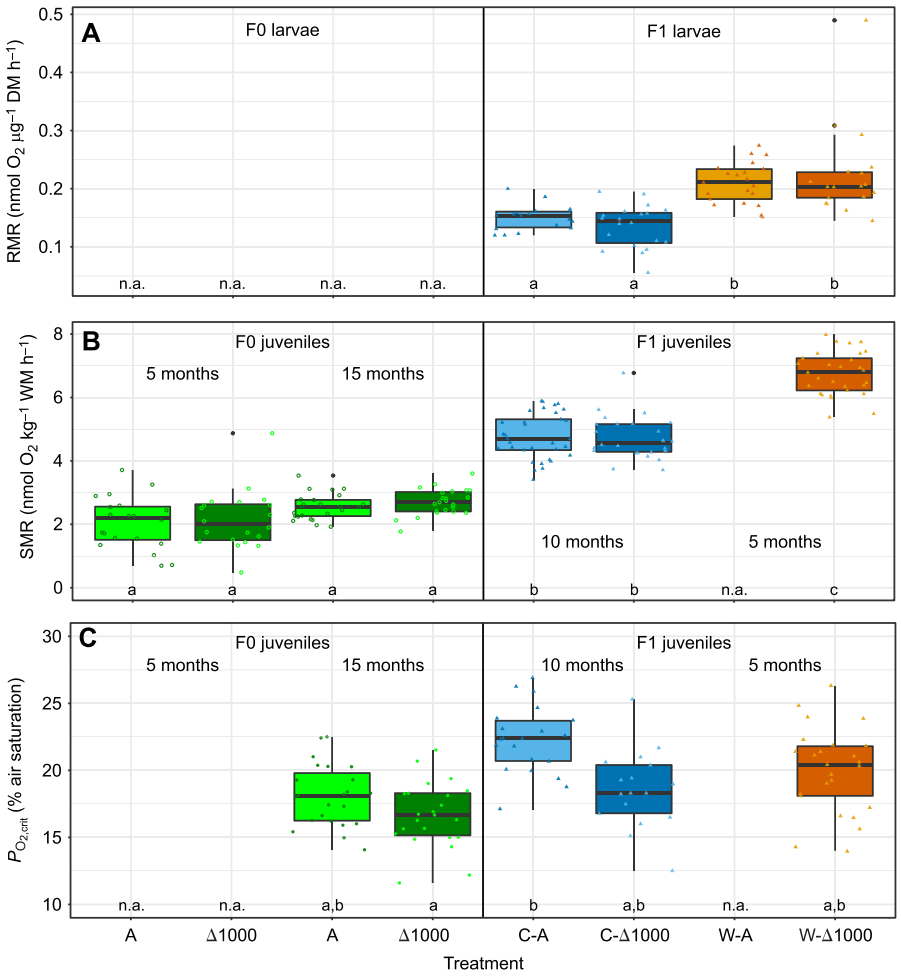

Fig. 4. Routine and standard metabolic rate and critical oxygen concentration of F0 and F1 larvae and juveniles. (A) Routine metabolic rate (RMR), (B) standard metabolic rate (SMR) and (C) critical oxygen concentration ($P_{O_2,crit}$). Box plots show median, 25th and 75th percentile, 1.5x interquartile range and outliers. Overlying symbols are the individual data points for each treatment. Metabolic rates were corrected with allometric scaling factors (0.89 and 0.98 for larvae and juveniles, respectively). Different letters indicate significant differences (LME, $P<0.05$; data of 15 month old F0 juveniles taken from Crespel et al., 2019). A, ambient P_{CO_2}; Δ1000, ambient+1000 µatm CO_2; C, cold life condition; W, warm life condition; n.a., treatment was not available or not measured in this state. n=20–35.

the age of ∼3000 dd (165, 140 and 181 dph for F0, F1 W and F1 C juveniles). Because of their high growth rates during the juvenile phase, F1 W-Δ1000 fish were largest at 3000 dd, while F0 and F1 C fish were smaller (WM and BL) but similar to each other. This matched the findings for SMR, which was not affected by OA and was higher in F1 W than in F1 C fish. Surprisingly, SMR was also higher in F1 C than in F0 fish. This difference might be explained by the different temperature life histories. While F0 fish had been raised at warmer temperatures and were acclimated to colder temperatures afterwards, F1 C fish had been reared at 15°C throughout their life, except for summer months, when temperatures reached up to 19°C. No detrimental effects on juvenile growth rate under OA were visible in the second generation of sea bass reared under OA conditions, as reflected by similar SMR and SGR between the A and Δ1000 condition. Because of the missing F1 W-A treatment, we cannot state whether the detrimental effects of OAW observed in F1 larvae persisted to the juvenile phase.

Ecological perspective

Larvae are not fully developed compared with later stages and are exposed to higher predation and starvation risks; as such, they had been thought to be more vulnerable to environmental stressors such as OAW (as reviewed in Houde, 2009). In this context, OAW could impact larval survival and recruitment success via different mechanisms. If OAW leads to faster growth rate and increased metabolic rate (as seen in this study between F1 W and F1 C larvae), larvae will need more food in a shorter time to support these growth rates; therefore, it is essential that they match adequate prey fields (prey abundance, size and quality). In our study, the larvae were fed *ad libitum* at both temperatures, supporting increased energetic

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demands for the high growth and metabolic rates at higher temperatures. However, in the ocean it is possible that food availability is not sufficient to support accelerated growth under OW. Bochdansky et al. (2005) showed that fish larvae with higher growth and metabolic rates died earlier when food was limited, but profited when fed at saturation level. In sea bass larvae, high growth rates were also only supported under high food ratios, but survival was not significantly decreased, even at one-eighth saturation ratio (Zambonino Infante et al., 1996). This might indicate that sea bass will not grow as fast as in our study under future OW scenarios if food is scarce, but might still survive to juvenile stage.

Besides food-related aspects, OAW can also have a large impact on larval behaviour and dispersal, which can later influence recruitment success. Sea bass spawn in the open ocean and larvae drift inshore (Jennings and Pawson, 1992). As with many temperate species, their swimming behaviour and its effect on dispersal have not been studied as extensively as for coral reef fish that have well-developed sensory abilities (hearing, olfaction, vision) and show directional swimming early on (as reviewed in Leis, 2018; Berenshtein et al., 2021 preprint). To the best of our knowledge, it seems that early sea bass larvae are more dependent on currents than on their swimming performance and that they are able choose a certain depth and therefore a certain current in the preferred direction (Jennings and Pawson, 1992). When drifting closer to the coast, sea bass larvae wait for certain cues from nursery areas, which are present from June onwards (Jennings and Pawson, 1992).

OW accelerates the development of sea bass larvae and therefore possibly alters the timing and spacing of dispersal. Studies have shown species-specific responses of fish behaviour to OA, OW and OAW, e.g. OW increased activity level in larval kingfish but not boldness, while OA had no effect on these behavioural traits (Laubenstein et al., 2019). Yet, OA decreased swimming duration and orientation in larval dolphinfish (Pimentel et al., 2014) and reversed orientation towards settlement habitat cues in barramundi (Rossi et al., 2015). To our knowledge, larval sea bass behaviour has not been measured under OAW yet. Consequently, because of the altered timing of larval development and in combination with the possibility of altered behaviour and impacted senses, reaching nursery areas might be challenging for sea bass larvae under OAW, especially if (1) food is not abundant and (2) cues are weaker and/or different as a result of the greater distance and/or earlier timing. Once the larvae enter the coastal areas and metamorphose, they are exposed to a more changing environment. Although this study confirmed that juvenile sea bass are less vulnerable to OAW than larval sea bass, food availability and behaviour will determine whether the observed increased growth under OAW in F1 will occur in the wild too. In a sister study on offspring of wild-caught European sea bass, OAW reduced digestive enzyme activity under restricted food ratios, resulting in severely reduced food conversion efficiency and reduced growth rates (Cominassi et al., 2020). Additionally, OA decreased the distance over which early juvenile sea bass sensed food or predator cues (Porteus et al., 2018) and juvenile sea bass behaviour was altered by OW, resulting in a decreased latency of the escape response and mirror responsiveness (Manciocco et al., 2015). Consequently, although faster larval (OW) and juvenile growth (OAW) as well as earlier metamorphosis (OW, OAW) are generally beneficial for larvae and early juveniles, many factors may modulate this effect and whether it will translate into higher larval survival, recruitment and increased growth rates in the wild. Further research should determine the effects of limited food under OAW on larval and juvenile growth and behaviour.

As the hypoxia tolerance of European sea bass juveniles was unaffected by OA, OW and OAW, they might cope well with upcoming hypoxia events in coastal areas. However, it is important to note here that we measured $P_{O_2,crit}$ only at SMR and thus may have estimated P_{O_2} effects too conservatively. Recent studies suggest that this $P_{O_2,crit}$ at SMR might not be the most ecologically relevant estimate (see Seibel and Deutsch, 2020, and references therein). Long-term survival of individuals and the population would require that the fish are able to digest food, grow and reproduce, which would require more energy than provided by SMR. Consequently, depending on the duration and intensity of hypoxia events, individuals might be able to survive in the short term, but other fitness-related traits such as growth might be affected in the long term.

Conclusion

We confirmed our hypotheses that OW increases growth and metabolism in the European sea bass, and that larvae as well as juveniles are resilient to OA if it occurs as a single stressor. We also confirmed that OAW has detrimental effects on larval growth. Our results, together with other findings on larval fish and European sea bass suggest that it is possible that under OAW, fewer individuals will reach metamorphosis, e.g. as a result of limited food to support high growth rates, different dispersal to nursery areas by altered developmental timing, changed behaviour or altered olfactory senses. However, those individuals that reach the juvenile phase might benefit from higher temperatures, because of increased performance.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.H., M.M., A.C., L.C., G.C., M.P., F.C.M.; Methodology: S.H., M.M., A.C., L.C., G.C., M.P., F.C.M.; Validation: S.H., M.M., A.C., G.C., F.C.M.; Formal analysis: S.H., M.M., A.C., L.L.K., L.C.; Investigation: S.H., A.C., L.L.K., L.C., G.C., M.P., F.C.M.; Resources: M.P., F.C.M.; Data curation: S.H., A.C.; Writing - review & editing: S.H., M.M., A.C., L.L.K., L.C., G.C., M.P., F.C.M.; Visualization: S.H., A.C.; Supervision: M.M., G.C., M.P., F.C.M.; Project administration: G.C., M.P., F.C.M.; Funding acquisition: G.C., M.P., F.C.M.

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Data availability

Datasets of growth, metabolic rates and water conditions during rearing are available from PANGAEA: https://doi.pangaea.de/10.1594/PANGAEA.941767

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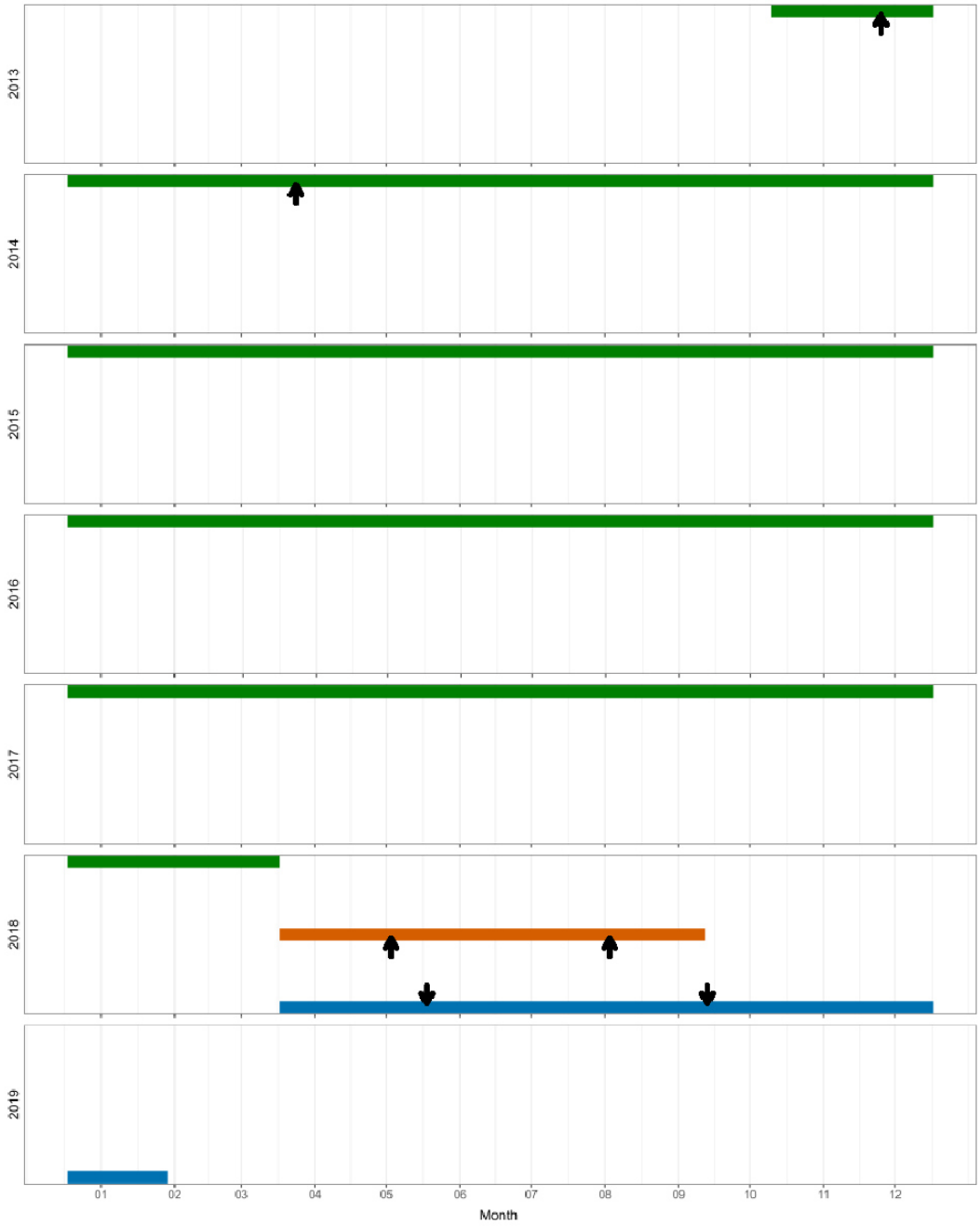

Fig. S1. Timeline of the rearing of the different treatment. Green (2013-2018): rearing of F0 fish; Orange (2018): rearing of F1-W fish; Blue (2018-2019): rearing of F1-C fish. Arrows indicate the time of metamorphosis from larvae to juveniles (first arrow per treatment) and when the fish reached the age of 3000 dd (second arrow per treatment). C- Cold life condition, W- Warm life condition.

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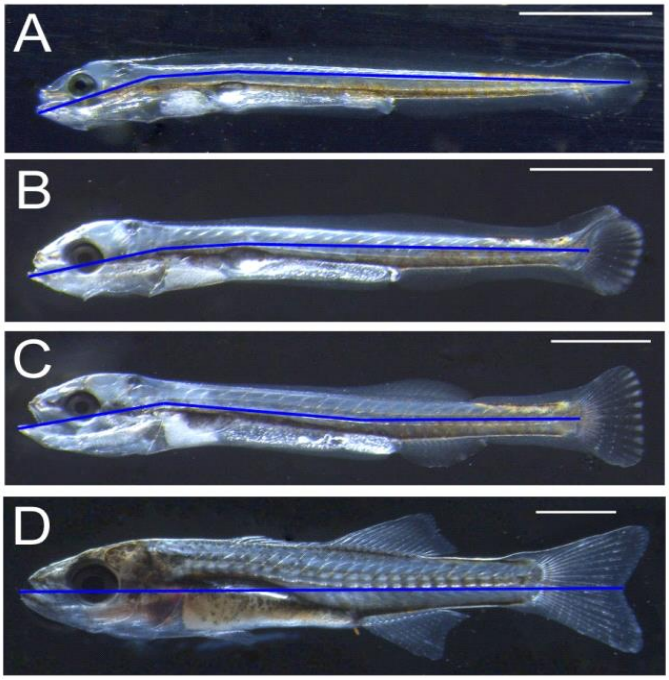

Fig. S2. Body length measurements in larvae at different developmental stages. A – pre flexion (about 300 dd), B – flexion (about 460 dd), D- post flexion (about 460 dd) and (post)metamorphosis (about 900 dd). Until post flexion the segmented line tool in the software ImageJ (Schneider, et al., 2012) was used to measure the length of the larva, afterwards the length of the larvae was measured as a straight line, as it would be done with callipers. The lines of the measurement are marked in blue.

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Table S1. Light intensity during rearing phase of European sea bass larvae. Age is given in days post hatch (dph). Light intensity was changed at the indicated days and remained identical during the light phase until the next increase.

Age [dph]	2	8	11	20	30	32	36	46
Light intensity [lux]	0	0-1	1	7	10	31	59	96

Table S2. Larval mortality in % in the different larval rearing tanks (n=3). A – Ambient PCO_2 andD1000 – ambient + 1000 µatm CO_2, T – temperature, Rep 1-3 – replicate tank 1-3.

	A			Δ1000		
T [°C]	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
15	73.5	28.8	83.1	67.9	55.0	47.2
20	96.4	76.2	25.8	59.3	52.5	53.7

Table S3. Biometrical data of larvae used for respiration measurements. Treatments: C – cold life condition (15°C), W – warm life condition (20°C), A – ambient PCO_2, Δ1000 – ambient PCO_2 + 1000 µatm, values are means ± s.e.m. Different letters indicate significant differences between groups (LME, P<0.05).

Treatment	n	Dry weight [mg]	Bodylength [mm]
C – A	18	2.87±0.51[a]	13.96±0.77[a]
C – Δ1000	20	2.95±0.46[a]	14.04±0.80[a]
W – A	21	2.51±0.43[a]	13.04±0.71[a]
W – Δ1000	18	1.70±0.53[a]	11.63±0.85[a]

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Table S4. Biometrical data of juveniles used for respiration measurements. Treatments: C – cold life condition (up to 18°C), W – warm life condition (up to 23°C), A – ambient PCO_2, Δ1000 – ambient PCO_2 + 1000 µatm, values are means ± s.e.m. Different letters indicate significant differences between groups (LME, P<0.05).

Generation	Treatment	Age [m]	n	Fish mass [g]	Forklength [mm]	Condition factor [-]
F0	C – A	6	20	5.06±0.24^{a}	-	-
F0	C – Δ1000	6	20	5.85±0.27^{a}	-	-
F0	C – A	18	24	81.80±2.60^{b}	18.11±0.18^{b}	1.37±0.02^{b}
F0	C – Δ1000	18	24	81.40±3.22^{b}	18.25±0.22^{b}	1.33±0.03^{b}
F1	C – A	10	33	15.00±0.69^{c}	10.86±0.14^{c}	1.14±0.02^{c}
F1	C – Δ1000	10	26	13.05±0.56^{c}	10.31±0.14^{c}	1.17±0.01^{c}
F1	W – Δ1000	5	29	15.73±1.01^{c}	11.04±0.20^{c}	1.12±0.02^{c}

4 Metabolome analysis

Changes in metabolites in liver, white muscle, heart and gill tissue of European sea bass raised under ocean acidification and warming conditions

This chapter is a manuscript draft, prepared for submission as followed:

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Authors: Sarah Howald, Christian Bock, Guy Claireaux, Louise Cominassi, Marta Moyano, Myron Peck, José-Luis Zambonino-Infante, Felix C. Mark

Changes in metabolites in liver, white muscle, heart and gill tissue of European sea bass raised under ocean acidification and warming conditions

Sarah Howald[1,2]*, Christian Bock[1], Guy Claireaux[3,4], Louise Cominassi[2,5], Marta Moyano[2,6], Myron Peck[2,7], José-Luis Zambonino-Infante[4], Felix C. Mark[1]

[1] Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Integrative Ecophysiology, 27570 Bremerhaven, Bremen, Germany

[2] Institute of Marine Ecosystem and Fisheries Science, Center for Earth System Research and Sustainability (CEN), University of Hamburg, 20146 Hamburg, Hamburg, Germany

[3] Université de Bretagne Occidentale, LEMAR (UMR 6539), 29280 Plouzané, Bretagne, France

[4] Ifremer, LEMAR (UMR 6539), Laboratory of Adaptation and Nutrition of Fish, Centre Ifremer de Bretagne, 29280 Plouzané, Bretagne, France

[5] Institute of Arctic Biology, University of Alaska, Fairbanks, PO Box 757000, Fairbanks, AK 99775, USA

[6] Center for Coastal Research, University of Agder, Postbox 422, 4604 Kristiansand, Norway

[7] Coastal Systems (COS), Royal Netherlands Institute for Sea Research (NIOZ), 1797 SZ 't AQ4 Horntje, Texel, The Netherlands

*Corresponding author

Email: sarahhowald@gmx.de

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4.1 List of abbreviations

Δ1000 – Acidification condition (ambient PCO_2 + 1000 µatm)

A – Ambient PCO_2 condition

AA – Amino acids

AAM – Intermediates of amino acid metabolism and amino acid derivates

BL – Body length

C – Cold life-conditioned group

CC – Intermediates of the citrate cycle

CH - Carbohydrates

dph – Days post hatch

EC – Energy compounds

HRMAS – high-resolution magic angle spinning

^{1}H-NMR – proton nuclear magnetic resonance spectroscopy

IPCC – Intergovernmental Panel on Climate Change

MS-222 – Tricaine methane sulfonate

OA – Ocean acidification

OAW – Ocean acidification and warming

OOA – Osmolytes and organic acids

OW – Ocean warming

PCA – Principle components analysis

PCO_2 – Partial pressure of CO_2

PLS-DA – Partial-least squares discriminant Analysis

SP – Intermediates of different sugar pathways

VIP – Variable importance in projection

W – Warm life-conditioned group

WM – Wet mass

4.2 Introduction

Metabolomics have been shown to be a valuable tool in assessing the effects of environmental stress in a variety of organisms, including marine fish (as reviewed in Lankadurai et al., 2013). The benefit of metabolomics studies is that changes are detected rapidly and quantitatively, additionally they are more sensitive than other 'omics' technologies, as changes in gene expression and protein production are amplified at the level of the metabolome. Therefore, they might indicate potential

ecosystem shifts earlier than other methods (Lankadurai et al., 2013). The ongoing climate change is leading to ocean acidification (OA) and warming (OW, together OAW) with possible implications for ectothermic organisms, such as fish. As fish are an important source of nutrition for humans all over the world, it is important to predict how climate change will affect fish stocks and aquaculture of economically important species. Metabolomics can be used to identify implications of fish caused by climate change.

The effects of OW, OA and OAW on fish are species and trait specific: In most species increasing temperature can improve growth performance and metabolism within their thermal window (e.g. Chauton et al., 2015; Baumann, 2019). OA often decreases (inland silverside juveniles, Baumann et al., 2012) or does not affect growth of juvenile fish (Atlantic halibut juveniles, Gräns et al., 2014). In combination, most effects of OAW are synergistic, e.g. OAW increased growth and survival, but decreased aerobic scope (European sea bass juveniles, Pope et al., 2014).

The capacity of the cardiovascular system to supply the tissues with oxygen seems to be the key process in determining the thermal sensitivity of a fish (Pörtner & Lannig, 2009). Consequently, the heart plays a key role in shaping an organism's tolerance to increased temperatures, especially under acute warming. The other important organ in terms of oxygen supply to the tissues is the gill. In addition to its function as the major organ for respiratory gas exchange, the gill is important in maintaining acid-base and osmotic balance and is the main organ for ammonia excretion (as reviewed in Mommsen, 1984). Due to its significant role in acid-base regulation the gill is an important organ in OA research. To maintain its functions the gill used a significant amount of the consumed oxygen, which can add up to about 7% of the total oxygen consumption of the fish (Mommsen, 1984). In addition to these two organs, responsible for oxygen supply to the organism, we decided to investigate the metabolic profiles of liver and white muscle after acclimation to OAW. The liver has several important functions, including the control of the metabolism, bile secretion and glycogen storage, which makes the liver an important hub for maintaining general homeostasis especially under environmental challenges.

Although the effects of OA, OW and OAW on fish have been investigated intensively during the last decades, studies on large economically important fish from temperate regions are still rare and usually concentrate on the whole organisms or on single tissues. The objectives of this study were: (1) to evaluate the metabolic profiles of liver, heart, white muscle and gill tissue of juvenile European sea bass and the differences between this tissues, (2) to examine the differences due to OA, OW, OAW in the metabolic profiles of these tissues at two time points and (3) to investigate the relationship between growth performance and changes in metabolic profiles at different OA, OW and

OAW scenarios. Therefore, we incubated larvae of European sea bass *Dicentrarchus labrax* from 2 dph until around 11 months of age under four OAW conditions, reflecting the predictions of the IPCC until 2100. The larvae were obtained from a wild broodstock of an aquaculture facility, to better reflect the capacities of wild sea bass. European sea bass is an economically important species, which is extensively used in aquaculture as well as in industrial and recreational fishing throughout its distribution range in the Mediterranean, the Black Sea and the North-eastern Atlantic from Norway to Senegal (Bjørndal & Guillen, 2018). European sea bass are highly tolerant to temperature and salinity changes (Dalla Via et al., 1998; Claireaux & Lagardère, 1999) and juveniles seem to possess some tolerance to OA, but OAW can lead to antagonistic reactions (Howald et al., 2022; 2019; Cominassi et al., 2020; 2019; Crespel et al., 2019). We obtained ^{1}H-NMR metabolic profiles from heart, liver, gill and white muscle tissues of juvenile sea bass. In addition growth was measured to determine whether changes in metabolic profiles due to OAW will translate into different growth performances. We hypothesize that (1) OW will increase growth rates in sea bass juveniles, which might be visible in increased protein synthesis in muscle and liver tissue, (2) we will not see severely reduced growth due to OA alone, but OA will lead to alterations in metabolites involved in maintaining homeostasis, such as organic acids and osmolytes and (3) the combination of OW and OA might lead to synergistic effects on later stages which will be visible in the metabolic profiles.

4.3 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).

All chemicals were purchased from Sigma-Aldrich, Germany, except for Tricaine methane sulphonate (MS-222), which was purchased from Pharma Q.

4.3.1 Animals and experimental conditions

Sea bass origin and larval rearing was described in detail in Howald et al. (2019), briefly: Larvae of a wild brood stock (origin: sea off Morbihan, France) were obtained from the aquaculture facility Aquastream (Ploemeur-Lorient, France) at 2 dph. Sea bass were reared from 3 dph onwards in six OAW conditions, following the predictions of the Intergovernmental Panel on Climate Change (IPCC, 2021) for the next 130 years: today's ambient situation in coastal waters of Brittany and the Bay of

Brest (A, approx. 650 µatm (cf. Pope et al., 2014; Duteil et al., 2016)) and a scenario according to SSP5-8.5, projecting a ΔPCO_2 of 1000 µatm (Δ1000, approx. 1700 µatm). Additionally, two different temperatures were applied on each OA condition to create a cold (C) and a warm (W) life condition scenario or four OAW conditions (C-A, C-Δ1000, W-A and W-Δ1000), respectively. During larval rearing, water temperatures were fixed to 15 and 20 °C for C and W larvae. During juvenile rearing water temperatures of C juveniles were adjusted to ambient temperature in the Bay of Brest during summer (up to 18 °C), but were kept constant at 15 °C, when ambient temperature decreased below these values. The W life condition was always 5 °C warmer than the C life condition. As larvae and post-larval juveniles would display different growth rates at the two different thermal scenarios, we adopted the concept of degree days (dph · T(°C)) as basis for comparison between these life conditions.

4.3.1.1 Juvenile rearing

Juvenile rearing was also described in detail in Howald et al. (2019), briefly: juveniles were transferred from larval to juvenile rearing at 50 dph and 65 dph for W and C life-conditioned fish, respectively. All juveniles from one condition were pooled in one round tank (0.67 m^3 volume and 0.65 m depth) and the juveniles with developed swim bladders were separated into duplicate tanks at 78 dph and 86 dph for W and C life-conditioned fish, respectively. The juveniles were fed daily with commercial fish food (Neo Start) by automatic feeders during day time, which was adjusted in size (1-3) and amount during the juvenile rearing time, as recommended by the supplier (Le Gouessant, Lamballe, France). Photoperiod was adjusted to natural conditions once a week, with slowly increasing light intensities in the juvenile rearing facilities during the first hour each morning. Water flow within the tanks was adjusted once a week, so that oxygen saturation levels were not below 90%, with having equal flow through rates in all tanks of one temperature.

4.3.1.2 Experimental conditions

Water preparation was described in detail in Howald et al. (2019), briefly water was pumped from the Bay of Brest into the aquaria, passing through a sand filter (~500 µm), a tungsten heater (Plate Heat Exchanger, Vicarb, Sweden), a degassing column, a 2 µm membrane filter and UV sterilization (PZ50, 75W, Ocene, France). During juvenile rearing PCO_2 conditions were regulated within a PVC column (one column for both replicate tanks): water arrived at the top of the column and left via the tank at the bottom. The CO_2-bubbler inside the column was adjusted by a flow control unit. Temperature and pH were checked each morning with a handheld WTW 3110 pH meter (Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW Sentix 41, NIST scale, calibrated daily) before feeding the fish. Total alkalinity was measured once a week following the protocol of

Anderson & Robinson (1946) and Strickland & Parsons (1972), as described in Howald et al. (2019) and sea water chemistry was calculated with the Microsoft Excel macro CO2sys (Lewis & Wallace, 1998) and the constants after Mehrbach et al. (1973, as cited in CO2sys) refit by Dickson and Millero (1987, as cited in CO2sys). 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. Mean water parameters during juvenile phase are displayed in Table 1. A full table of all water parameters during larval and juvenile rearing is available at PANGAEA (Howald et al., 2020).

4.3.2 Growth

Body length (BL) and wet mass (WM) were measured approx. every 3 – 4 weeks. Early juveniles were starved for one day prior to growth samplings. Later on two days of starving were put into practice, to make sure that digestive tracts were empty. Juveniles were caught from their tank and anaesthetized with MS-222 (Pharma Q). Concentration of anesthetic was adjusted to reach a loss of equilibrium within less than 5 minutes, typically 0.2 g l^{-1}. WM and BL were directly determined with a precision balance (Sartorius MC1 AC210P) and callipers. If tissue samplings were done on the same day, dissections were carried out subsequently, see below. For all sampling, only the morning hours were used, to avoid diurnal artefacts in data. If tissue samplings were conducted, this meant that three tanks were done on one day and the other three tanks the following day.

4.3.2.1 Data handling

Mean specific growth rates (SGR [% day^{-1}]) of each tank were calculated after Sutcliffe (1970) with the following formula:

$$SGR = 100 \cdot (e^{g} - 1)$$

The instantaneous growth coefficient (g) was calculated as followed:

$$g = \frac{\ln S_1 - \ln S_0}{\Delta t}$$

With: S_0 and S_1 – initial and final size (BL or WM) and Δt – time between the two measurements [days]. Initial and final sizes were calculated for three quantiles (0.05, 0.5 and 0.95) for each tank (ecdf function in R).

Q_{10} was calculated with the following formula:

$$Q_{10} = \left(\frac{SGR_W}{SGR_C}\right)^{\left(\frac{10}{T_W - T_C}\right)}$$

With: SGR – specific growth rate, T – temperature, W and C as subscripts for W and C life condition.

4.3.3 Metabolic profiling

4.3.3.1 Tissue sampling

Dissections for metabolic profiling of different tissues took place during at 2900 and 3350 dd (09. and 10.06.2016 and 30.06. and 01.07.2016 for W and 28. and 29.07.2016 and 18. and 19.08.2016 for C life-conditioned fish, respectively). After measuring and weighing the fish, heart, liver and gills were dissected completely, followed by dissection of a piece of white muscle (approx. 1x1.5 cm). The whole gill apparatus was dissected and cleaned in fresh ice-cold phosphate buffered saline afterwards. The tissues of four fish were pooled from the warm life conditions to have sufficient (heart) tissue for the metabolite extraction and six fish per pool for the cold life conditions. Dissections, as well as storage of tissues until pool was complete, were done on ice. The tissue was weighed by weighing empty and loaded tubes. Afterward the tissue was frozen in liquid nitrogen and stored and transported at -80 °C. The time from catch of the fish to dissection was about 5 to 12 minutes and about 3-8 min from dissection to freezing of tissue.

4.3.3.2 NMR Sample preparation

Extracts of polar metabolites were gained from methanol-chloroform-water-extraction: Frozen tissue samples were powdered with a mortar and pestle under liquid nitrogen. The pre-weighed (~70 mg of heart tissue and ~140 mg of gill, liver and muscle tissue) frozen tissue samples were added to homogenization tubes containing ceramic beads, 400 µl ice-cold methanol and 125 µl ice-cold Milli-Q water. To avoid thawing, groups of 12 samples were prepared and stored on ice until they were mixed for one cycle of 20 s at 6000 rpm in a precooled Precellys tissue homogenator (0-4 °C, Bertin Instruments, France). After homogenization, 400 µl ice-cold chloroform and 400 µl ice-cold Milli-Q water were added and the tube was mixed thoroughly. Samples were left on ice for 10 min followed by centrifugation for 10 min at 3000 g at 4 °C. The upper methanol layer, containing the polar metabolites, was carefully transferred into a new 1.5 ml Eppendorf cup and dried by vacuum centrifugation (RVC 2–18 HCl, Christ GmbH, Germany) at room temperature. The pellet was stored at -20 °C until assessment of metabolic profiles.

The polar metabolite pellet was resuspended in deuterated water (D_2O) containing 0.05 wt% 3-(trimethylsilyl)propionic-2,2,3,3,d_4 acid (TSP) as a marker. D_2O provides a deuterium lock for the NMR

spectrometer, while the TSP acts as an internal standard and as a chemical shift reference (delta = 0.0ppm). The volume of D_2O was adjusted according to tissue weight: 50 µg heart tissue and 100 µg liver, gill and muscle tissue were resuspended in 100 µl D_2O. After resuspension, 70 µl were transferred to a standard zirconium rotor for high-resolution magic angle spinning proton nuclear magnetic resonance spectroscopy (HRMAS ^{1}H-NMR).

4.3.3.3 NMR spectroscopy acquisition and processing

Untargeted NMR spectra were measured in an ultra-shielded vertical 9.4 T NMR spectrometer (Avance III HD 400 WB, Bruker-BioSpin GmbH, Germany) at a proton frequency of 400 MHz at 10 °C controlled with the complementary software TOPSPIN 3.2. All samples were analysed using one dimensional HRMAS ^{1}H-NMR spectroscopy with a 1D Carr-Purcell-Meiboom-Gill (CPMG) pulse train including f1 pre-saturation (Bruker protocol cpmgpr1d) with a spectral width of 8803 Hz and 70k data points. 32 scans were acquired for each spectrum.

4.3.3.4 NMR data analysis

Metabolites were qualitatively and quantitatively assigned with the software Chenomx NMR Suite (version 8.0, Chenomx Inc., Canada). A line broadening of 0.5-1 Hz was applied prior to Fourier transformation. All spectra were then calibrated and shim corrected for phase and baseline referenced to the internal TSP standard, followed by operator-controlled phase adjustments and baseline corrections within Chenomx NMR Suite. Spectra were transferred to the profiler within Chenomx NMR Suite, where metabolites were identified using the incorporated data base of Chenomx NMR Suite, as well as an in-house database. Metabolite concentration was quantified based on the concentration of the internal standard TSP by manually fitting NMR peak integrals to specific metabolites. Prior to further analysis concentrations were normalized on tissue weight.

4.3.4 Statistical analysis

Statistics were performed with R (growth data, R Core Team, 2020) and MetaboAnalyst 5.0 (metabolite data, see further Pang et al., 2021; Xia et al., 2009). Significance for all statistical tests was set at $p < 0.05$.

4.3.4.1 Growth data

All data were tested for outliers (Nalimov test), normality (Shapiro-Wilk's test) and homogeneity (Levene's test). The datasets did not meet the assumptions for ANOVA, therefore all data were fitted to linear mixed effects models (LME models, ''lme'' function of the ''nlme'' package, package Pinheiro et al., 2017). Temperature, PCO_2 concentration, age and their interactions were included as fixed

effects in the model for BL and log-transformed WM over time. BL and WM at 2900 and 3350 dd were analyzed with temperature, PCO_2 concentration and their interactions as fixed effects. Rearing tank was included as a random effect in all models. In case of heterogeneity of data, variance structures were included in the random part of the model. The best variance structure was chosen according to lowest Akaike information criteria (AIC) values. After fitting fixed and random effects, a backwards model selection process was applied to determine the significant and fixed variables and interactions. If significant effects were detected in the linear mixed effect models, posthoc Tukey tests were performed with the "lsmeans" function ("lsmeans" package, Lenth, 2016). All graphs are produced from the lsmeans-data with the "ggplot2" package (Wickham, 2016). All data are shown as lsmeans ± s.e.m. (standard error of the mean).

4.3.4.2 Metabolic profiles

The statistical analysis of the metabolic profiles of juvenile European seabass was done with MetaboAnalyst version 5.0 (Pang et al., 2021). The different tissues were analyzed individually. Normalization was done with log-transformation and pareto-scaling within MetaboAnalyst. Outliers were identified by visual analysis of heatmaps and verified with principle components analysis (PCA) and subsequently removed from the dataset. The cleaned dataset without the outliers was used for all further analyses. The variable importance in projection (VIP Score) of the partial-least squares discriminant Analysis (PLS-DA) was used to determine significant metabolites as well as differences between groups. Since the number of variables was less than 100, we applied a VIP scores cutoff of 1.0. Metabolites which were significant in the VIP scores were cross checked with significance analysis of microarray (SAM) and the ANOVA function was used to determine significant differences between treatment groups in these metabolites.

4.4 Results

4.4.1 Growth

Juvenile sea bass had higher growth rates in the warm life condition than in the cold life condition: SGR ranged from 2.74 to 3.05 and 2.40 to 2.50 % day^{-1} for juvenile WM and 0.79 to 0.88 and 0.62 to 0.68 % day^{-1} for juvenile BL in W and C life-conditioned fish, respectively, (Table S 1). The higher growth rates in W juveniles resulted in Q_{10} of 1.30 to 1.66 and 1.48 to 2.25 for juvenile WM and BL (Table S 1). Consequently, C juveniles were significantly smaller than W juveniles at 2900 (Figure 4C and D, Table S 2) and also when compared at the same age (in dph, Figure 4A and B, Table S 2).

Significance of OA, OW and OAW on growth was tested with LME, F- and p-values are summarized in Table S 2.

4.4.2 Metabolic profiling

Typical ^{1}H-NMR spectra of aqueous extracts of liver, white muscle, heart and gill tissue of European sea bass juveniles are presented in Figure 5, peaks are labelled with the corresponding metabolite. We identified 63 unique metabolites in the ^{1}H-NMR spectra, with 46, 35, 33 and 33 metabolites present in liver, white muscle, heart and gill tissue samples, respectively (Table 2). The identified metabolites were classified in amino acids (AA), metabolites involved in amino acid metabolism and amino acid derivates (AAM), organic acids and osmolytes (OOA), carbohydrates (CH), energy compounds (EC), as well as intermediates of different sugar pathways (SP) and of the citrate cycle (CC). Some metabolites did not fit in these groups and were groups together (Other). All identified metabolites occurred in all samples of the respective tissue, regardless of treatment group. Eighteen metabolites were present in all tissue samples, including AAs, OOAs, ECs and succinate (CC) and malonate (Other). AA present in all spectra included alanine, glutamine, glycine, leucine and valine. OOA present in all spectra were acetate, betaine, choline, dimethylamine (DMA), lactate, O-phosphocholine, sn-glycero-3-phosphocholine, taurine and trimethylamine-N-oxide (TMAO). EC present in spectra of all tissues were AMP and creatine. The most prominent metabolite in spectra of all tissues was the OOA taurine, with two large triplet peaks at 3.3 and 3.4 ppm. The chemical shifts of all other metabolites are listed in Table 2. In general the spectra of liver tissues contained a higher variability of CHs and SPs than the spectra of other tissues. The spectra of muscle tissue contained a higher variability of ECs. There was no metabolite group with higher variety in heart or gill tissue in comparison to other tissues.

4.4.2.1 Metabolite profiles of liver tissue

In addition to the dominating signals of taurine, the spectra of liver tissue (Figure 5A, E and I) were dominated by signals of AAs and AAMs as well as CHs and SPs. Dominating AAs and AAMs included metabolites such as alanine, glutamate, glutamine, glycine, glycylproline, guanidoacetate, leucine, threonine and valine. Signals of CHs and SPs included 2-phosphoglycerate, fructose, glucose, glucose-1-phosphate, glucose-6-phosphate, glucarate and ribose.

Metabolites only present in liver tissue samples, but not in samples of the other tissues were citrate, fructose, glucarate, gluconate, glucose-1-phosphate, glucoronate, GTP, hydroxyacetone, isocitrate, N,N-dimethylglycine, O-acetylcarnitine, pyruvate, S-adenosylhomocysteine, threonine and xanthine.

4.4.2.2 Metabolite profiles of white muscle tissue

The spectra of white muscle tissue were dominated by AAs, OOAs and ECs. Dominating AAs in white muscle tissue spectra were alanine, glutamine, glycine, leucine, lysine and valine. In addition to the dominating OOA taurine, lactate and TMAO were also dominating in spectra of white muscle tissue. All ECs found in muscle tissue spectra were dominating the region of their chemical shift, namely ADP, AMP, ATP, creatine and creatine phosphate. Metabolites only present in white muscle tissue samples, but not in samples of the other tissues were ADP, ATP, proline, xanthine and π-methylhistidine.

4.4.2.3 Metabolite profiles of heart tissue

Comparable to the spectra of white muscle tissue the spectra of heart tissue were dominated by AAs, OOAs and ECs. The dominating AAs in heart tissue spectra were alanine, glutamate, glycine, leucine and valine. Similar to white muscle tissue, the OOAs taurine, lactate and TMAO dominated the spectra of heart tissue. In contrast to muscle tissue, we found only three ECs in heart tissue spectra (AMP, creatine and creatine phosphate), but all of them dominated the region of their chemical shift. No metabolites were only present in the spectra of heart tissue, but anserine, creatinine, ethanolamine and O-phosphoserine were only present in heart and white muscle tissue.

4.4.2.4 Metabolte profiles of gill tissue

The spectra of gill tissue were dominated bei AAs, ECs and OOAs. All AAs present in gill tissue samples were dominating the region of their chemical shift, namely alanine, aspartate, glutamate, glutamine, glycine, histidine, leucine, lysine, serine and valine. In comparison to white muscle and heart tissue spectra, the ECs creatine and creatine phosphate were dominating the spectra of gill tissue. In addition to lactate, taurine and TMAO, which were dominating metabolites in other tissues, the OOAs ascorbate and dimethylamine were dominating the spectra of gill tissue.

Metabolites only present in gill tissue samples, but not in samples of the other tissues were aspartate, hypoxanthine, inosine, malate, O-acetylcholine and serine.

4.4.2.5 Effects of OW, OA and OAW on metabolic profiles

PLS-DAs were visually inspected and revealed a clear differentiation between the C life condition and the W life condition in all tissues (Figure 6A-D), although the difference between the W-A condition and the C life conditions appeared to be less pronounced in heart tissue. The effects of OA on the metabolic profiles of the different tissues were not as strong and uniform as the effects of OW: A and Δ-1000 conditions were not significantly different in heart and gill tissue, neither in C nor in W life

condition. In liver and white muscle tissue the A and Δ-1000 conditions appeared to be different, especially in W life condition. Significant metabolites discriminating the treatments were ranked on the basis of the variable importance in projection (VIP, Figure 7E-F) score with a cut off of 1.0 and verified with SAM.

In liver tissue, the difference between the C and the W life condition was due to higher concentrations of alanine (AA), O-phosphocholine (OOA) and glucose (CH), as well as lower concentrations of glutamate (AA), carnitine (AAM, only in A treatment), N,N-dimethylglycine (AAM), S-adenosylhomocysteine (AAM, only in Δ1000 treatment), choline (OOA, only in A treatment), myo-inositol (OOA, only in Δ1000 treatment), citrate (CC), succinate (CC, only in Δ1000 treatment) and O-acetylcarnitine (Other, only in A treatment) in C compared to W life condition, respectively. The difference between liver tissues of fish from A and Δ1000 treatments was due to higher concentrations of choline (OOA, only in W life condition), threonate (OOA, only in C life condition) and glucose-1-phosphate (CH, only in W life condition) and lower concentrations of glutamate (AA, only in C life condition, myo-inositol (OOA, only in W life condition), succinate (CC, only in W life condition) and O-acetylcarnitine (Other, only in C life condition) in A compared to Δ1000 treatments, respectively.

Pyruvate (SP), gluconate (SP), myo-inositol (OOA) and glycine (AA) were significant in the PLSDA and the SAM, but not in the ANOVA. Considering the PLSDA, they were all higher in C than in W life-conditioned fish with no effect of OA.

In white muscle tissue, the difference between the C and the W life condition was due to higher concentrations of O-phosphoserine (AAM), TMAO (OOA), DMA (AAM), taurine (OOA) and glucose (CH) and lower concentrations of glycine (AA), choline (OOA), sn-glycero-3-phosphate (OOA, only in A treatment) and xanthine (Other) in C in comparison to W life condition, respectively. The difference between white muscle tissue of fish from A and Δ1000 treatment was due to higher concentrations of lysine (AA, only in W life condition) and DMA (OOA, only in W life condition) and lower concentrations of glycine (AA, only in C life condition) and xanthine (Other, only in W life condition) in A compared to Δ1000 treatments, respectively. Betaine (OOA), π-Methylhistidine (AAM) and ADP (EC), all being higher in W-Δ1000 than in other treatments, were significant in PLSDA and SAM but not in ANOVA.

Only few metabolites contributed with significant differences to the differentiation between the treatments in heart tissue: the concentrations of glutamate (AA), glycine (AA), O-phosphocholine (OOA) and taurine (OOA) were lower in heart tissue of fish from W compared to heart tissue of fish from C life condition. The concentration of glutamate (AA) was also lower in heart tissue of fish from

C-A compared to heart tissue of fish from Δ1000 treatment. Guanidoacetate (AAM), dimethylamine (OOA), fumarate (CC) and malonate (Other) were significant in PLSDA and SAM but not in ANOVA. There was no obvious pattern in these metabolites between treatments: guanidoacetate seemed to be higher in C-A, while dimethylamine seemed to be lower in C-A compared to other treatments. Fumarate seemed to be lower in W-Δ1000, while malonate seemed to be higher in W-Δ1000 compared to other tissues.

The concentrations of alanine (AA) and TMAO (OOA) were significantly higher in gill tissue of C compared to gill tissue of W life-conditioned fish and the concentration of sn-glycero-3-phosphate was significantly lower in gill tissue of C compared to gill tissue of W life-conditioned fish. Additionally, in the PLSDA and SAM analyses, acetate (OOA, only in A treatment) and ascorbate (OOA) were higher in gill tissue of W compared to gill tissue of C life-conditioned fish, while O-phosphocholine (OOA) was lower in gill tissue of W compared to gill tissue of C life-conditioned fish. Ascorbate (OOA) and inosine (EC) were higher in gill tissue of Δ1000 fish compared to gill tissue of A fish. AMP (EC) was lower in gill tissue of Δ1000 fish compared to gill tissue of A fish.

4.5 Discussion

The aim of this study was to evaluate growth performance and metabolic profiles of gill, heart, liver and white muscle tissue of juvenile European sea bass in the light of OA, OW and OAW. This study focuses on major patterns in the metabolic profiles and cannot explain all discovered differences in detail because of the complexity of the total metabolic profiles. It is to our knowledge the first study to analyse metabolic profiles of four tissues of the same pool of fish within one experiment.

4.5.1 Comparison of metabolic profiles of different tissues of European sea bass juveniles with present literature

We grouped the identified metabolites into different classes to facilitate the detection of differences between tissues as well as between OAW treatments. We could identify twelve amino acids (AA) and eleven metabolites involved in amino acid metabolism or amino acid derivates (AAM), thirteen osmolytes and organic acids (OOA), five carbohydrates (CH), as well as five intermediates of different sugar pathways (SP), seven energy compounds (EC), five intermediates of the citrate cycle (CC) and five miscellaneous metabolites, which did not fit in these groups (Other). However, we did not find all metabolites in each tissue.

4.5.1.1 Amino acids

The AAs alanine, glutamine, glycine, leucine and valine were present in all tissue extracts and are generally found in ^{1}H-NMR spectra of marine fish liver (Bankefors et al., 2011; Palma et al., 2019), white muscle (Mannina et al., 2008; Bankefors et al., 2011; Fuentes et al., 2010), heart (Li et al., 2014, except for glycine) and gill (Rebelein et al., 2018; Capello et al., 2016), respectively. The same holds for glutamate, lysine and threonine in liver tissue (Bankefors et al., 2011; Palma et al., 2019), histidine, lysine and proline in white muscle (Mannina et al., 2008; Bankefors et al., 2011; Fuentes et al., 2010), and glutamate and histidine, in heart tissue (Li et al., 2015). Gill tissue contained the highest number of AAs, adding aspartate, glutamate, lysine and serine to the AAs present in all tissues, which is in line with current literature (Rebelein et al., 2018; Capello et al., 2016).

4.5.1.2 Osmolytes and organic acids

Most of the identified OOAs, namely acetate, betaine, choline, dimethylamine, lactate, O-phosphocholine, sn-glycero-3-phosphocholine, taurine and TMAO, were present in all tissue extracts and are generally found in ^{1}H-NMR spectra of marine fish liver (Bankefors et al., 2011; Palma et al., 2019; Li et al., 2015; Teng et al., 2013; Abro et al., 2014), white muscle (Mannina et al., 2008; Bankefors et al., 2011; Melis & Anedda, 2014, except for dimethylamine, O-phosphocholine and sn-glycero-3-phosphocholine), heart (Li et al., 2014, only choline, lactate, O-phosphocholine, taurine and TMAO) and gill (Rebelein et al., 2018; Capello et al., 2016), respectively. The same holds for myo-inositol in both liver and gill tissue (Teng et al., 2013; Rebelein et al., 2018).

4.5.1.3 Carbohydrates

No CH or SP was present in all tissues. Glucose was present in liver and white muscle extracts and is generally found in ^{1}H-NMR spectra of these tissues (Palma et al., 2019; Bankefors et al., 2011; Mannina et al., 2008).

4.5.1.4 Energy compounds

Only the ECs AMP and creatine were present in all tissue extracts and are generally found in ^{1}H-NMR spectra of marine fish liver (Bankefors et al., 2011; Palma et al., 2019), white muscle (Mannina et al., 2008; Bankefors et al., 2011), heart (Li et al., 2014, except for glycine) and gill (Rebelein et al., 2018; Capello et al., 2016), respectively. The same holds for GTP in liver tissue, creatine phosphate in heart and gill tissue and inosine in gill tissue (Teng et al., 2013; Li et al., 2014; Rebelein et al., 2018; Capello et al., 2016) and ADP, ATP and creatine phosphate in white muscle tissue (Melis & Anedda, 2014; Mannina et al., 2008).

4.5.1.5 Intermediates of the citrate cycle

Succinate is the only CC metabolite that was present in all tissues extracts and is generally found in ^{1}H-NMR spectra of marine fish liver (Bankefors et al., 2011; Palma et al., 2019), white muscle (Mannina et al., 2008; Bankefors et al., 2011) and gill tissue (Rebelein et al., 2018), respectively. The same holds for citrate and fumarate in liver tissue (Li et al., 2015; Palma et al., 2019) and fumarate in white muscle tissue (Mannina et al., 2008)

4.5.1.6 Miscellaneous metabolites

Malonate was present in all tissues and is generally found in studies on liver and gill tissue (Rebelein et al., 2018; Capello et al., 2016; Li et al., 2015), as well as O-acetylcarnitine in liver tissue (Ong et al., 2009). The absence of hypoxanthine, inosine and xanthine is a good indicator for tissue freshness (Reale et al., 2008). Nevertheless, those metabolites were found in low concentrations in spectra of gill (hypoxanthine and inosine) and white muscle (xanthine) and are generally found in ^{1}H-NMR spectra of freshly sampled gill and white muscle tissue (Rebelein et al., 2018; Capello et al., 2016; Mannina et al., 2008). Tissue freshness is further verified by the absence of trimethylamine (TMA) in all tissues. Processes in dead tissues of fish include the oxidation of TMAO to TMA (Savorani et al., 2010). Low or non-detectable quantities of hypoxanthine, inosine, xanthine and TMA indicate that the metabolic profiles gained from tissue extracts in this study reflect the processes in the living organism.

4.5.2 Comparison of tissues

Depending on their function, the tissues in this study contained different metabolic profiles. The liver has several important functions, including the bile secretion and glycogen storage. The liver is also important for protein synthesis. Another important function of liver tissue is the storage and detoxification of toxicants. The liver is the major storage site for lipids in fish. The white muscle tissue is used for mostly anaerobic locomotion, for example, during foraging and escape. The heart is an important part of the cardiovascular system, which is responsible for the supply of the tissues with oxygen and nutrients, while simultaneously transporting carbon dioxide and waste products away. The gill's main function is the respiratory gas exchange, but it is also important in maintaining acid-base and osmotic balance as well as excretion of nitrogenous waste.

Fitting its function in protein synthesis the liver profile was dominated by AAs and AAMs as well as CHs and SPs. AAs are the building blocks for proteins and their dominating presence in the liver profile was expectable considering the importance of protein synthesis in liver tissue. The same holds

for the presence of metabolites involved in glycolysis, which is an important metabolic pathway in liver tissue to fuel protein synthesis.

TMAO concentrations were much lower in liver tissue than in white muscle, heart and gill tissue. This might be due to its physiological functions, which are amongst others: regulation of osmotic pressure, stabilization of proteins and regulation of bond formation in proteins (Sotelo & Rehbein, 2000). White muscle and heart tissue consist mainly of proteins, consequently stabilizing metabolites should be found in these tissues in higher amounts than in other tissues. The role of TMAO in osmoregulation explains its high occurrence in gill tissue, as osmoregulation is one of the main functions of this organ. Higher concentrations of TMAO in white and red muscle tissue compared to liver tissue had been found in other marine fish before (Sotelo & Rehbein, 2000 and references therein).

Glycine is a building block of most proteins. Its presence in all examined tissues is therefore not surprising. Glycine concentrations were much higher in white muscle tissue than in liver, heart and gill tissue. Glycine concentrations were also higher in white muscle tissue than in red blood cells, gill and red muscle tissue of Atlantic charr (Bystriansky et al., 2007). Taurine concentrations were higher than glycine concentrations in all tissues in this study, as well as in red blood cells, gill and red muscle tissue of Atlantic charr, but were lower in white muscle tissue of Atlantic charr (Bystriansky et al., 2007). However, although taurine concentrations were higher than glycine concentrations in white muscle tissue of European sea bass, the ratio between taurine and glycine concentrations was several magnitudes lower in white muscle than in the other tissues. Taurine is involved in osmoregulation and membrane stability, while glycine is important for collagen synthesis. Therefore the different ratios between the different tissues are not surprising. As white muscle tissue has fewer mitochondria and therefore less membrane area than the other tissues, while it contains more collagen than the other tissues, the ratio between taurine and collagen should be different.

Although the heart muscle is working continuously in contrast to white muscle tissue, which is more used for sudden movements, the ^{1}H-NMR spectra of these two tissues were relatively similar and both dominated by AAs, OOAs and ECs. High concentrations of AAs are characteristic for white muscle tissue (Carpene et al., 1998). As the fish used in this study were juvenile fish, they were still growing. As the matrix of muscle tissues, heart muscle as well as white muscle, are different proteins, protein synthesis is an important metabolic pathway to allow growth of these tissues. Due to being necessary for growth as well as generally being characteristic for muscle tissues, AAs are an important metabolite type in these tissues. The other important metabolite types in heart and white muscle tissue are ECs. The protein synthesis and movement of heart and white muscle need energy.

The metabolic profiles of those two tissues reflect their energy generation: The heart is a highly aerobic tissue, therefore energy for cellular processes is provided by aerobic pathways. In white muscle, energy is provided mostly via anaerobic processes, therefore lactate was dominating the spectra of white muscle, reflecting the importance of anaerobic metabolism in this tissue. Another metabolite, which was quite different in concentration between those two muscle tissues, was taurine, which is important for different physiological functions, such as cellular homeostasis, membrane stabilization and heart rate (Fugelli & Thoroed, 1990). The first two are important in all tissues and explain the dominating presence of taurine in all spectra. The latter is only important in heart tissue and explains that the concentration of taurine is 1.5-2 times higher in heart tissue than in liver, white muscle and gill tissue.

Similarly to heart and white muscle tissue, the ^{1}H-NMR spectra of gill tissue were dominated by AAs, OOAs and ECs, but in contrast to heart and white muscle tissue, the OOAs were the dominating metabolites in terms of concentration and variety of metabolites. This was an expectable result considering the importance of maintaining osmotic balance in fish via gill tissue.

4.5.3 Effect of ocean acidification and warming on juvenile sea bass

OW led to increased growth at age in W compared to C life-conditioned fish, even the employment of degree days (dd) did not straighten this picture: fish in the C life condition were smaller than fish in the W life condition on both sampling dates, Figure 4. The PLSDA of the metabolic profiles indicated clear differences between the fish from C life condition and fish from W life condition in all tissues (Figure 6). OA as a single driver did not lead to changes in growth performance. Increased growth under OW conditions and unaffected growth under OA conditions had been observed for juvenile European sea bass in different other studies before (e.g. Howald et al., 2022; Crespel et al., 2019) and confirmed our expectations and hypothesis 3. Notably, we could not observe reduced growth in W-Δ1000 compared to C-A fish, as it was observed in Cominassi et al. (2020). This was surprising, as the fish in this study originated from the same rearing and experiments as the fish in Cominassi et al. (2020), except that the fish in this study stayed in the big rearing tanks until sampling, while the fish in the study of Cominassi et al. (2020) were reared separately from the start of their experiment in smaller tanks (277 and 367 dph for Wild W and Wild C juveniles, respectively). They discussed the possibility that cannibalism occurred in the big rearing tanks, in which the fish of this study were reared their entire life. Cannibalism in reared sea bass juveniles was observed before (Hatziathanasiou et al., 2002) and tanks would affect the population in the tanks in at least two ways, first the small individuals will not survive and second, the surviving individuals will profit from the higher amount of nutrition, which additionally has an ideal composition, supporting the higher

growth rates at warmer temperatures. Cominassi et al. (2020) found reduced food conversion efficiency and reduced growth in sea bass juveniles under restricted food rations, in combination with possibly altered swimming ability in the warmth (Cominassi et al., 2019), decreased ability to sense prey and predator cues under OA (Porteus et al., 2018) and altered behaviour under OW (Manciocco et al., 2015), the observed high growth rates of this study might not translate into high growth rates in nature.

For further analysis of the effects of OAW on juvenile European sea bass (including the metabolic profiles), see chapter 5.5. In this chapter, I discuss how the findings of the different experiments of this thesis fit together and how they are supported by the findings in the metabolite profiles of the different tissues.

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4.7 Competing interests

The authors declare no competing or financial interests.

4.8 Author contributions

Conceptualization: SH, CB, GC, MM, MP, JLZI, FCM

Data curation: SH

Formal analysis: SH, CB, FCM

Funding acquisition: MP, GC, FCM

Investigation: SH

Methodology: SH, CB, FCM

Project administration: SH, MM, GC, MP, FCM

Resources: CB, GC, MP, FCM

Software: SH, CB

548 Supervision: CB, FCM

549 Validation: SH, CB, FCM

550 Visualization: SH

551 Writing -original draft: SH

552 Writing - review & editing: SH, CB, GC, MM, MP, JLZI, FCM

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557 4.10 Data availability

558 Datasets of growth, metabolic rates and water conditions during rearing are available online from
559 PANGAEA (www.pangaea.de).

560 4.11 References

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4.12 Figures and Tables

Table 1 Water parameters during juvenile phase of European sea bass rearing: Juvenile period from 04.03.2016 (46 dph, ~900 dd) to 24.10.2016 (280 dph, ~5900 dd) and 18.03.2016 (60 dph, ~900 dd) to 08.02.2017 (387 dph, ~6200 dd) for warm (W) and cold (C) life-conditioned fish respectively. Means ± s.e.m. over all replicate tanks per condition. Temperature (Temp.), pH (free scale), salinity, oxygen (during juvenile rearing) and total alkalinity (TA) were measured weekly; PCO_2 was calculated with CO2sys; sea water (SW) measurements were conducted in 2017 and 2018; A – Ambient PCO_2, Δ500 – ambient + 500 µatm CO_2, Δ1000 – ambient + 1000 µatm CO_2.

Treatment	pH_{free} [-]	Temp. [°C]	Salinity [psu]	O_2 [% airsat.]	TA [µmol L^{-1}]	PCO_2 [µatm]
C-A	7.97±0.01	16.0±0.2	34.2±0.1	90.9±0.5	2396±18	655±18
C-Δ1000	7.55±0.01	16.1±0.2	34.2±0.1	90.9±0.6	2399±19	1841±40
W-A	7.92±0.01	21.9±0.2	35.0±0.2	90.2±0.9	2418±12	788±22
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

716 **Table 2 Metabolites found in aqueous extracts of the different tissue samples of juvenile European sea bass grouped by**
717 **metabolite type or pathway. The column chemical shift indicates the most prominent peaks of the corresponding**
718 **metabolite, with s – single peak, d – doublet, t – triplet and m – multiplet. The tissue columns indicate whether the**
719 **metabolite was present in the respective tissue or not.**

Metabolite	Chemical shift [ppm]	Liver	White muscle	Heart	Gill
Amino acids					
Alanine	1.5 (d), 3.8 (m)	X	X	X	X
Aspartate	2.7 (m), 2.8 (m), 3.9 (m)				X
Glutamate	2.1 (m), 2.3 (m), 2.4 (m), 3.8 (m)	X		X	X
Glutamine	2.1(m), 2.2 (m), 2.4 (m), 2.5 (m), 3.8 (m)	X	X	X	X
Glycine	3.6 (s)	X	X	X	X
Histidine	7.1 (s), 7.8 (s)		X	X	X
Leucine	1.0 (2xd), 1.7 (3xm)	X	X	X	X
Lysine	1.7 (m), 1.9 (2xm), 3.0 (m)	X	X		X
Proline	2.0 (m), 2.1 (m), 3.3 (m)		X		
Serine	3.8 (t), 3.9 (m), 4.0 (m)				X
Threonine	1.3 (d), 3.6(d)	X			
Valine	1.0 (2xd)	X	X	X	X
Amino acid metabolism and derivates					
Anserine	3.8 (s)		X	X	
Carnitine	2.4 (m), 2.5 (m), 3.2 (s)	X	X	X	
Ethanolamine	3.1 (m), 3.8 (m)		X	X	
Glycylproline	2.0 (2xm), 3.9 (m)	X			X
Guanidoacetate	3.8 (s)	X	X	X	
Hydroxyacetone	2.1 (s), 4.4 (s)	X			
N,N-Dimethylglycine	2.9 (s), 3.7 (s)	X			
N-Acetylcysteine	2.1 (s), 2.9 (2xm)				X
O-Phosphoserine	3.9 (m), 4.1 (m)		X	X	
S-Adenosylhomocysteine	6.1 (d), 8.3 (s), 8.4 (s)	X			
π-Methylhistidine	3.7 (s)		X		
Organic acids and Osmolytes					
Acetate	1.9 (s)	X	X	X	X
Ascorbate	3.7 (m), 3.8 (m), 4.0 (m), 4.5 (m)	X		X	X
Betaine	3.3 (s), 3.9 (s)	X	X	X	X
Choline	3.2 (s)	X	X	X	X
Creatinine	3.0 (s), 4.0 (s)		X	X	
Dimethylamine	2.7 (s)	X	X	X	X
Lactate	1.3 (d), 4.1 (m)	X	X	X	X
myo-Inositol	3.3 (m), 3.5 (m), 3.6 (m), 4.1 (m)	X			X
O-Phosphocholine	3.2 (s)	X	X	X	X
sn-Glycero-3-phosphocholine	3.2 (s)	X	X	X	X
Taurine	3.3 (t), 3.4 (t)	X	X	X	X
Trimethylamine N-oxide	3.3 (s)	X	X	X	X
Threonate	3.6 (m), 3.7 (m), 4.0 (m), 4.0 (d)	X		X	

720

721 **Continuation Table 2**

Metabolite	Chemical shift [ppm]	Liver	White muscle	Heart	Gill
Carbohydrates					
Fructose	3.5-4.1 (14xd&t&m)	X			
Glucose	3.2-3.9 (12xt&m), 4.7 (d), 5.3 (d)	X	X		
Glucose-1-phosphate	3.4-3.9 (6xt&m), 5.4 (m)	X			
Glucose-6-phosphate	3.3-4.0 (10xt&m), 4.7 (d), 5.2 (d)	X		X	X
Ribose	3.5-4.2 (20x), 4.9 (d), 5.2 (s), 5.4 (d)	X		X	
Energy compounds					
ADP	6.2 (d), 8.2 (s), 8.5 (s)		X		
AMP	6.2 (d), 8.2 (s), 8.6 (s)	X	X	X	X
ATP	6.2 (d), 8.2 (s), 8.5 (s)		X		
Creatine	3.0 (s), 3.9 (s)	X	X	X	X
Creatine phosphate	3.0 (s), 3.9 (s)		X	X	X
GTP	5.9 (d), 8.1 (s)	X			
Inosine	6.1 (d), 8.2 (s), 8.3 (s)				X
Intermediates of different sugar pathways, e.g. Glycolysis					
2-Phosphoglycerate	3.8 (m), 3.9 (m), 4.5 (m)	X	X	X	
Glucarate	3.9 (m), 4.1 (3xm)	X			
Gluconate	3.7-4.1 (6xm)	X			
Glucuronate	3.3-3.7 (7xd&t&m), 4.1 (d), 4.7 (d), 5.2 (d)	X			
Pyruvate	2.3 (s)	X			
Intermediates of the Citrate Cycle					
Citrate	2.5 (d), 2.7 (d)	X			
Fumarate	6.5 (s)	X	X	X	
Isocitrate	2.4 (m), 2.6 (m), 3.0 (m)	X			
Malate	2.4 (m), 2.7 (m)				X
Succinate	2.4 (s)	X	X	X	X
Other					
Hypoxanthine	8.2 (2xs)				X
Malonate	3.1 (s)	X	X	X	X
O-Acetylcarnitine	3.2 (s)	X			
O-Acetylcholine	2.1 (s), 3.2 (s)				X
Xanthine	7.9 (s)		X		

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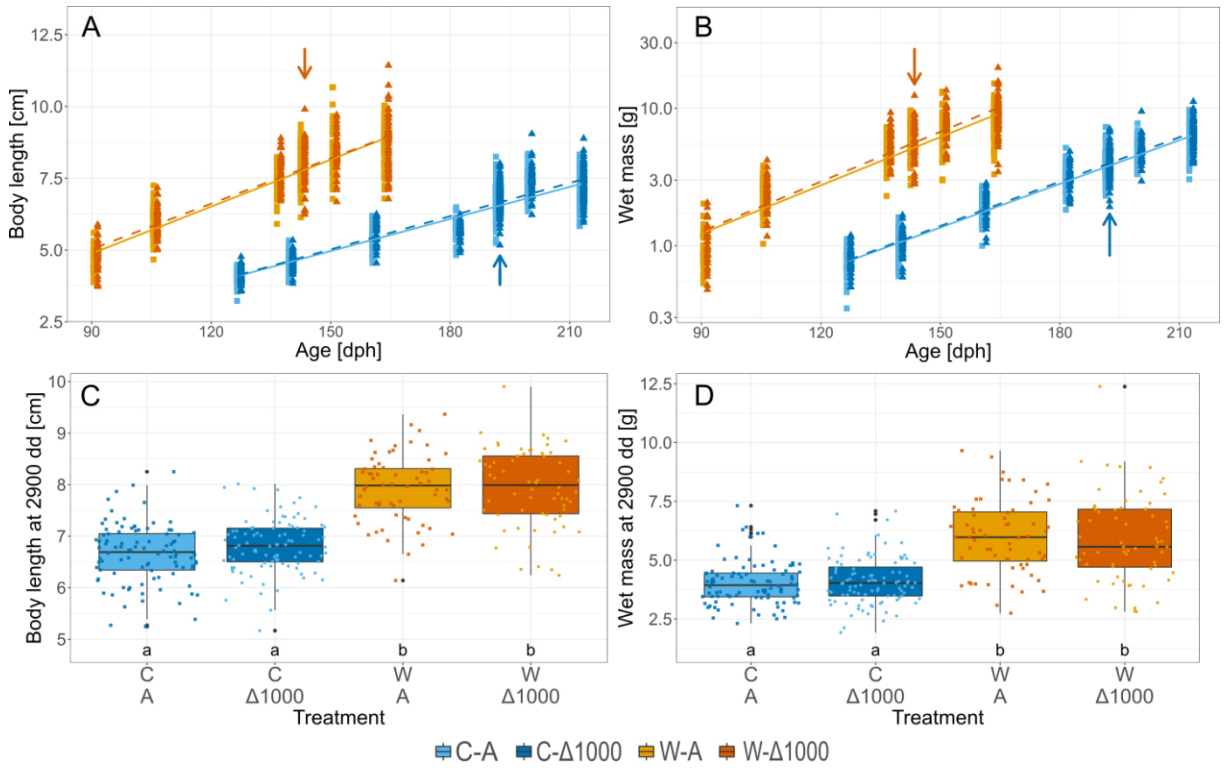


Figure 4 Growth of European sea bass juveniles. Subfigure A and B: Growth over time with linear regression lines. Shown are individual data points of body length (A) and wet mass (B). W juveniles grew significantly faster than C juveniles and no differences were observed between PCO_2 treatments [linear mixed effects model (LME), P<0.05]. All data were tested with LMEs, F- and p-Values are summarized in Table S 2. Arrows indicate the data points at 2900 dd, data for different PCO_2 conditions of the same age are slightly moved for better visibility. Subfigure C and E: Body length (C) and wet mass (D) at approx. 2900 dd. Boxplots show median, upper and lower quartiles and 1.5x interquartile range. Overlying symbols are the individual data points for each treatment, different letters indicate significant differences (LME, P<0.05). C – cold life condition (15-18 °C), W – warm life condition (C + 5 °C), A – Ambient PCO_2 (~650 µatm CO_2), Δ1000 – A + 1000 µatm CO_2, n=40-96.

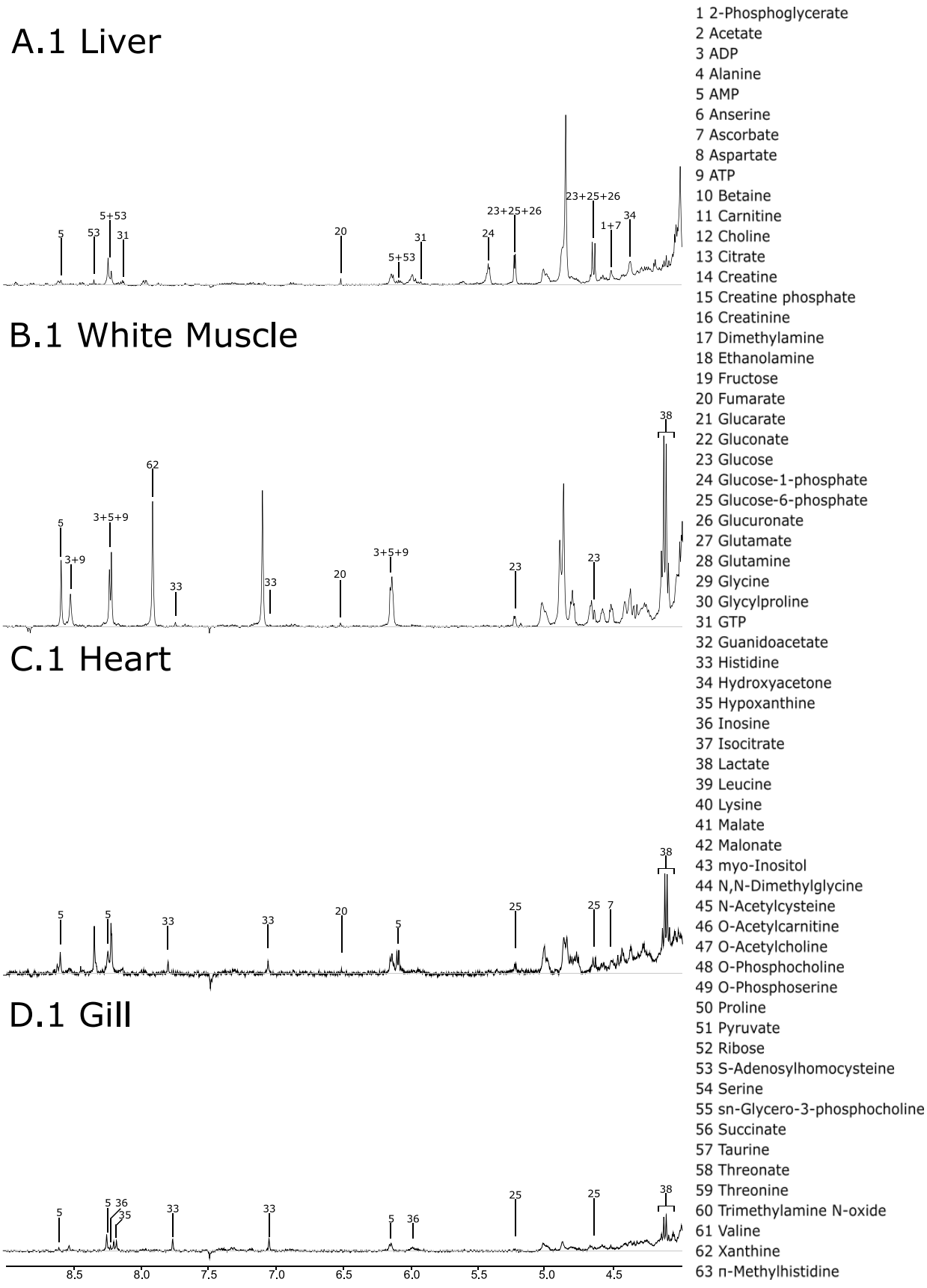
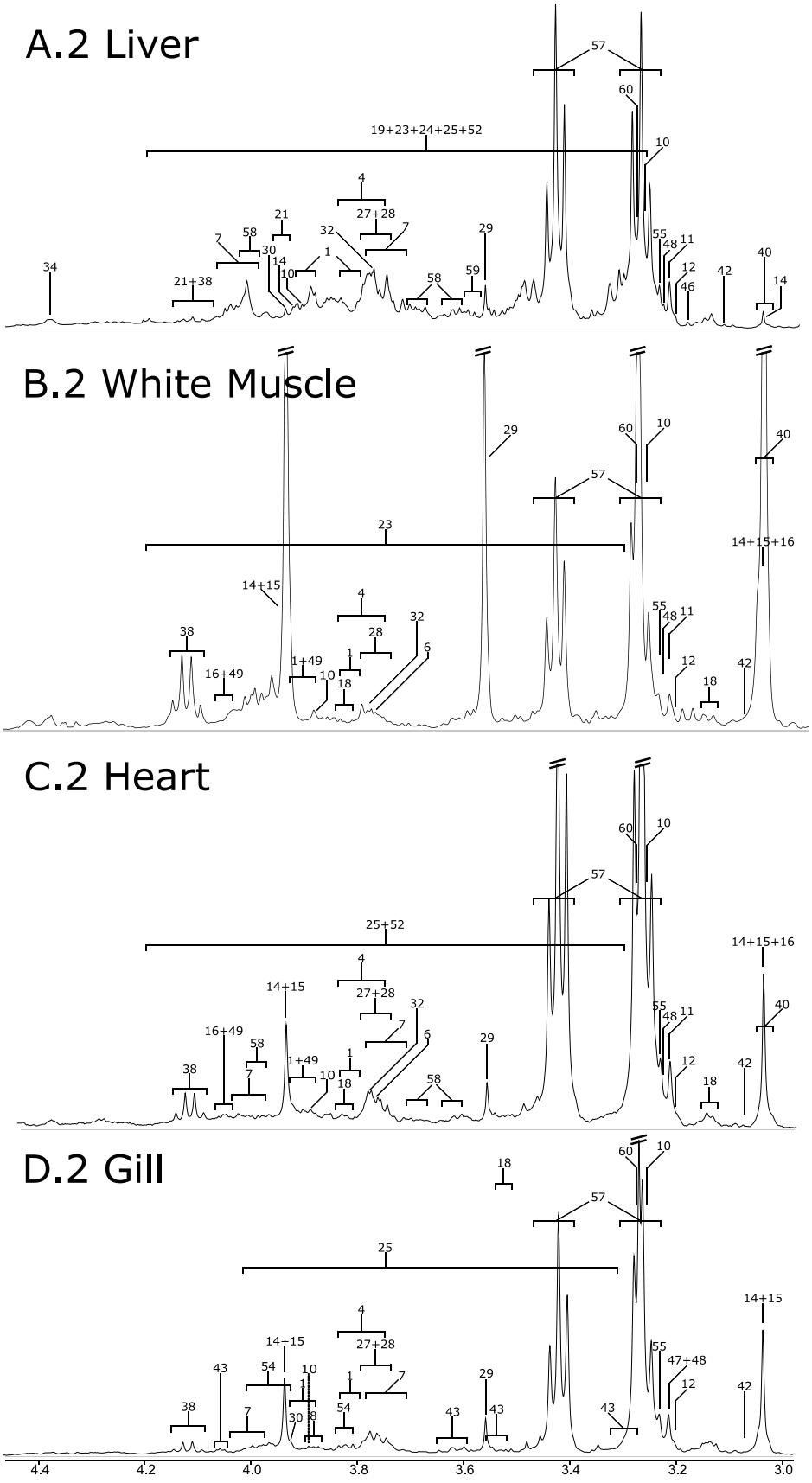
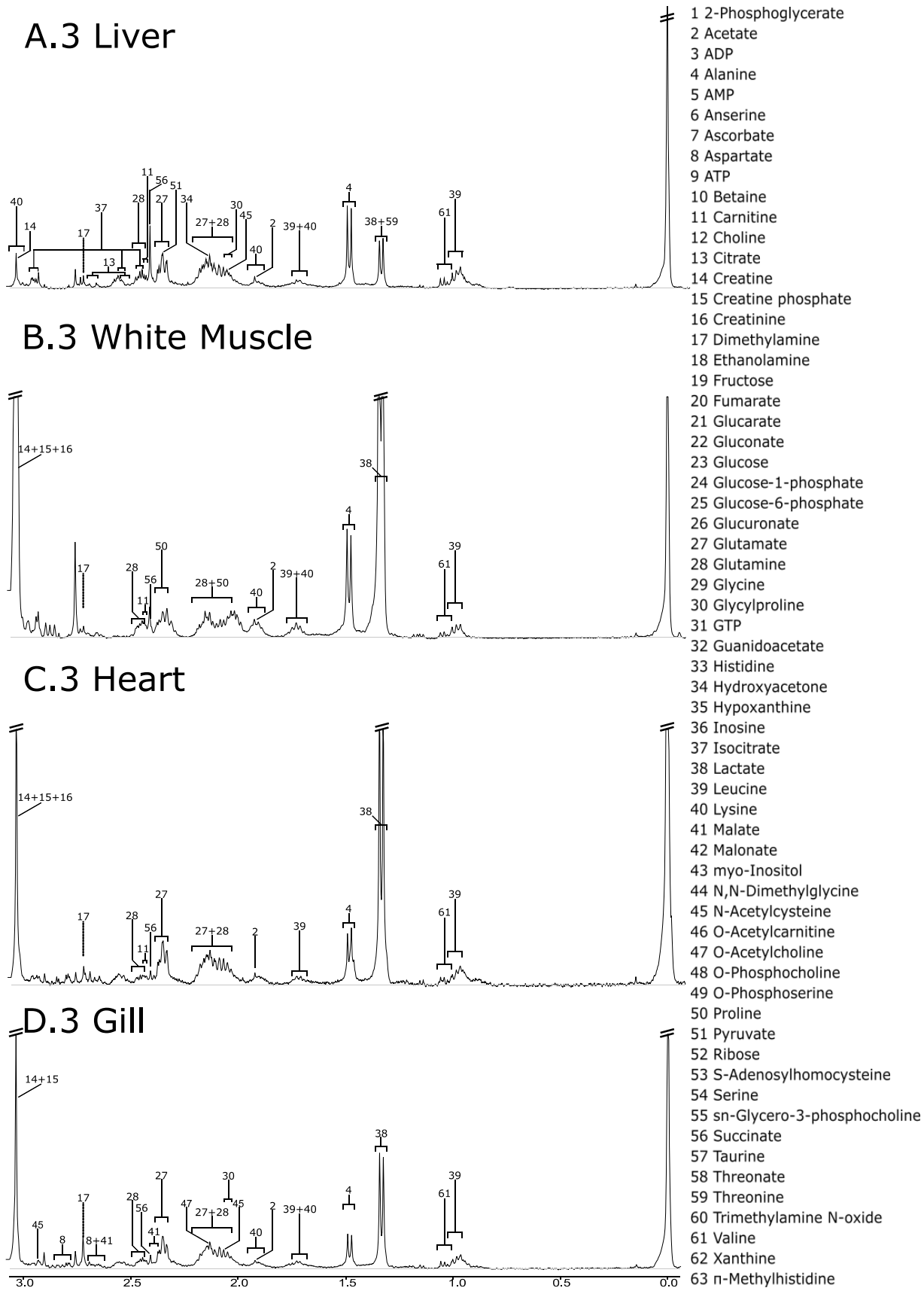


Figure 5 Representative ^{1}H-NMR spectra of liver (A), white muscle (B), heart (C) and gill (D) tissue extracts from European sea bass juveniles. Vertical scale in A.1-D.1 and A.3-D.3 is magnified 3 times with respect to A.2-D.2 to better represent all peaks. The most left and right peaks of these spectra is the most right and left peak of the spectra in A.1-D.1 and A.3-D.3, respectively and labelled in both spectra.

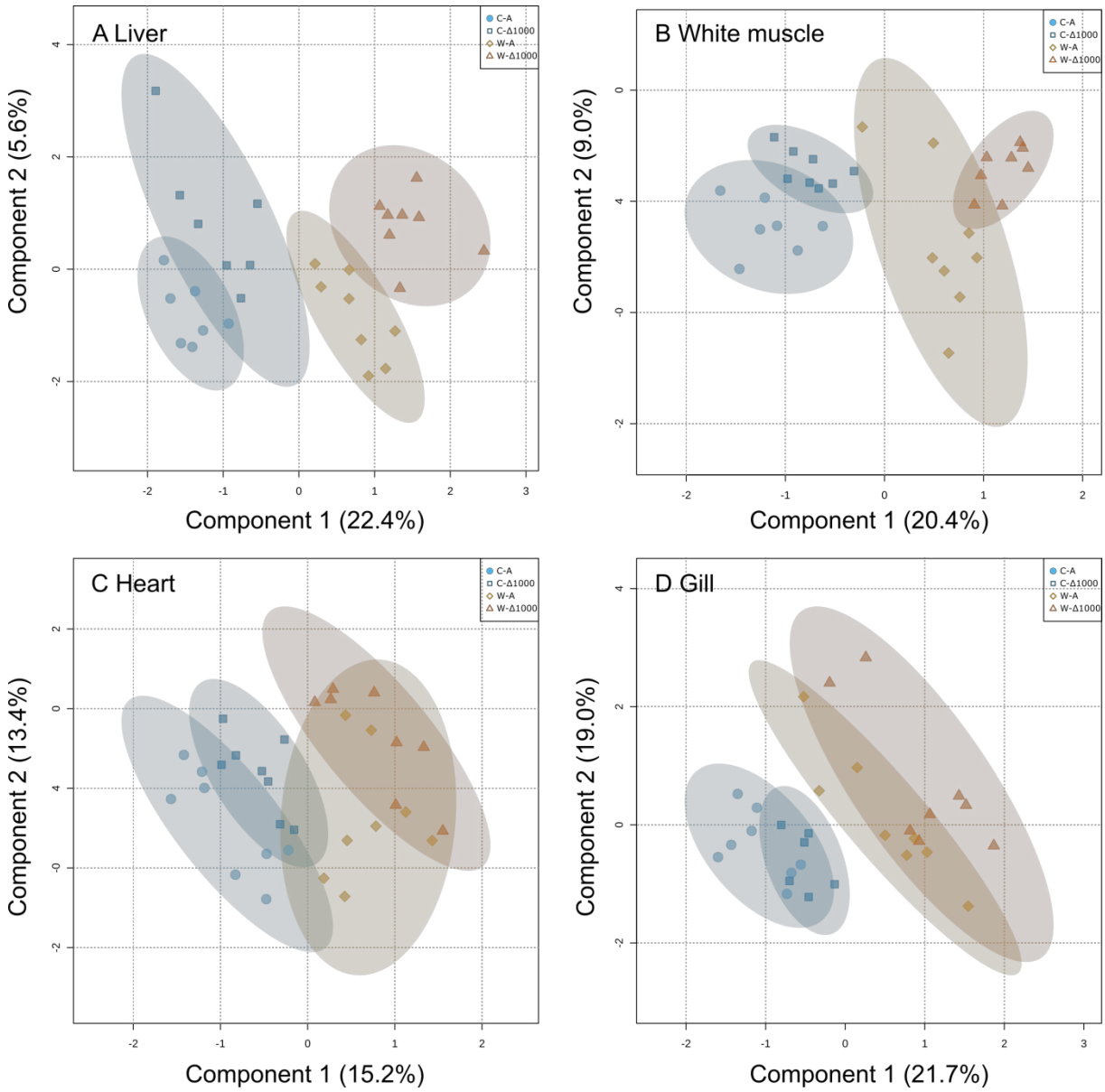


1 2-Phosphoglycerate
2 Acetate
3 ADP
4 Alanine
5 AMP
6 Anserine
7 Ascorbate
8 Aspartate
9 ATP
10 Betaine
11 Carnitine
12 Choline
13 Citrate
14 Creatine
15 Creatine phosphate
16 Creatinine
17 Dimethylamine
18 Ethanolamine
19 Fructose
20 Fumarate
21 Glucarate
22 Gluconate
23 Glucose
24 Glucose-1-phosphate
25 Glucose-6-phosphate
26 Glucuronate
27 Glutamate
28 Glutamine
29 Glycine
30 Glycylproline
31 GTP
32 Guanidoacetate
33 Histidine
34 Hydroxyacetone
35 Hypoxanthine
36 Inosine
37 Isocitrate
38 Lactate
39 Leucine
40 Lysine
41 Malate
42 Malonate
43 myo-Inositol
44 N,N-Dimethylglycine
45 N-Acetylcysteine
46 O-Acetylcarnitine
47 O-Acetylcholine
48 O-Phosphocholine
49 O-Phosphoserine
50 Proline
51 Pyruvate
52 Ribose
53 S-Adenosylhomocysteine
54 Serine
55 sn-Glycero-3-phosphocholine
56 Succinate
57 Taurine
58 Threonate
59 Threonine
60 Trimethylamine N-oxide
61 Valine
62 Xanthine
63 π-Methylhistidine

Continuation Figure 5 Representative ^{1}H-NMR spectra of liver (A), white muscle (B), heart (C) and gill (D) tissue extracts from European sea bass juveniles.



Continuation Figure 5 Representative ^{1}H-NMR spectra of liver (A), white muscle (B), heart (C) and gill (D) tissue extracts from European sea bass juveniles.



746

747 **Figure 6 Partial Least Squares - Discriminant Analysis (PLS-DA): 2D-Scores plots of European sea bass juveniles. Chosen**
748 **are components 1 and 2, the explained variances are shown in brackets. Liver (A), white muscle (B), heart (C) and gill (D)**
749 **tissue were sampled at 2900dd. The shaded areas indicate the 95% confidence regions, overlying symbols are the**
750 **individual data points for each treatment. C – cold life condition (15-18 °C), W – warm life condition (C + 5 °C), A –**
751 **Ambient PCO_2 (~650 µatm CO_2), Δ1000 – A + 1000 µatm CO_2, n=6-8.**

752

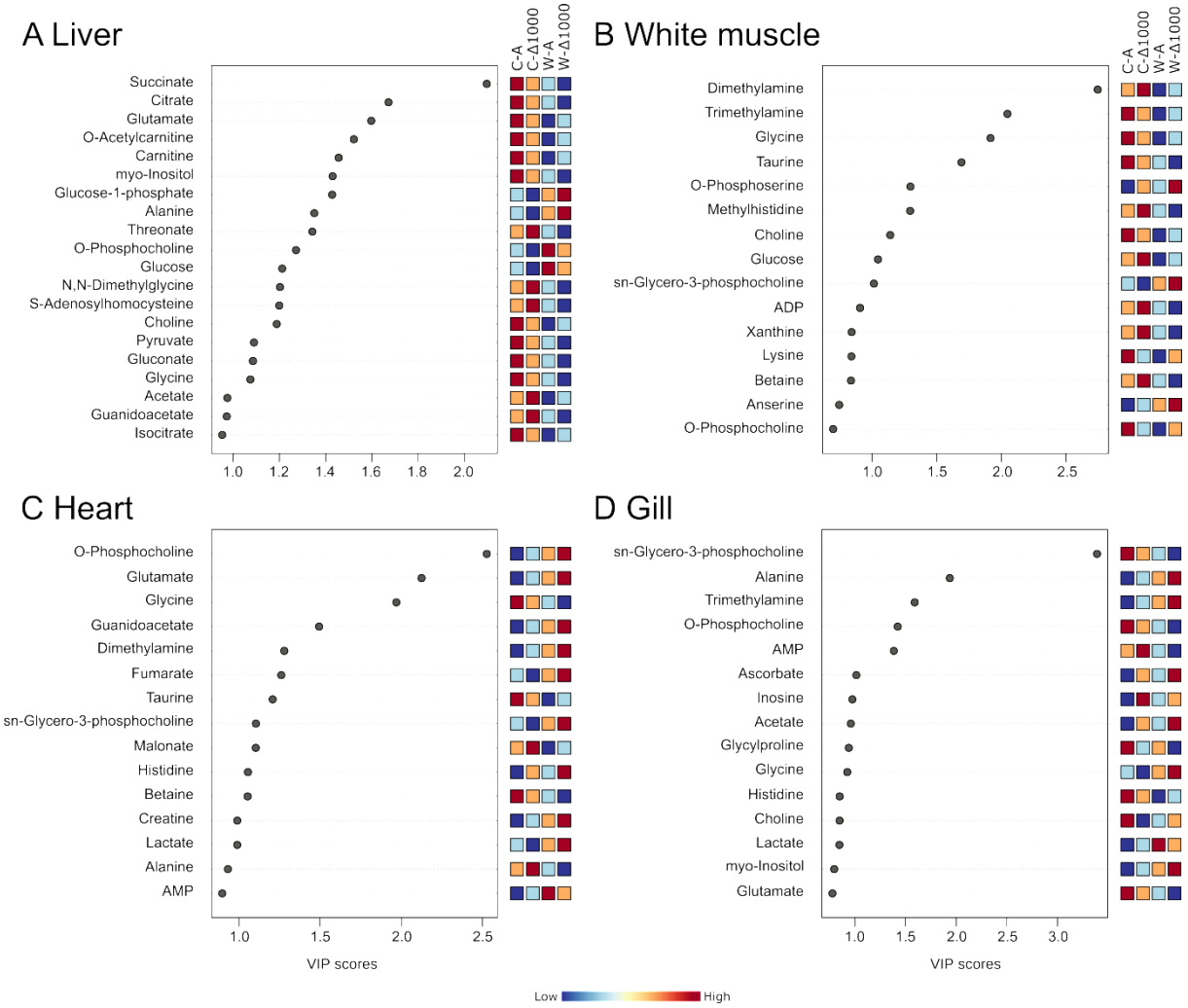


Figure 7 Important features contributing to component 1 of the Partial Least Squares - Discriminant Analysis (PLS-DA) of European sea bass juveniles. A cutoff of 1 was applied for the Variable Importance in Projection (VIP) score. Liver (A), white muscle (B), heart (C) and gill (D) tissue were sampled at 2900dd. C – cold life condition (15-18 °C), W – warm life condition (C + 5 °C), A – Ambient PCO_2 (~650 µatm CO_2), Δ1000 – A + 1000 µatm CO_2, n=6-8.

759 ## 4.13 Supplement

760 **Table S 1 Specific growth rates (SGR) and their respective Q10 of wet mass and body length of juvenile European sea**
761 **bass. SGR [% day^{-1}] and Q10 [-] are given for 0.05, 0.5 and 0.95 quantile of the cohort. Means ± s.e. over duplicate tanks**
762 **per condition. C – cold life condition (15-18 °C), W – warm life condition (C + 5 °C), A – Ambient pCO_2 (~650 µatm CO_2),**
763 **Δ1000 – A + 1000 µatm CO_2.**

	0.05 Quantile	0.5 Quantile	0.95 Quantile
Wet mass			
SGR C-A [% day^{-1}]	2.43±0.13	2.40±0.15	2.46±0.01
SGR C-Δ1000 [% day^{-1}]	2.50±0.18	2.50±0.11	2.47±0.14
SGR W-A [% day^{-1}]	2.90±0.04	2.97±0.01	2.74±0.08
SGR W-Δ1000 [% day^{-1}]	3.05±0.35	2.95±0.32	3.00±0.42
Q_{10} Amb [-]	1.52	1.66	1.30
Q_{10} Δ1000 [-]	1.60	1.49	1.59
Body length			
SGR C-A [% day^{-1}]	0.62±0.01	0.65±0.05	0.68±0.01
SGR C-Δ1000 [% day^{-1}]	0.65±0.06	0.66±0.04	0.68±0.04
SGR W-A [% day^{-1}]	0.88±0.01	0.88±0.02	0.84±0.05
SGR W-Δ1000 [% day^{-1}]	0.85±0.16	0.79±0.16	0.80±0.19
Q_{10} Amb [-]	2.25	2.03	1.67
Q_{10} Δ1000 [-]	1.85	1.56	1.48

764

765

766 **Table S 2 F- and p-values of fixed effects from the linear mixed models on juvenile European sea bass growth. OA – ocean**
767 **acidification – effect of the PCO_2-Treatment, OW – ocean warming – effect of the temperature treatment, OAW – ocean**
768 **acidification and warming – interaction effects of temperature and PCO_2-Treatment.**

	OA		OW		OAW	
	F-value	p-Value	F-value	p-Value	F-value	p-Value
Body length						
over time	2.76	0.17	445.79	<0.0001	0.04	0.84
at 2900 dd	0.55	0.50	97.42	0.0006	0.35	0.59
Wet mass						
over time	17.49	0.01	1446.33	<0.0001	0.76	0.43
at 2900 dd	0.01	0.92	57.79	<0.0001	0.09	0.78

769

Table S 3 Biometrical data of juveniles used for metabolomics: Treatments: C – cold life condition (15-18 °C), W – warm life condition (C + 5 °C), A – Ambient PCO_2 (~650 µatm CO_2), Δ1000 – A + 1000 µatm CO_2, CF – condition factor, values are means ± standard error.

Treatment	n	Wetmass [g]	Bodylength [cm]	CF [-]
C-A	96	4.02±0.09	6.66±0.06	1.55±0.03
C-Δ1000	96	4.11±0.10	6.81±0.05	1.76±0.01
W-A	64	5.96±0.19	7.92±0.08	1.62±0.03
W-Δ1000	64	5.91±0.23	7.93±0.09	1.58±0.02

5 General Discussion

5.1 Is mitochondrial performance impaired in juvenile European sea bass heart mitochondria after long-term acclimation to OAW or after acute temperature changes?

The cardiovascular system is an important part in the interplay of the different organs in general, but especially when it comes to temperature tolerance. Higher temperatures increase metabolic processes and therefore result in higher oxygen demands. The cardiovascular system needs to supply sufficient oxygen to the tissues so that the organism is able to withstand increased temperatures. At some point the cardiovascular system is no longer able to meet these demands. The thermal sensitivity of fish is therefore connected to the capacity of oxygen supply by their cardiovascular system (Pörtner & Lannig, 2009). As the heart is a highly oxygen dependent tissue, it is believed that the capacity of its mitochondria to produce ATP might define the upper thermal tolerance of an organism. Consequently, studies on heart mitochondria after acute temperature change, as well as long-term temperature acclimation, were done in significant amounts before (e.g. Fangue et al., 2009; Shama et al., 2014 and references therein) and mitochondrial capacity was found to be impaired at elevated temperatures (Fangue et al., 2009; Hilton et al., 2010; Mark et al., 2012; Iftikar & Hickey, 2013). As described before, future ocean conditions will not only be warmer, but also more acidic. Although juvenile and adult fish possess well-developed acid-base regulating mechanisms (for review, see Heuer & Grosell, 2014), these mechanisms need energy to function. In consequence, OA might add additional stress when combined with OW, as it increased the energy and therefore the oxygen demand of an organism. While studies on the effects of temperature on mitochondria are numerous, studies investigating the effects of OA or OAW on mitochondria fewer in number (e.g. Strobel et al., 2013; Leo et al., 2017). In Publication I, I examined the effect of long-term acclimation to OAW, as well as acute temperature change, on heart mitochondria. For this purpose I determined mitochondrial respiratory capacities in permeabilized heart fibres of juvenile European sea bass from group 3 (Wild). The juvenile European sea bass had been reared under the six described OAW conditions since 2dph (Figure 3). At the time of the experiments the juvenile fish were approx. 7 months old.

As observed in other studies before, acute warming impaired mitochondrial capacities. In the case of the examined juvenile European sea bass mitochondria of my study, increased LEAK respiration decreased mitochondrial efficiency after acute warming of heart fibres from C-life-conditioned fish.

Increased LEAK respiration is a sign of impaired mitochondrial membrane integrity, which results in higher needs of oxygen for the same amount of ATP production. Using more oxygen to produce the same amount of ATP, brings the cardiovascular system closer to its limits. This was shown by Ekström et al. (2017), who observed heart failure after acute warming compromised mitochondria. The high thermal tolerance of juvenile European sea bass (Dalla Via et al., 1998; Claireaux & Lagardère, 1999) seemed to contradict the results on mitochondrial properties. But although European sea bass is able to survive under a broad range of temperatures, it has been shown before that arrhythmia occurred in European sea bass acclimated to 17 °C already at 22 °C, although their CT_{max} was as high as 30 °C (Anttila et al., 2017). We concluded that large acute temperature changes of 5 °C could challenge juvenile European sea bass heart mitochondria when combined with additional drivers such as OA.

Acclimation to long-term warming restored mitochondrial capacities and even more, mitochondria from W life-conditioned fish displayed increased mitochondrial efficiency in comparison to C life-conditioned fish. This was expressed by higher respiratory control ratios in W life-conditioned fish compared to C life-conditioned fish. This gives rise to the suggestion that the chosen C condition is not the optimal temperature for juvenile European sea bass, probably backed by the origin of this species in warmer environments. Higher RCR can translate to higher growth rates, which was the case in all experiments of the FITNESS project, W juveniles were bigger than C juveniles under *ad libitum* food availability in Publication I and Chapter 4, but also in Cominassi et al. (2019; 2020) and Cominassi et al. (in prep.).

The effect of OA on the mitochondrial metabolism was not as clear as the effect of acute or long-term warming. If OA was applied as a single driver on C life-conditioned fish, mitochondrial properties were not affected. However, if OA was combined with the drivers long-term and acute warming, CII of the electron transport system was inhibited. Some of the effects of increased sea water PCO_2, such as lower intracellular pH, can be counteracted by fish. The intracellular pH is buffered via raised intracellular bicarbonate concentrations (Strobel et al., 2013). Bicarbonate, on the other hand, can act as an inhibitor of mitochondrial citrate synthase and succinate dehydrogenase and compensation after acclimation to hypercapnia was observed in rodents (Simpson, 1967; Wanders et al., 1983), but is not universal in fish (Strobel et al., 2012; 2013a,b). Although there might have been indications for impaired mitochondria, e.g. reduction of CII in W-Δ1000 fish after acute temperature change, overall mitochondrial efficiency of heart mitochondria of juvenile European sea bass was not compromised. It might be possible that European sea bass is able to use anaplerotic mechanisms, such as decarboxylation of aspartate and glutamate, which both feed into the Krebs cycle and therefore stimulate CI, to overcome the inhibitory effects of OA on CII (Langenbuch & Pörtner, 2002; Strobel et al., 2013). In Chapter 4, I determined metabolic profiles of heart tissue of

European sea bass of the same group (Wild) as the ones used in this study. The potential of such mechanisms in European sea bass will be discussed in the light of the metabolic profiles in chapter 5.5.

The conclusion from this study on juvenile European sea bass mitochondria was that European sea bass seem to profit from higher temperatures, when OW is the only driver applied. European sea bass also seems able to cope with the inhibiting effects of OA on CII when faced with OA as a single driver, but might be challenged when those drivers occur collectively with the addition of acute temperature change.

5.2 Did OAW lead to synergistic effects on growth rates of larval and juvenile European sea bass?

I determined growth of two groups of European sea bass during my thesis, namely F1 (group 2, Figure 3) and Wild (group 3) and received the growth data of F0 (group 1) from former experiments. In Publication II I discussed and compared the growth of F0 and F1. F0 was raised under two different OA conditions. F1 was raised under the respective OA condition as their parents, expanded by two OW conditions, resulting in four OAW conditions. In Chapter 4, I determined growth of the Wild batch of European sea bass and used the same four OAW conditions that were used in Publication II. During the experiments on group 3 (Figure 3, Publication I and Chapter 4) of this project, larval growth was followed and analysed throughout larval phase (Figure 8, Table 3 and additional information in appendix A1). Due to the design of the publications and the manuscript, these results did not fit into any of them, but provide additional insight into the effects of OAW on successive generations, which shall be interpreted in this General Discussion.

As temperature was only applied to F1 and Wild fish, the effect of OW on growth of larval and juvenile European sea bass was only determined in those two groups. Cold life-conditioned fish displayed specific growth rates (SGR) from 8.19 to 10.29 and 7.85 to 9.75 % day^{-1} for larval dry mass (DM), 1.89 to 2.56 and 2.14 to 2.43 % day^{-1} for larval body length (BL) in Wild and F1 fish, respectively (Table 3 for Wild larvae, Table S 1 in Chapter 4 for Wild juveniles and Table 3 in Publication II for F1 larvae). SGR in cold juveniles ranged from 2.74 to 3.05 % day^{-1} for wet mass (WM) and 0.79 to 0.88 % day^{-1} for BL in Wild fish, respectively (Table 3 for Wild larvae, Table S 1 in Chapter 4 for Wild juveniles and Table 3 in Publication II for F1 larvae and juveniles). The SGRs of W life-conditioned fish were higher than those of C life-conditioned fish: 9.56 to 14.81 and 11.67 to 14.76% day^{-1} for larval DM, 2.27 to 3.38 and 2.88 to 3.50 % day^{-1} for larval BL in Wild and F1 fish, respectively. SGR in W juveniles ranged from 2.40 to 2.50 % day^{-1} for juvenile WM and 0.62 to 0.68 % day^{-1} for juvenile BL in Wild fish,

respectively. The difference in SGR between C and W life-conditioned fish was displayed in Q_{10} values of 1.30-2.43 (Table 3 for Wild larvae, Table S 1 in Chapter 4 for Wild juveniles and Table 3 in Publication II for F1 larvae). These higher SGRs in W life-conditioned fish compared to C life-conditioned fish resulted in bigger Wild and F1 larvae and Wild juveniles in W condition than in C condition when compared at the same age in days post hatch (dph). However, as temperature is not only affecting growth rates, but also the pace of development, we additionally compared the different treatments at similar age in degree days (dd): 900 dd equals 45 dph at 20 °C and 60 dph at 15 °C. In this case, the size of larvae at the end of the larval period was similar in W-A and C-A larvae in Wild and F1 (Figure 8 for Wild larvae and Figure 2 and 3 in Publication II for F1 larvae). The picture changed for juvenile fish: W juveniles were not only bigger when compared at the basis of dph but also when compared at the basis of dd in Wild fish (Figure 4). We could not determine the effect of OW as a single driver on F1 juveniles, as the F1 W-A treatment was missing.

Table 3 Specific growth rates (SGR) and their respective Q_{10} of larval dry mass and body length. SGR [% day^{-1}] and Q_{10} [-] are given for 0.05, 0.5 and 0.95 quantile of the cohort. Means ± s.e. over all replicate tanks per condition. A – Ambient pCO_2, Δ1000 – ambient + 1000 µatm CO_2, C – cold life condition, W – warm life condition.

Treatment	n	0.05 Quantile	0.5 Quantile	0.95 Quantile
Larval dry mass				
SGR C-A [% day-1]	3	8.37±0.46	8.30±0.39	8.55±0.02
SGR C-Δ1000 [% day-1]	3	8.19±0.67	8.70±0.08	9.00±0.05
SGR W-A [% day-1]	3	9.56±0.33	11.33±0.98	12.47±0.29
SGR W-Δ1000 [% day-1]	3	10.72±1.15	11.68±1.10	12.44±0.96
Q_{10} Amb [-]		1.30	1.86	2.13
Q_{10} Δ1000 [-]		1.71	1.80	1.91
Larval body length				
SGR C-A [% day-1]	3	2.05±0.17	2.35±0.13	2.38±0.11
SGR C-Δ1000 [% day-1]	3	1.89±0.11	2.03±0.03	2.15±0.04
SGR W-A [% day-1]	3	2.27±0.09	2.66±0.21	2.82±0.16
SGR W-Δ1000 [% day-1]	3	2.61±0.30	2.69±0.28	2.89±0.16
Q_{10} Amb [-]		1.22	1.28	1.40
Q_{10} Δ1000 [-]		1.90	1.75	1.80

Having two experiments in which I surveyed growth of European sea bass, which both showed that OW increases growth at age in dph as well as in dd in Wild and F1 larvae and Wild juveniles, respectively, strengthens the informative value. Increased growth and accelerated development at higher temperatures was already observed in other studies on European sea bass (Ayala et al., 2001; 2003). As the studies of Ayala et al. (2001; 2003) came from an aquacultural background, their interpretation of these high growth rates and faster development and whether these results are positive or not might be different, as it is more important that fish in aquaculture facilities grow fast than that they are able to flee from predators. In my project, I wanted to add some pieces to the puzzle whether European sea bass is vulnerable to climate change and which underlying mechanisms motivate this estimate. Therefore, it would be bold to state that European sea bass larvae will profit

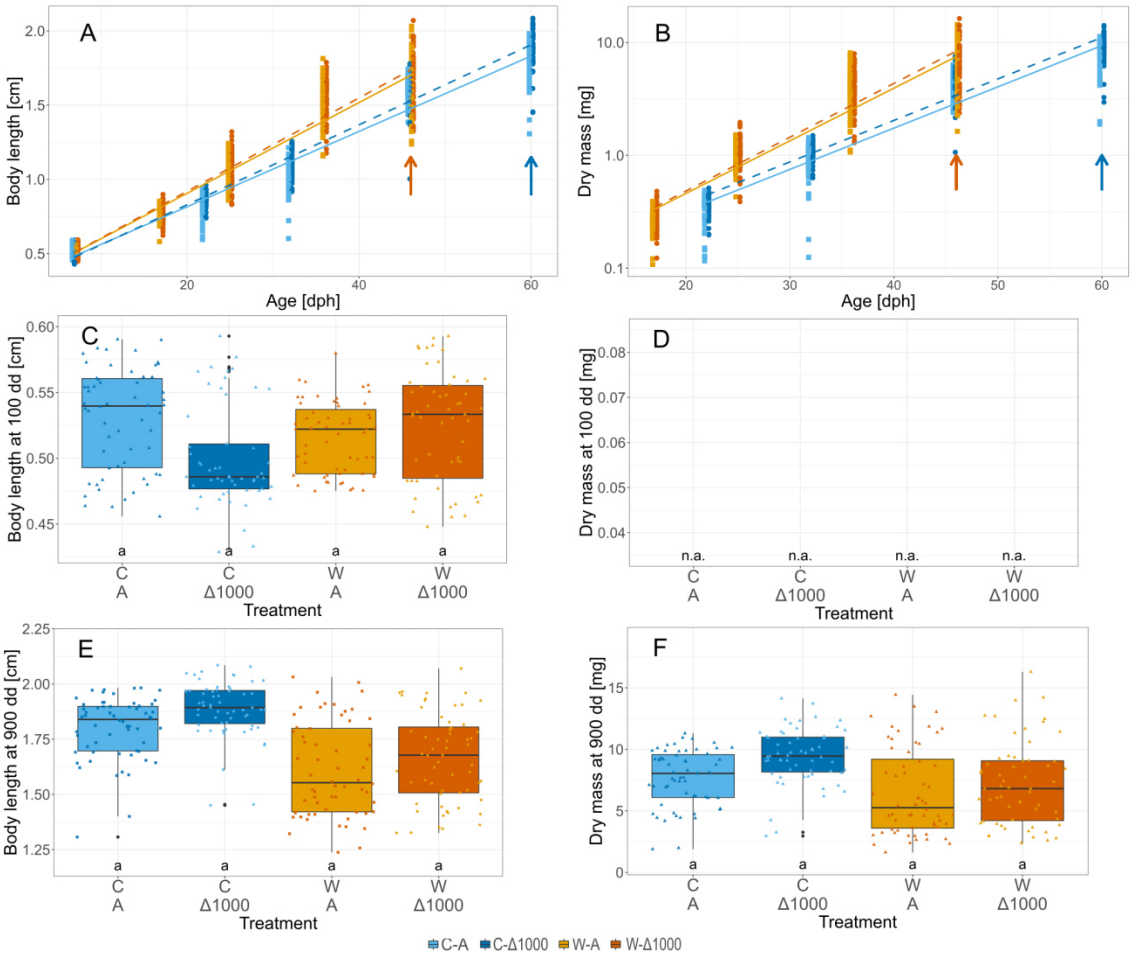


Figure 8 Growth of European sea bass larvae of group 3 (Wild). Subfigure A and B: Growth over time with linear regression lines. Shown are individual data points of body length (A) and dry mass (B). W larve grew significantly faster than C larvae and no differences were observed between pCO_2 treatments [linear mixed effects model (LME), P<0.05]. All data were tested with LMEs, F- and p-Values are summarized in Table A 1. Arrows indicate the data points at 100 dd (mouthopening) and 900 dd (metamorphosis), data for different pCO_2conditions of the same age are slightly moved for better visibility. Subfigure C to E: Body length (C, E) and dry mass (D, F) at approx. 100 dd (C, D) and 900 dd (E, F). Boxplots show median, upper and lower quartiles and 1.5x interquartile range. Overlying symbols are the individual data points for each treatment, different letters indicate significant differences (LME, P<0.05). C – cold life condition, W – warm life condition, A – Ambient pCO_2, Δ1000 – ambient + 1000 µatm CO_2, n=40-60.

from higher temperatures as their development and growth is accelerated by higher temperatures in an ecological point of view. Although faster growth at larval and juvenile stage is usually thought to be beneficial, as it means for the organism that predation pressure is lower, it is necessary to take a look at the whole picture before suggesting an ecological prognosis. As my thesis was embedded in a big project, I can profit from results of other studies embedded in the same project and working on the same groups of fish. We found that the faster growth of Wild W larvae in comparison to Wild C larvae came at the cost of impaired swimming capacity (Cominassi et al., 2019) and Moyano et al. (2017) found that the upper thermal limit of Wild W-A larvae was 27 °C. The upper thermal temperature of 27 °C in larvae of the W life condition in combination with the findings of arrhythmia at 22 °C and CT_{max} at 30 °C in another study on European sea bass juveniles acclimated to 17 °C (Anttila et al., 2017) and impaired mitochondria under acute warming in Wild W-Δ1000 juveniles suggest that European sea bass larvae will be challenged when faced with more drivers than just ocean warming. One combination of OW with an additional driver is OAW, which effects on growth are discussed below, further additional drivers comprise acute warming and food availability. As European sea bass are spawning offshore, larvae are developing in waters with relatively stable conditions (Thompson & Harrop, 1987), climate change is not only leading to generally higher ocean surface temperatures, but also to an increase in heat waves (IPCC, 2021). If those heat waves occur, the relatively low CT_{max} of W acclimated larvae (Moyano et al., 2017) and the findings of arrhythmia (Anttila et al., 2017) at just 22 °C in juvenile European sea bass propose challenges for European sea bass larvae when hit by heat waves. The second driver which larvae might face due to increased SGR and development is food scarcity. Food scarcity could be provoked by both accelerated development as well as higher growth rates: Accelerated development might lead to mismatches in larval and larval food abundance, resulting in generally lower availability of food. Increased growth rates on the other hand increase also the demand of food. The larvae within the FITNESS project were fed *ad libitum*, allowing them to grow as fast as they did. Even if larval and food abundance are not mismatched, it is not certain that food abundance meets the increased needs of faster growing larvae. It was shown before that food rations impacted larval growth rates and survival (Zambonino Infante et al., 1996; Bochdansky et al., 2005). We tested the effect of restricted food rations on Wild juveniles and found that food scarcity combined with OAW severely impaired European sea bass juveniles (Cominassi et al., 2020). Consequently, although faster larval growth and development, as found in Wild and F1 larvae, as well as faster juvenile growth, as found in Wild juveniles, can be beneficial for larvae and early juveniles, it seems unlikely that it will translate into advantages when European sea bass faces other drivers than “just” ocean warming. Nevertheless, I could confirm my first hypothesis that growth rates of larvae and juveniles will be increased under OW.

OA as a single driver on Cold life-conditioned European sea bass larvae and juveniles did not affect SGR or size at age (dph and dd) in F0, F1 and Wild. The absence of vulnerability of European sea bass larvae and juveniles to OA was supported by the findings of other experiments within the FITNESS project. OA as a single driver applied on C larvae and juveniles did not affect: larval swimming performance (Wild, Cominassi et al., 2019), mitochondrial capacity of permeabilized juvenile heart fibres (Wild, Publication II), larval RMR (F1, Publication II), juvenile SMR (F0 and F1, Publication II), juvenile PO_{2crit} (F0 and F1, Publication II), food consumption rate and food conversion efficiency (Wild, Cominassi et al., 2020), larval survival (F0, Crespel et al., 2017) and metabolic profiles of liver, white muscle, heart and gill tissue (Wild, Chapter 4). In contrast skeletal mineralization was increased and skeletal deformities were decreased under OA in F0 larvae (Dahlke et al., 2016). The findings of my and the other studies of the FITNESS project are in line with the general resilience of European sea bass juveniles to abiotic environmental conditions (e.g. Dalla Via et al., 1998; Claireaux & Lagardère, 1999), as well as recent studies on European sea bass ability to cope with OA (Pope et al., 2014; Montgomery et al., 2019). These findings confirmed my second hypothesis that OA will not affect growth rates of larval and juvenile European sea bass.

In addition to OA as a single driver, OAW was applied as a multiple driver on F1 and Wild larval and juvenile European sea bass. The picture was not equal for these groups: The observed differences in growth and size at age in Wild larvae and juveniles were only due to temperature, no differences were observed which were due to OA or the interaction of the two drivers (Figure 4 and 8). In F1 on the other hand, we could not observe effects of OA on F1 C larvae and they were of comparable size to F1 W-A at metamorphosis. But F1 W-Δ1000 larvae were significantly smaller at metamorphosis than F1 C and F1 W-A larvae (Figure 2B and E and Table 4 in Publication II). Due to the missing F1 W-A treatment, we could only compare F1 C-A with F1 C-Δ1000 and F1 C-A with F1 W-Δ1000 to determine the effects of OA and OAW respectively. However, it was not possible to detect the effect of OW as a single driver in F1 and therefore not possible to determine if the negative effects of OAW on larval growth in F1 W persisted into the juvenile phase.

It was interesting to observe that the effect of OA and OW as single drivers on different traits of larval and juvenile physiology was quite similar in groups 1-3 throughout the FITNESS project, except for growth under OAW. The observed decrease in size at age in dph as well as in dd in W-Δ1000 larvae compared to W-A larvae was only observed in the F1 group and not in the Wild groups. It was also not observed in Wild juveniles of F1 and could not be determined in F1 juveniles, due to the missing F1 W-A treatment. We used Wild juveniles for an experiment with restricted food rations (Cominassi et al., 2020). When the juveniles were separated into smaller tanks at 277 and 367 dph for Wild W and Wild C juveniles, there was neither a difference in size between Wild C-A and Wild C-

Δ1000 nor between Wild W-A and Wild W-Δ1000. Although no size difference was observed at the beginning of the feeding trial, confirming the findings in all groups except F1 larvae, Wild W-Δ1000 showed significantly reduced growth rates under *ad libitum* feeding. Those reduced growth rates were linked to reduced food consumption and reduced food conversion efficiency caused by lower specific activity of digestive enzymes (Cominassi et al., 2020). We discussed the probability of cannibalism within the big rearing tanks during general larval and juvenile rearing as a cause for the observed difference in growth within the feeding trial even under *ad libitum* feeding, which was not seen during the regular growth analysis of the general rearing tanks. Cannibalism occurs in juvenile European sea bass reared in aquaculture (Hatziathanasiou et al., 2002) and would affect results on growth in two ways: (1) small individuals would not survive, as they are consumed by bigger animals, resulting in the impression that this population is growing faster than a population without cannibalism. (2) Feeding on conspecifics results in higher amounts of nutrition which also has the ideal composition supporting the higher growth rates. Except for the feeding trial, larvae and juveniles were fed *ad libitum* during the FITNESS project. However, *ad libitum* was determined after the recommendations of the food supplier. It would therefore be possible that this ration was not sufficient for *ad libitum* feeding, leaving some range for cannibalism, masking reduced growth in other W-Δ1000 treatments. When trying to explain why those masking processes did not work in F1 larvae but did work in Wild larvae, one should keep in mind that those were larvae from two generations successively reared under OA conditions combined with OW in the second generation. It was found in other studies that OA and OW as single drivers alter European sea bass behaviour, e.g. OA decreased the distance over which food and predator cues were sensed, decreasing their chances to detect food or predators (Porteus et al., 2018) and OW resulted in a decreased latency of the escape response and mirror responsiveness (Manciocco et al., 2015). F1 was the only group, which was reared during embryonal development under OA conditions and the only group that was reared as a successive generation under OA conditions. It is possible that the negative effects of OAW on larvae of European sea bass are less severe when applied only from 2 dph onwards. Therefore, Wild larvae could have been able to compensate the negative effects of our OAW exposures, resulting in unimpaired growth rates. Developmental acclimation during embryonal phase and the history of F0 could have increased the effects of OAW on F1 larvae, resulting in a severity that could no longer be compensated. Different effects of OA had been observed on European sea bass that could lead to reduced growth rates, especially when they occur together. Although I have not measured food consumption or behaviour of F1 larvae, it is possible that both were altered under OAW. Lower food consumption was already observed in Wild W-Δ1000 juveniles fed with food pellets and might have been more prominent in F1 W-Δ1000 larvae, e.g. due to altered behaviour. F1 larvae were fed with

live artemia nauplii, which they had to hunt, if OA had decreased their ability to sense food (as seen in Porteus et al., 2018), this would affect their food consumption. In addition to decreased food consumption, decreased food conversion efficiency as observed in Wild W-Δ1000 juveniles (Cominassi et al., 2020) and impaired mitochondrial capacities as observed in Wild W-Δ1000 juveniles under acute temperature change (Publication I) could be explanations for the observed lower growth rates under OAW only in F1 larvae but not in Wild larvae. While I had to reject hypothesis 3 for the Wild larvae and juveniles, I could confirm it for F1 larvae. F1 larvae displayed synergistic effects of OAW on growth rates, as F1 W-Δ1000 showed lower growth rates than F1 W-A larvae and were smallest at metamorphosis. This was not observed in Wild larvae and juveniles and could not be tested in F1 juveniles, as F1 W-A treatment was missing in juveniles.

5.3 Did OAW lead to synergistic effects on metabolic rates of larval and juvenile European sea bass?

OW increased RMRs in F1 W-A European sea bass larvae in comparison to F1 C-A larvae with a Q_{10} of 2.24. This reflects the expected Q_{10} of 2-3 for biological processes. I could not determine the effect of OW as a single driver on SMR or PO_{2crit} in juvenile European sea bass, as the F1 W-A treatment was missing. Therefore, I could only confirm part of my first hypothesis: larval RMR was increased under OW.

OA as a single driver did not affect metabolism of European sea bass larvae or juveniles in terms of larval RMR, juvenile SMR and juvenile PO_{2crit}. It was generally thought that fish larvae might be more vulnerable to OA than other life stages and might therefore show decreased growth rates under OA. It was already shown before that European sea bass larvae do not display impaired growth under OA (Pope et al., 2014; F0 in Crespel et al., 2017). In line with these findings, this study shows that the maintained growth rates of European sea bass larvae under OA are not due to increased metabolic rates, which means that larval European sea bass are able to cope with OA without spending additional energy on compensatory processes in comparison to their counterparts under ambient PCO_2 conditions. The insensitivity of juvenile European sea bass to OA was expected, as juvenile European sea bass tolerate a wide range of temperatures and salinities (Dalla Via et al., 1998; Claireaux & Lagardère, 1999). Therefore, these results confirmed my second hypothesis for this question that larval and juvenile metabolic rates will not be impaired by OA. Additionally, my results also confirmed another hypothesis: Montgomery et al. (2019) determined the PO_{2crit} in European sea bass after acute increase of PCO_2 and hypothesized that the 20 % decrease in PO_{2crit}, which they

observed, will vanish after long-term acclimation to OA. As I could not find effects of OA on PO_{2crit} after long-term acclimation I can confirm this hypothesis.

Although OAW did not increase larval RMR to higher values than OW as a single driver, the picture between F1 W-A larvae was still different to that of F1 W-Δ1000 larvae. F1 W-A larvae increased growth and metabolic rate due to increased temperature in a pace that allowed them to reach the same size at metamorphosis as F1 C larvae, although the larval duration was much shorter in F1 W-A larvae compared to F1 C larvae. F1 W-Δ1000 larvae displayed similar RMR than F1 W-A larvae, but had significantly lower growth rates than F1 W-A larvae, resulting in the smallest larvae in F1 W-Δ1000 at metamorphosis compared to all other treatments. This suggests that F1 W-Δ1000 allocated energy to different regulatory processes or that their energy production was not as efficient as in the other groups. Decreased mitochondrial efficiency and therefore decreased energy production efficiency could be caused by increased mitochondrial LEAK respiration rates. I observed increased LEAK respiration rates in Wild C juveniles under acute warming but they were compensated in Wild W juveniles after long-term acclimation to OW. Yet, it is possible that due to the higher vulnerability of larvae, mitochondria of F1 W-Δ1000 larvae were less efficient than those of F1 W-A larvae, i.e. by displaying higher LEAK respiration rates. This would mean that F1 W-Δ1000 larvae with the same RMR as F1 W-A larvae would have a lower energy budget for processes such as growth. In addition they might need to spend additional energy on processes to maintain homeostasis, again cutting the energy budget for growth. SMR of juvenile European sea bass was higher in F1 W-Δ1000 treatments than in both F1 C treatments. As the F1 W-A treatment was missing in juvenile European sea bass, it was not possible to determine whether the increase in SMR was due to OW alone and whether OAW impaired F1 W-Δ1000. Consequently I had to reject the third hypothesis for larvae, as larval metabolic rates were not lower in F1 W-Δ1000 larvae than in other treatments. I could not answer this hypothesis for the juveniles, as F1 W-A was missing in the juveniles. Similarly, for the fourth hypothesis, I could confirm that neither OA nor OAW affected juvenile PO_{2crit}, but could not determine the effect of OW as a single driver.

5.4 Did transgenerational plasticity in European sea bass after acclimation of F0 to OA improve growth and metabolic rates of F1 under OAW?

My thesis was embedded in the FITNESS (Fish Transgenerational adaptive Strategies to ocean acidification and warming) project. As the name of the project indicates, one goal of this project was to determine the transgenerational plasticity of European sea bass on several physiological traits

important to an organism's fitness, such as SMR. For this purpose, F0 was reared under two OA conditions from 2 dph in October 2013 to maturation and finally breeding in March 2018 (Figure 3). Between F0 and F1, group 3 (Wild) was reared under six OAW conditions. Throughout all determined traits, OW had higher effects than OA. Additionally, as described in chapter 1, it appears crucial, to apply not only single drivers, but multiple drivers to conduct reasonable climate change research. Consequently, the F1 generation was reared under the same OA conditions as their respective parents with the addition of the same two temperatures, which were applied on Wild fish, resulting in four OAW conditions in F1. For the determination of parental and therefore transgenerational effects, it would have been necessary to rear F1 fish in a full factorial design: Group A: F0 A – F1 A, Group B: F0 A- F1 Δ1000, Group C: F0 Δ1000 – F1 A, Group D: F0 Δ1000- F1 Δ1000. Applying an OW scenario on the full factorial transgenerational design would have resulted in eight different groups, which was not possible in terms of larval count, space and manpower. Therefore the four OAW conditions as described above were applied to F1. This meant that the fish were reared only in the OA treatment of their respective parents and group B and C of the full factorial transgenerational design were missing in the design of this study. However, the decision to apply two temperatures instead of having a full factorial transgenerational design allowed testing the effect of multiple drivers (OA and OW) on the metabolism of European sea bass and although not allowing to test for transgenerational plasticity, it still allowed determining effects on fish raised over successive generations under OA conditions.

This study was to my knowledge the first that determined the effects of OA on European sea bass or other long-lived teleosts over successive generations. OAW only decreased larval growth in F1 W-Δ1000 larvae and not in F0 Δ1000 larvae, although they had similar thermal histories. This could be due to (1) parental provisioning: when parents have been reared under the same conditions, egg size and composition could vary (Munday, 2014) resulting in improved or impaired performance of F1. I did not measure provisioning or other parental effects directly, as I did not measure egg size or composition and did not incubate F1 in a full factorial design. However, I measured larval size at mouth opening in F1, up to this point larvae depended solely on yolk sac reserves. Using this landmark as indirect indicator of parental provisioning did not indicate difference between treatments. Therefore parental effect might seem less likely to be the cause of the difference between F0 and F1. (2) Differences in the incubation protocol: F0 larvae were incubated under OA conditions from 2 dph onwards, while F1 was incubated from fertilization onwards to OA, with the addition of OW from 2 dph onwards. It is possible that OA affected embryogenesis, e.g. Atlantic cod, embryos showed reduced hatching success and oxygen consumption rates when exposed to OAW (Dahlke et al., 2016). To summarize, different effects of OAW on larvae of F0 and F1 can be due to

parental or developmental effects and more research is necessary to determine the underlying mechanisms.

In juvenile European sea bass, growth rates of F1 W-Δ1000 fish were higher than those of F0 and F1 C fish. This matched SMR which were also higher in F1 W-Δ1000 juveniles than in F0 and F1 C. An interesting observation was that SMR was also higher in F1 C juveniles than in F0 juveniles. This was probably due to their different temperature life histories: while F1 C juveniles were reared at 15-18 °C throughout their life, F0 juveniles were reared at higher temperatures during larval development and were acclimated to colder temperatures from metamorphosis onwards. As the F1 W-A treatment was missing, I could not test whether the detrimental effects on larval growth persisted into juvenile phase.

5.5 Were OAW induced changes in growth, metabolic rates and mitochondrial function visible at the metabolite level?

One of the scopes of my thesis was to compare the results from other experiments within the FITNESS project to the findings on metabolome level in juvenile European sea bass. For this purpose I determined metabolic profiles of liver, white muscle, heart and gill tissue of juvenile European sea bass from group 3 (Wild). I used only four of the six OAW conditions of this group: C-A, C-Δ1000, W-A and W-Δ1000. It was possible to determine differences in the metabolic profiles of the different tissues fitting their different functions (Chapter 4).

Due to the long duration of the project, it was possible to look at different physiological traits in European sea bass and how they were affected by OAW. While my thesis focussed on the aerobic metabolism at different levels of biological organisation, Louise Cominassi centred her thesis on development and swimming capacity combined with one experiment where food rations were limited.

5.5.1 Metabolomics of liver tissue

Metabolic profiles of liver tissue originating from juveniles from the four OAW treatments showed significant differences, resulting in a clear separation of metabolic profiles of liver tissue from Wild C fish and liver tissue from Wild W fish in the PLS-DA (Figure 6). The observed higher glucose concentrations (Figure 7 and 9) in liver tissue of Wild W fish in comparison to liver of Wild C fish could indicate (A) a shift from lipid to carbohydrate metabolism in Wild W fish and increased glycolysis or (B) the exact opposite: decreased glycolysis. In case A, glucose would be high, as it would be provided to the cell via utilization of glucose storages, such as glycogen, to fit the needs of

increased glycolytic activity. In case B, glucose would be high, because the decreased activity of a metabolic pathway would result in an accumulation of its starting metabolites, in this case glucose. Results from this and other studies indicate that European sea bass juveniles increased glycolytic activity in warm-acclimated individuals. A shift from lipid to carbohydrate metabolism due to warm acclimation was already observed in other fish (*Pachycara brachycephalum*, warm acclimation to 5 °C, Windisch et al., 2011). Glycolysis is an effective aerobic metabolic pathway to generate ATP, an increase in glycolysis would therefore (1) provide additional energy for processes such as protein and lipid synthesis and (2) increase metabolic rates. Lower concentrations of free amino acids in combination with increased growth rates indicate increased protein synthesis in W life-conditioned fish, as AAs are the building blocks for proteins. Lower choline concentrations combined with higher O-phosphocholine concentrations indicate increased activity of the glycerophospholipid metabolism, which finally results in the generation of phosphatidylcholine, the major phospholipid of eukaryotic cell membranes. This means that in W life-conditioned fish, carbohydrates are used in high amounts to generate energy for increased protein and lipid synthesis in liver tissue. The more pronounced change of these pathways and consequently the higher protein and lipid synthesis is reflected in the higher SGR and higher condition factor of fish from the W life condition in comparison to fish from the C life condition, 1.43-1.67 and 1.21-1.26, respectively. Although I did not determine metabolic rates in Wild fish, I determined SMR of F1 C-A, C-Δ1000 and W-Δ1000 juveniles. SMR was increased in F1 W-Δ1000 juveniles in comparison to both F1 C groups, supporting the notion of increased glycolysis in liver tissue. For further studies, it would be interesting to also measure the non-polar metabolites to gain insight into the lipid metabolism. Muscle and liver belong to the major storage sites for lipids (Sheridan, 1988), information about different depots might give further insight into the capacities to cope with additional energetic limitations, e.g. due to food deprivation. It would also be interesting to measure glycogen concentrations to compare storage capacities of this metabolite at the different treatments.

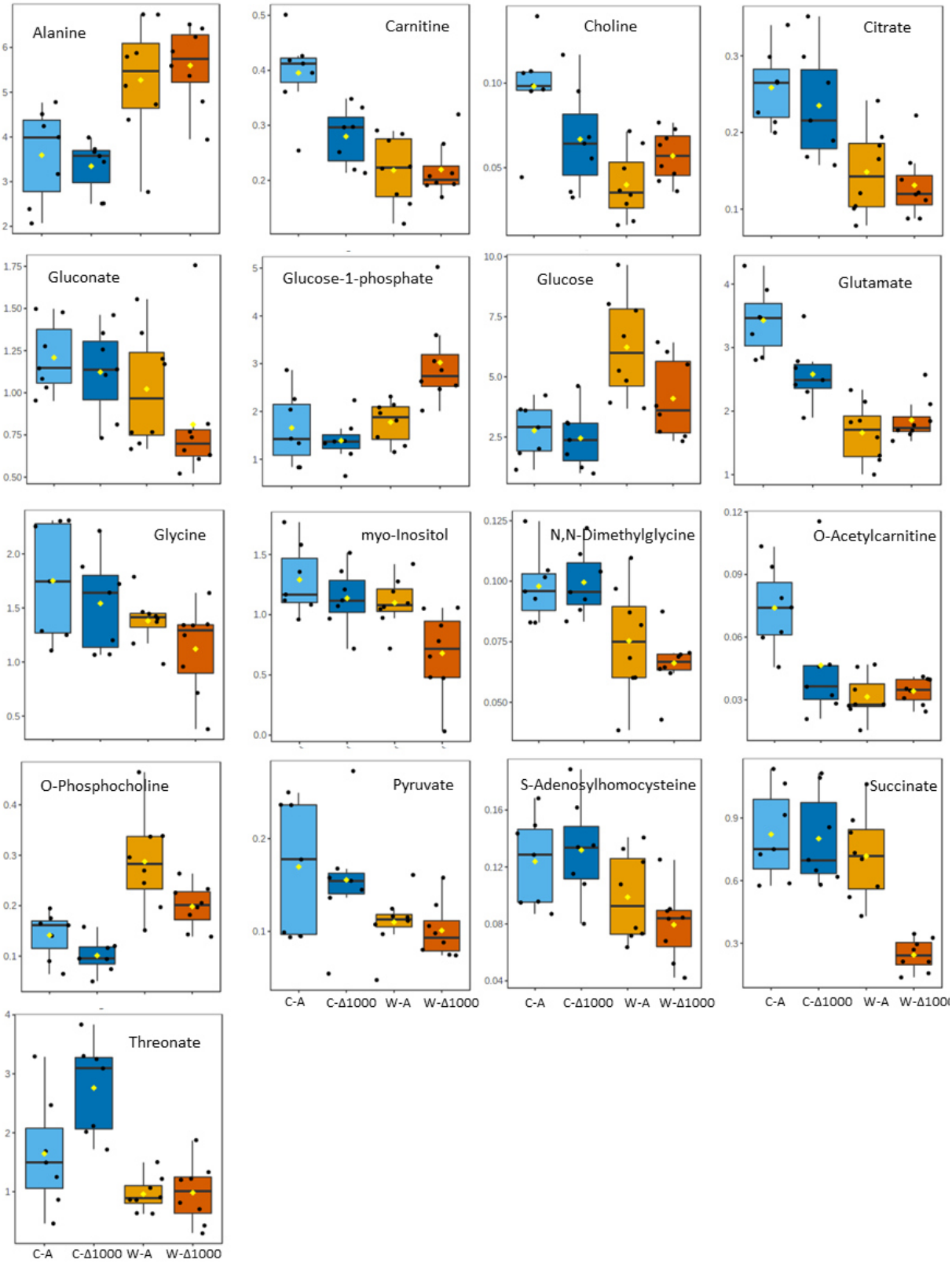


Figure 9 Absolute concentrations (µmol g^{-1} tissue weight) of significantly altered metabolites in liver tissue of Wild European sea bass juveniles as identified by PLS-DA. Boxplots show median, upper and lower quartiles and 1.5x interquartile range. Overlying symbols are the individual data points for each treatment. C – cold life condition (15-18 °C), W – warm life condition (C + 5 °C), A – Ambient PCO_2 (~650 µatm CO_2), Δ1000 – A + 1000 µatm CO_2, n=6-8.

5.5.2 Metabolomics of white muscle tissue

Glucose concentrations in white muscle tissue were also higher in Wild W juveniles than in Wild C juveniles (Figure 7 and 10), as observed in liver. Therefore, one would expect that these higher glucose concentrations translate to increased glycolytic activity within both tissues supporting higher protein and lipid synthesis and ultimately causing the higher SGR in Wild as well as F1 W juveniles in comparison to Wild and F1 C juveniles. Interestingly this didn't seem to be the case in white muscle tissue of European sea bass juveniles. It rather seemed that glycolysis was decreased in white muscle tissue of Wild W juveniles in comparison to Wild C juveniles: In contrast to liver tissue and all other investigated tissues, I could not detect glucose-6-phosphate in white muscle tissue. Higher concentrations of glucose-6-phosphate seemed to indicate increased glycolytic acitivty within the cell, as glucose-6-phosphate is produced during glycolysis and is, in contrast to glucose, not able to pass the cell membrane. The absence of detectable concentrations of this metabolite in white muscle tissue might be an indication that glycolysis is not increased in white muscle tissue under OW. In contrast to metabolites indicating glycolysis, lactate concentrations were higher in muscle tissue than in all other tissue, reflecting the importance of anaerobic metabolism in this tissue. However, lactate concentrations were not significantly different between OAW treatments, indicating that anaerobic metabolism is important in white muscle tissue, but not altered by OAW. In addition to those findings that indicate stable rates of glycolysis and anaerobic energy production between OAW conditions, the accompanying findings indicate that protein and lipid synthesis is not as increased in white muscle tissue as in liver tissue as well: Although choline was lower in Wild W juveniles, I could not find higher O-phosphocholine concentrations, which would accompany lower choline concentrations if they were induced by increased phosphoglycerolipid synthesis. Additionally, the only temperature affected AAs in white muscle tissue were glycine and lysine. Glycine concentrations were higher and lysine concentrations were lower in W compared to C life-conditioned fish and in case of lysine only in W-Δ1000 in comparison to all other treatments. This indicated that protein synthesis in white muscle tissue was not increased in W acclimated fish in comparison to C acclimated fish. These findings from the metabolic profiles indicate that protein and lipid synthesis is not increased in white muscle of Wild W fish compared to Wild C fish. They also confirm that glycolysis plays a minor role as energy source in white muscle tissue compared to liver tissue of W life-conditioned fish. It is possible that liver and white muscle tissue do not share the same fractions of SMR in W life-conditioned fish than they do share in C life-conditioned fish. In fact, it was found for marine stickleback that CCO activity in liver mitochondria plays a key role in setting the metabolic rate of the whole fish (Cominassi et al., 2022), resulting in higher fractions of liver respiration on

whole organism respiration at higher temperatures. It is possible, that similar processes occurred in European sea bass, provisioning the liver with sufficient energy for increased protein synthesis, while energy production and protein synthesis are not increased in white muscle tissue under OW. This undisturbed protein synthesis in white muscle tissue might explain our findings of swimming performance in Wild larvae: we observed a trade-off between growth and swimming performance in Wild larvae (Cominassi et al., 2019). Increased growth due to increased protein and lipid synthesis in liver tissue without simultaneously increased protein synthesis in white muscle tissue might explain altered condition factor as well as altered swimming performance in W compared to C life-conditioned fish.

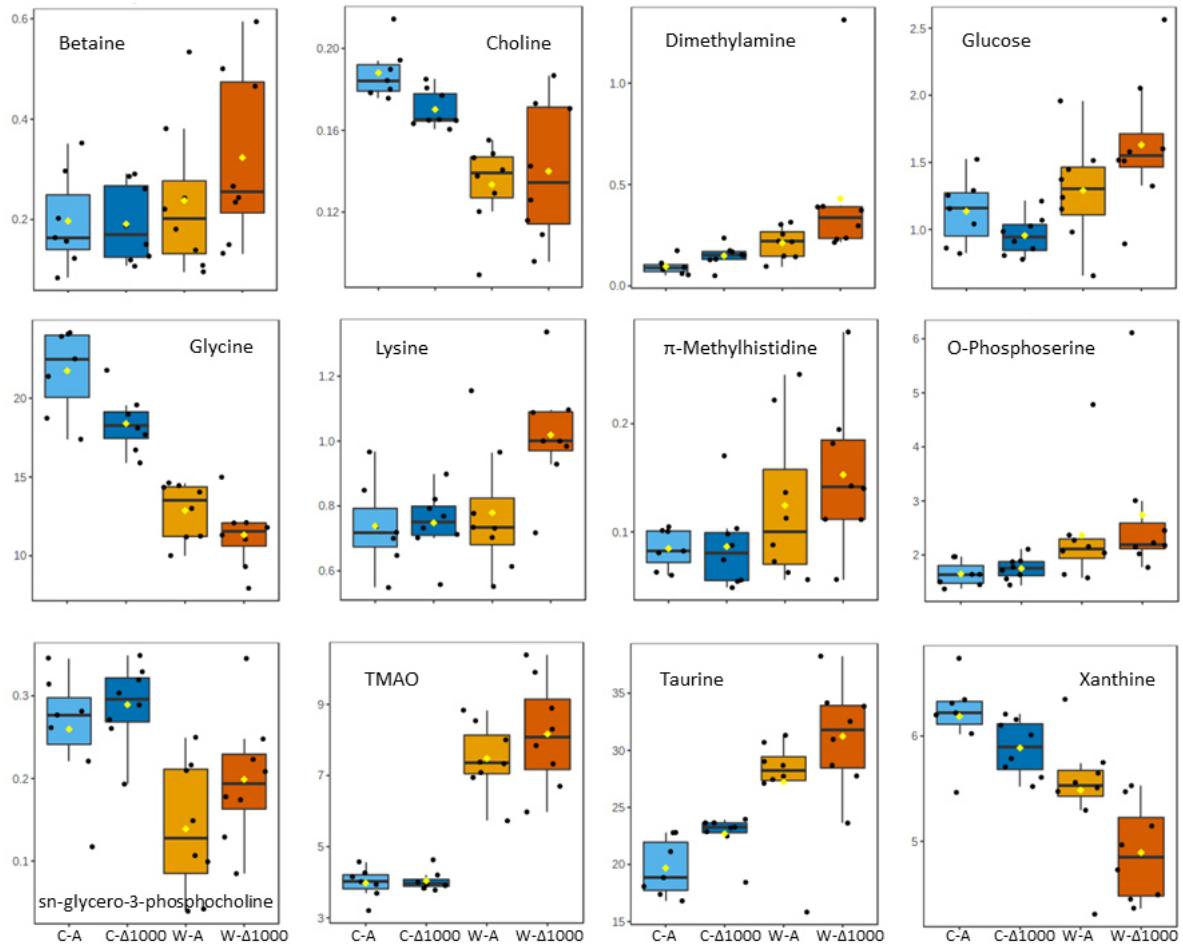


Figure 10 Absolute concentrations (µmol g^{-1} tissue weight) of significantly altered metabolites in white muscle tissue of Wild European sea bass juveniles as identified by PLS-DA. Boxplots show median, upper and lower quartiles and 1.5x interquartile range. Overlying symbols are the individual data points for each treatment. C – cold life condition (15-18 °C), W – warm life condition (C + 5 °C), A – Ambient PCO_2 (~650 µatm CO_2), Δ1000 – A + 1000 µatm CO_2, n=6-8.

5.5.3 Metabolomics of heart tissue

The capacity of the cardiovascular system to supply the tissues with oxygen seems to be the key process in determining the thermal sensitivity of a fish (Pörtner & Lannig, 2009). Consequently, the

heart plays a key role in shaping an organism's tolerance to increased temperatures, especially under acute warming. Decreased concentrations of O-phosphocholine, sn-glycero-3-phosphocholine and taurine in heart tissue of Wild W compared to Wild C European sea bass (Figure 7 and 11) oppose the increased activity of the glycerophospholipid metabolism in liver. Taurine is involved in diverse physiological functions, including cellular homeostasis, membrane stabilization and heart rate (Fugelli & Thoroed, 1990). The decreases in these three metabolites might indicate membrane decomposition. This contradicts the findings of mitochondrial capacities in permeabilized heart fibres, where Wild W juveniles displayed increased respiratory control ratios of mitochondria in comparison to Wild C juveniles. It therefore seems more plausible that decreases in these metabolites indicate that membranes are in a steady state. It could be interesting to determine the composition of membrane lipids.

Measurements of mitochondrial capacities in heart mitochondria of Wild juveniles showed that heart mitochondria performed better in W life-conditioned fish than in C life-conditioned fish. However, when mitochondria of W-Δ1000 fish were facing an acute temperature decrease, respiration rates of CII were inhibited. CII catalyses the oxidation of succinate to fumarate. Although, CII respiration rate was not reduced at the acclimation temperature in W-Δ1000 juveniles, fumarate is significantly reduced in W-Δ1000 heart tissue in comparison to all other treatments, possibly indicating an impairment of CII.

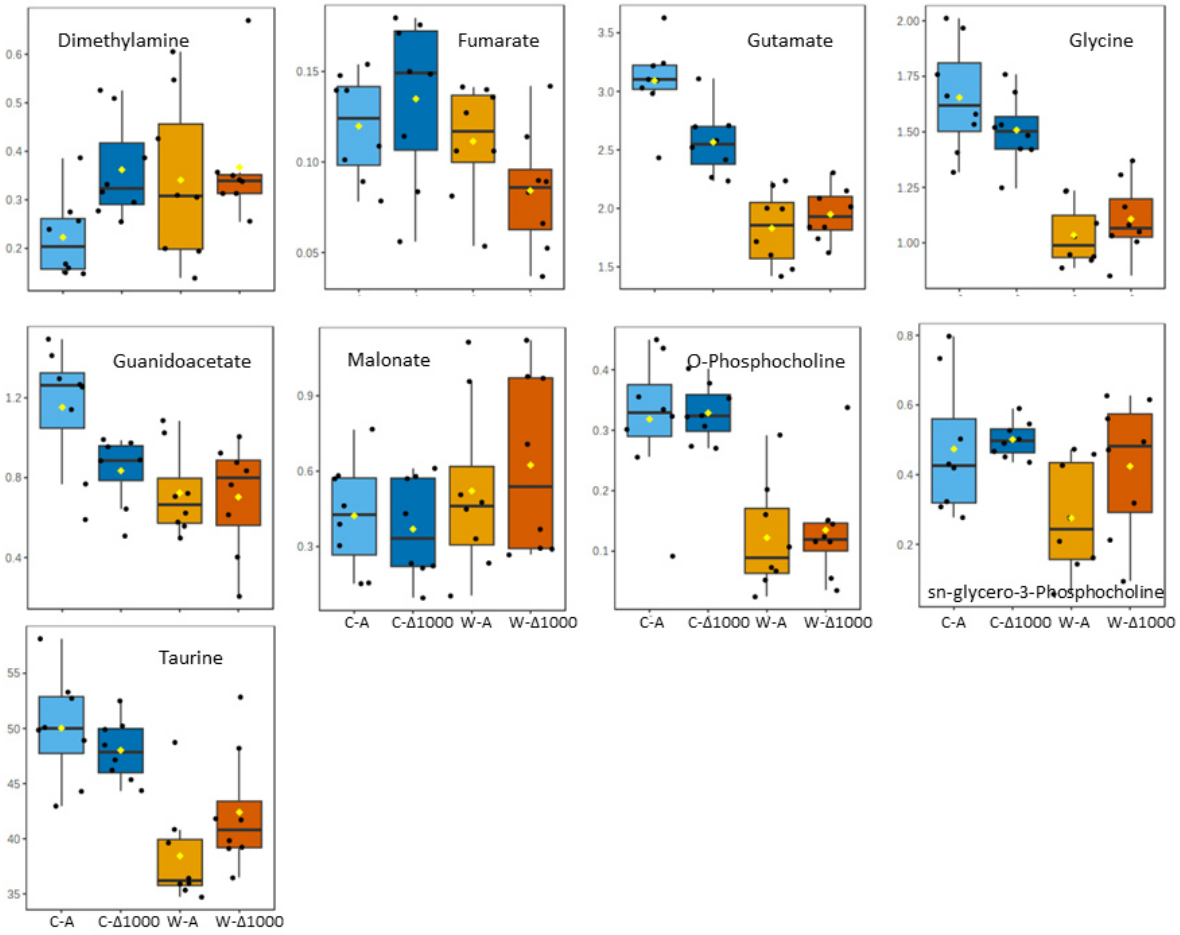


Figure 11 Absolute concentrations (µmol g^{-1} tissue weight) of significantly altered metabolites in heart tissue of Wild European sea bass juveniles as identified by PLS-DA. Boxplots show median, upper and lower quartiles and 1.5x interquartile range. Overlying symbols are the individual data points for each treatment. C – cold life condition (15-18 °C), W – warm life condition (C + 5 °C), A – Ambient PCO_2 (~650 µatm CO_2), Δ1000 – A + 1000 µatm CO_2, n=6-8.

5.5.4 Metabolomics of gill tissue

The other important organ in terms of oxygen supply to the tissues is the gill. In addition to its function as the major organ for respiratory gas exchange, the gill is important in maintaining acid-base and osmotic balance and is the main organ for ammonia excretion (as reviewed in Mommsen, 1984). Due to its significant role in acid-base regulation the gill is an important organ in OA research. To maintain its functions the gill used a significant amount of the consumed oxygen, which can add up to about 7% of the total oxygen consumption of the fish (Mommsen, 1984). The gill tissues displayed increased ascorbate and acetate (only in Wild C, Figure 7 and 12) concentrations after long-term acclimation to OA. Higher concentrations of OOAs under OA could be due to alterations of OOA concentration as a mechanism to maintain osmotic balance: Ions are actively excreted via the gill's surfaces and small organic osmolytes are used as compatible solutes to maintain homeostasis. As the gills are in direct contact with the water, they are much more involved in osmoregulatory processes than liver, heart and white muscle. Consequently, metabolic profiles of these tissues were not

significantly altered due to OA. However, alterations in single metabolites spread over groups and tissues were observed and reflect alterations in the steady-state within these tissues due to OA.

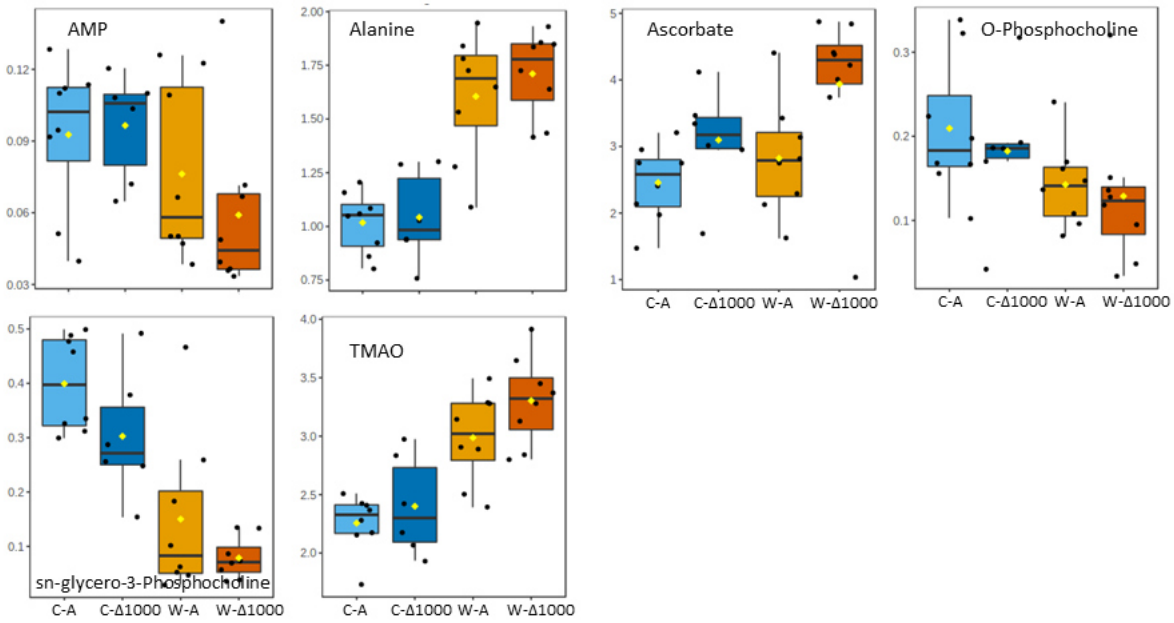


Figure 12 Absolute concentrations (µmol g^{-1} tissue weight) of significantly altered metabolites in gill tissue of Wild European sea bass juveniles as identified by PLS-DA. Boxplots show median, upper and lower quartiles and 1.5x interquartile range. Overlying symbols are the individual data points for each treatment. C – cold life condition (15-18 °C), W – warm life condition (C + 5 °C), A – Ambient PCO_2 (~650 µatm CO_2), Δ1000 – A + 1000 µatm CO_2, n=6-8.

5.6 Future Perspectives

I found increased growth and accelerated development in W life-conditioned larvae in comparison to C life-conditioned larvae in all my experiments on larval European sea bass. As described and discussed in the chapter 2 to 5, smaller animals at certain developmental stages were observed in other studies on larval fish before (e.g. Seikai et al., 1986; Minami & Tanaka, 1992; Fuiman et al., 1988, 1998). Only few fish seem to increase size at elevated temperature at certain developmental stages, (e.g. starry flounder, Policansky, 1982). Differences in size at certain developmental stages induced by different rearing temperatures usually occur quite early in the development of the fish and increase during ontogeny. Therefore, it might be worthy to incubate the fish from embryonal stage under increased temperatures, which was not feasible with my setup. Incubating just the eggs and not their parents under increased temperatures would mean an acute temperature change for the eggs directly after fertilization. Acute temperature changes often have stronger effects on marine organisms than long-term acclimation, e.g. while acute temperature increase led to impaired mitochondria in permeabilized heart fibres in publication I, long-term acclimation to OW led to improved mitochondrial performance in W compared to C fish. Consequently an acute temperature

change during embryonal phase might induce results, which do not reflect OW, which is a long-term process. Therefore the OW and OAW treatments in groups 2 and 3 of the FITNESS project were started from larval phase onwards by increasing the temperature over several days to minimize acute effects, starting at 2 dph. A slow increase over several days would not have been possible during the five days of embryonal development. Therefore, to avoid biases due to acute warming during embryonic stage, the temperature during embryonal development was set to 15 °C for all treatments in group 2 and 3. As the temperature increase for the OW and OAW treatment started as early as 2 dph, it was still some days prior to mouth opening, so the early larvae still depended on provisioning from the egg chorion. Nevertheless, future OW and OAW studies should include breeding at higher temperatures. This would avoid acute temperature change during embryonal ontogeny while still allowing for OW and OAW conditions during this stage. However, these kinds of experiments on large fish such as European sea bass are costly in terms of money, space and man-power, as this would mean for experiments with four OAW conditions to already rear the broodstock fish and the eggs under these four conditions. Rearing broodstock fish under two OA conditions as in Publication II was already intensive work, which was only possible due to the big collaboration in this project, rearing them additionally at two different temperatures would not have been possible.

In addition to OW and OAW at earlier development salinity and nutrition should be taken into account when determining the ability of European sea bass to survive under future conditions in our oceans. European sea bass late larvae and early juveniles move and live close to the coast, where they develop and grow to maturation within the following years (Dando & Demir, 1985). It has been shown in early studies on European sea bass that European sea bass late larvae as well as European sea bass juveniles of different sizes and ages can grow well at reduced salinities and might even profit from lower salt concentrations, although an interaction between temperature and salinity exists (e.g. Chervinski, 1975; Alliot et al., 1983). For the best predictions of European sea bass capacity to cope with climate change, it might be considered in future studies to combine different salinities with the different OAW scenarios, as it might play a role whether the European sea bass juveniles are coping with OAW in estuaries or in sea water. As full factorial designs with several drivers within the same experiment would result in a high number of treatments, it might be worth to compare control conditions to conditions with all drivers combined. This would not allow to determine whether effects are additive, antagonistic or synergistic, but would allow reasonable predictions of the capacities to cope with future conditions. In addition to salinity in the environment of juvenile European sea bass, food availability plays an important role, too. Sea bass in the bay of Mont-St-Michel feed today in the food-rich saltmarshes during high tide (Laffaille et al., 2001). If food availability in these salt marshes decreases accompanied by higher demands due to increased growth

rates, juvenile European sea bass might suffer from starvation. Altered food availability can also arise from differently timed development under increased temperature. While today the availability of food at the right sizes matches the timing of larval development, it might be possible that the needs of larvae and the availability of food at certain sizes might be shifted under future conditions. Therefore, not only experiments on larval development under OAW and food deprivation are necessary to predict larval survival in future oceans but also the effects of OAW on the abundance and timing of larval food.

The decision to work on polar metabolites resulted in findings about the amino acid and energy metabolism, as well as the structure of cellular membranes. In the future the addition of the non-polar metabolites might give further insights into the metabolic changes caused by OA, OW and OAW. Other studies observed increasing fat storage after cold acclimation, e.g. Zhang et al. (2021) in European sea bass and Brodte et al. (2007) in boreal eelpout (*Zoarces viviparus*). The difference between these studies and my study is probably due to the fact that I acclimated the cold life-conditioned fish at ambient temperature and not below as they did. However, it might be worth measuring the fat content of European sea bass liver to test whether fat content in liver tissue is lower in W life-conditioned fish than in C life-conditioned fish and whether the profile of the lipids has changed with temperature.

5.7 Conclusion

Although this thesis raised several new questions, which should be answered in future OAW research on European sea bass, it also added some new pieces to the puzzle how large temperate fish species might react to climate change. For European sea bass from the northern distribution range of this species, I can conclude that its capacity to thrive under future OAW conditions highly depends on availability of sufficient food to supply its increased growth rates and counteract negative effects, such as decreased food conversion efficiency, decreased swimming performance and possibly altered behaviour.

Juvenile European sea bass would benefit from increased temperatures if their environment is providing them with sufficient food to support their high growth rates. The physiological traits which I determined indicated that juvenile European sea bass aerobic metabolism is only challenged when faced with another driver in addition to OAW. Increased temperatures led to increased growth rates in juvenile European sea bass, which would mean that they literally grow out of the mouths of their predators and therefore reduce predation pressure. I could not observe detrimental effects of OAW on SMR, which was higher in juveniles under OAW than in juveniles from ambient conditions. Long-

term acclimation to higher temperatures increased mitochondrial functionality as seen in higher respiratory control ratios. However, I have seen that CII respiration in heart mitochondria of juveniles from OAW condition was inhibited when facing an acute temperature change, so an additional driver. To summarize, the aerobic metabolism as well as growth rates was increased in warm acclimated juvenile European sea bass, while OA did not have any effects, if not combined with an additional driver. This was not the case for physiological traits related to nourishment. Cominassi et al. (2020) showed decreased food consumption, impaired food conversion efficiency and decreased growth rates in fish acclimated to the OAW treatment. Although there are indices that European sea bass juveniles could be challenged under OAW, especially with additional drivers, they might benefit from high growth rates. Those high growth rates were observed in W life-conditioned fish in comparison to C life-conditioned fish and would translate to reduced predation pressure. However, high growth rates require that food availability meets the demand.

The picture for European sea bass larvae is less clear and less positive as for the juveniles: Comparable to juvenile European sea bass, larval European sea bass might benefit from increased growth and accelerated development, which would also reduce predation pressure. On the other hand this increased growth and accelerated development came at a cost in larvae, which may threaten the survival of the individuals. Cominassi et al. (2019) observed decreased swimming performance, due to altered muscle development in OW and OAW larvae. Additionally, larval resilience to OAW seemed to be impaired in the second generation raised under OA conditions with the addition of OW in F1. Although RMR was not impaired in OW and OAW larvae, size at metamorphosis was significantly decreased in OAW. This means that these larvae do not display beneficial growth performance, but might suffer from the same costs of high temperature as the larvae raised just at OW and not OAW.

To conclude this work, some pieces of the puzzle of European sea bass metabolism have been added to the big picture. This work has shown that research on large temperate fish species with long generation times is necessary. To gain meaningful insights into the physiology in a changing climate it is crucial to combine at least both drivers OA and OW, however, it would be reasonable to add other drivers such as food limitation, as negative effects of OAW could be compensated under *ad libitum* feeding, and to look at the potential of successive generations to cope with the conditions of their parents, as detrimental effects of larval growth only occurred in the second generation. With looking at “just” OAW, the European sea bass might cope well under future conditions, if it survives the challenges larval phase and finds sufficient food to meet its demands in juvenile phase. However, additional drivers on top of warmer and more acidic oceans might add challenges.

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A Appendix

A1. Additional Materials and Methods information and results to growth data of larval Wild fish

The material and methods information are described in detail in 4.4 Materials and Methods of Chapter 4. This section provides additional information about larval rearing and sampling of larvae for growth analyses.

Larval rearing

Larval rearing was performed in a temperature-controlled room using black 35 L tanks initially stocked with *ca*. 5000 larvae tank^{-1}. The division into the experimental tanks took place at 3 dph (21.01.2016). During the following three days, the temperature for the warm condition was increased stepwise, 1 °C during the first day and 2 °C during each of the following days. The PCO_2 conditions were applied directly after division into the experimental tanks. Starting at 7 dph (mouth opening), larvae were fed with live artemia, hatched from High HUFA Premium artemia cysts (Catvis, AE 's-Hertogenbosch, Netherlands). Until 33 dph the artemia were fed to the larvae 24h after rearing cysts in sea water, afterwards the artemia nauplii themselves were fed with cod liver oil and dry yeast after 24 h and fed to the larvae after 48 h. The artemia were transferred to the larval rearing tanks from two storage tanks (one for each temperature) with peristaltic pumps, their concentration in the tanks was maintained high during the day, to allow *ad libitum* feeding, excess artemia left the tank via the waste water outflow. The 15 h photoperiod in the larval rearing room lasted from 7 am to 10 pm, the light intensity increased progressively during the larval rearing period from total darkness to 96 lux. To work in the larval rearing facilities, headlamps were used (set to lowest light intensity). Larval mortality was 10-80 %, without pattern for condition (data not shown). Water surface was kept free of oily films using a protein skimmer. Density of larvae was continuously reduced for different samplings, such as growth or metabolic profiling. Additionally, at approx. 750 dd, 300 larvae per tank were removed to decrease density in the tank, which corresponds to 38 dph and 49 dph for warm and cold life condition, respectively.

Larval growth

Larval were sampled at approx. 100 (mouth opening), 300, 500, 700 and 900 dd (metamorphosis). At the first sampling point, 20 larvae per tank were anaesthetized in ice cold water in the morning and photographed in groups of 4-6 larvae (Olympus E410, Olympus SE & Co.KG, Hamburg, Germany). The software ImageJ (Schneider et al., 2012) was used to determine BL of larvae, see Figure S1 in

Publication II on the definition of BL. At each of the following sampling point, 20 larvae per tank were anaesthetized with MS-222 (50 mg l^{-1}) in the morning prior to feeding and directly photographed individually with a microscope (Leica M165C), BL was again determined with ImageJ. Afterwards the larvae were stored in the fridge, until the afternoon, when they were dipped in deionised water, dried and weighed on a high precision balance. The larvae were than frozen in liquid nitrogen and stored and transported at -80 °C until dry mass measurements.

Table A 1 F- and p-values of fixed effects from the linear mixed models on growth of larval European sea bass.

	PCO_2 Treatment		Temperature		PCO_2:Temperature	
	F-value	p-Value	F-value	p-Value	F-value	p-Value
Larval growth						
Dry mass						
at metamorphosis	1.1668	0.2327	3.1502	0.1138	0.2779	0.6124
over time	5.938	0.016	30.087	0.0001	1.970	0.182
Body length						
at mouth opening	0.538	0.5975	0.077	0.787	0.701	0.515
at metamorphosis	1.1438	0.3161	9.8259	0.0139	0.0641	0.8065
over time	5.266	0.023	12.295	0.004	0.266	0.771

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Declaration of individual contributions to chapters with multiple authors

The work of my dissertation was split in two peer-reviewed publications as well as one manuscript draft under preparation for publication. The concept of the studies in Chapters 2 to 4 was developed by myself together with Dr. Louise Cominassi and the supervisors Dr. Felix Mark, Prof. Dr. Myron Peck, Prof. Dr. Guy Claireaux, Dr. Marta Moyano, Dr. Christian Bock and Dr. José-Luis Zambonino-Infante. In the following section, the contributions of each author of the publications are described.

Publication I

Howald, S.; Cominassi, L.; LeBayon, N.; Claireaux, G. and Mark, F.C. (2019). Future ocean warming may prove beneficial for the northern population of European sea bass, but ocean acidification will not. J.Exp.Biol. 222, jeb213017. (doi:10.1242/jeb.213017)

I codesigned this study with GC and FM. I shared rearing the fish as well as keeping the treatments under surveillance with LC and NLB. I conducted the experiments and the analyses of the results, including the writing of the R codes for statistical analysis, with advice from FM and GC. I was the principal athor of the manuscript, from the first draft to the final accepted version. FC and GC contributed with substancial advice to the first draft of the manuscript. All coauthors gave feedback to the manuscript. GC and FM acquired the funds and and carried out the supervision of the project. FM carried out the project administration.

Publication II

Howald, S.; Moyano, M.; Crespel, A.; Kuchenmüller, L.;Cominassi, L.; Claireaux, G.; Peck, M.A.; Mark, F.C. (2022): Effects of ocean acidification over successive generations decrease resilience of larval European sea bass to ocean acidification and warming, but juveniles could benefit from higher temperatures in the NE Atlantic. J.Exp.Biol. 225, jeb243802. (doi:10.1242/jeb243802)

I codesigned the experiments on F1 with MM, LC, MP, GC and FM. The experiments on F0 were codesigned by AC, MM, MP, GC and FM. I shared rearing the F1 fish as well as keeping the

treatments under surveillance with LC. For F0 fish, this was mainly done by AC. I conducted the experiments on F1 and AC conducted the experiments on F0. AC preprepared the results on F0 for statistical analyses and I preprepared the results on F1 and did the analyses of the results, including the writing of the R codes for statistical analysis, with advice from FM and MM. I was the principal athor of the manuscript, from the first draft to the final accepted version. FC and MM contributed with substancial advice to the first draft of the manuscript. All coauthors gave feedback to the manuscript. GC, MP and FM acquired the funds and and carried out the project administration. FM, MM, GP and MP supervised the project.

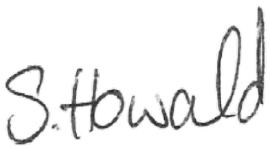
Chapter 4 – Metabolome analysis

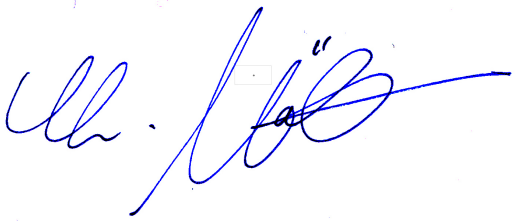
This chapter is a manuscript draft, prepared for submission as followed:

Titel: Changes in metabolites in liver, white muscle, heart and gill tissue of European sea bass raised under ocean acidification and warming conditions

Authors: Sarah Howald, Christian Bock, Guy Claireaux, Louise Cominassi, Marta Moyano, Myron Peck, José-Luis Zambonino-Infante, Felix C. Mark

I codesigned the experiments on with MM, CB, MP, GC, JLZI and FM. I shared rearing the fish as well as keeping the treatments under surveillance with LC. I conducted the experiments and did the analyses of the results, including the writing of the R codes for statistical analysis, with advice from FM and CB. I was the principal athor of the manuscript, from the first draft to the final accepted version. FC and CB contributed with substancial advice to the first draft of the manuscript. GC, MP and FM acquired the funds and and carried out the project administration. FM and CB supervised the project.





Sarah Howald

(Doctoral candidate)

Prof. Dr. Möllmann

(First supervisor)

Eidesstattliche Versicherung

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

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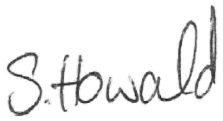
Osterholz-Scharmbeck, 30.12.2022	Sarah Howald 
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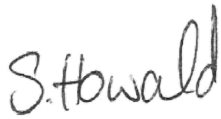
Osterholz-Scharmbeck, 30.12.2022	Sarah Howald 
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