# European sea bass, *Dicentrarchus labrax*, in a changing ocean

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## Abstract

Ocean acidification, caused by rising concentrations of carbon dioxide ($CO_2$), is widely considered to be a major global threat to marine ecosystems. To investigate the potential effects of ocean acidification on the early life stages of a commercially important fish species, European sea bass (*Dicentrarchus labrax*), 12,000 larvae were incubated from hatch through metamorphosis under a matrix of two temperatures (17 and 19 °C) and two seawater $pCO_2$s (ambient and 1,000 µatm) and sampled regularly for 42 days. Calculated daily mortality was significantly affected by both temperature and $pCO_2$, with both increased temperature and elevated $pCO_2$ associated with lower daily mortality and a significant interaction between these two factors. There was no significant $pCO_2$ effect noted on larval morphology during this period but larvae raised at 19 °C possessed significantly larger eyes and lower carbon:nitrogen ratios at the end of the study compared to those raised under 17 °C. Similarly, when the incubation was continued to post-metamorphic (juvenile) animals (day 67-69), fish raised under a combination of 19 °C and 1,000 µatm $pCO_2$ were significantly heavier. However, juvenile *D. labrax* raised under this combination of 19 °C and 1,000 µatm $pCO_2$ also exhibited lower aerobic scopes than those incubated at 19 °C and ambient $pCO_2$. Most studies investigating the effects of near-future oceanic conditions on the early life stages of marine fish have used incubations of relatively short durations and suggested these animals are resilient to ocean acidification. Whilst the increased survival and growth observed in this study supports this view, we recommend more work is required to investigate whether the differences in juvenile physiology observed in this study manifest as negative impacts in adult fish.

## 1 Introduction

Ocean acidification is widely considered as a major threat to marine ecosystems globally (Wood et al., 2008; Doney et al., 2009; Dupont and Pörtner, 2013; Kroeker et al., 2013). Caused by rising concentrations of carbon dioxide ($CO_2$) in the atmosphere, which recently (9th May 2013) exceeded 400 ppm for the first time since records started in 1958 (Showstack, 2013; Mauna Loa Observatory, Hawaii), this phenomenon has led to a 30% increase in the acidity of surface oceans over the past 200 years (Feely et al., 2009; Dupont and Pörtner, 2013). Assuming anthropogenic $CO_2$ emissions continue unabated, atmospheric concentrations of $CO_2$ are projected to reach ca. 940 ppm by 2100 (Vuuren et al., 2011; RCP

8.5 emission scenario), resulting in a concurrent shift in seawater carbonate chemistry and a further decrease in surface ocean pH (Meehl et al., 2007). Ocean acidification therefore poses a significant challenge to marine organisms globally, and poignantly, this process is occurring against a background of warming. Sea surface temperatures have increased by 0.74 °C over the past 100 years and global surface temperatures are projected to increase by a further 1-4 °C by the year 2100 (Meehl et al., 2007).

Whilst the body of literature that has investigated the impact of decreased seawater pH on marine organisms continues to grow exponentially (Gattuso and Hansson, 2011), there is still a dearth of information for other taxa, especially for fish (see recent meta-analysis of ocean acidification studies by Kroeker et al., 2013). Whilst it is undeniably important to study the effects of ocean acidification on groups such as calcifying invertebrates, which are hypothesised to be particularly vulnerable, there is also a pressing need to understand how this environmental change will impact on fish (Bignami et al., 2013), which are important sources of dietary protein globally (FAO, 2012) and a vital economic resource for countries and communities worldwide.

Although relatively few studies have examined the influence of near-future ocean acidification on marine fish, this field has yielded interesting, often seemingly contradictory, results with decreased seawater pH being shown to impact survival (Baumann et al., 2011), growth (Munday et al., 2009a; Frommel et al., 2011, 2013; Bignami et al., 2012), tissue health (Frommel et al., 2011), swimming ability (Munday et al., 2009b) and behaviour (Simpson et al., 2011; Nilsson et al., 2012; Domenici et al., 2012; Chivers et al., 2013). These variable effects often occur within the very same studies, highlighting a pressing need for further investigations into the responses of marine fish to ocean acidification across a wide range of species and life history stages.

Adult and juvenile fish possess competent physiological processes that enable these organisms to acclimate to changing environmental conditions (Claiborne et al., 2002) and to seemingly cope with very high $pCO_2$ or correspondingly low water pH (Holeton et al., 1983). However, it is hypothesised that early life stages are more vulnerable to environmental challenges because they possess higher surface area to volume ratios and have not yet fully developed the homeostatic mechanisms present in adult fish (Hurst et al., 2013). This hypothesis has been supported by experimental work. For example, incubating newly fertilised eggs (<24 h old) of the estuarine fish *Menida beryllina* (reared under 30 ppt salinity)

under a range of $CO_2$ concentrations (~390 to ~1,100 ppm) until ca. 1 week post-hatch revealed a consistent decline in both larval survival and standard length with increasing $CO_2$ concentration (Baumann et al., 2011). Thus, understanding the impact of ocean acidification on these early stages is crucial to accurately project the likely sensitivity of commercially important fish species to changing environmental conditions (Pankhurst and Munday, 2011).

The European seabass, *Dicentrarchus labrax*, is an important species for fisheries, and aquaculture in particular. In the decade between 2002 and 2011, global *D. labrax* landings totalled 103,476 t, equivalent to *ca.* 10% of global aquaculture production over the same period (Fisheries Aquaculture Information Statistics Service, 1999, Fishstat – see http://www.fao.org/fishery/statistics/software/fishstatj/en). Therefore the potential effects of near-future oceanic conditions on *D. labrax* could have clear ecological and economical ramifications. In this study we investigated the effects of near-future warming (+2 °C) and increased $pCO_2$ (1,000 µatm, selected to match RCP 8.5 emission scenario; Vuuren et al., 2011) on the early life stages of this species measuring larval survival, development rate and morphology, as well as juvenile development and metabolic rate. For comparison, measurements of $pCO_2$ recorded as a partial pressure in seawater in µatm typically differ from atmospheric measurements in ppm by <3% at 500 ppm and <5% at 800 ppm (see Branch et al., 2013). This study raised large numbers (12,000 initially) of a commercially important finfish species from hatching through their entire larval stage under the multiple stressors/drivers (see Boyd and Hutchins, 2012) of near future temperature and $pCO_2$.

## 2 Materials and Methods

Throughout the following, experimental time is abbreviated to the format $d_x$, indicating day at time *x*.

### 2.1 Systems

Incubations were carried out in 4 independent systems with experimental conditions following a matrix of 2 temperatures (17 °C and 19 °C) and 2 $pCO_2$s (ambient and 1,000 µatm) adjusted via injection of compressed $CO_2$ gas (Fig. 1). Seawater $pCO_2$ was maintained via a computerised feedback system which monitored seawater $pH_{NBS}$ and regulated the addition of $CO_2$ (Fig. 1). The adjustment of *p*CO2 was undertaken in the header tanks flowing into the stocked experimental tanks. Inevitably, and reflecting the situation in nature, $pCO_2$

conditions become elevated around animals that are locally contributing to the dissolved $CO_2$ in their environment through respiration. Thus ambient conditions were observed to be *ca.* 200 µatm $pCO_2$ higher than would be expected from purely atmospheric conditions, which was attributed to respiration. It was not possible to adjust the *p*CO2 in the experimental tanks because the necessary rates of bubbling and agitation would be detrimental to the welfare of the animals.

Experimental tanks were maintained at a salinity of 28.17 ± 0.22 (mean ± 1 standard deviation, SD) measured according to the practical salinity scale, and held within a 12 h light: 12 h dark photoperiod (median light = 6.5 µmol photon $m^{-2}$ $s^{-1}$, range = 4.2 – 12.4 µmol photon $m^{-2}$ $s^{-1}$). Temperature and $pH_{NBS}$ were measured in each experimental tank daily using a WTW type pH/Cond 340i probe, calibrated daily using a NIST/DIN-traceable calibration (WTW technical buffers at 7.0 and 10.0). Total alkalinity (TA), was measured in each header tank less frequently (typically twice a week).

## 2.2 Water chemistry

TA was measured using open-cell pentiometric titration (Total Alkalinity AS-ALK2 Gran Titration System, Apollo SciTech Inc., Bogart, Georgia, USA). The hydrochloric acid used for titration was validated using certified reference material from the laboratory of Andrew Dickson (SCRIPPS Institution of Oceanography, batch 108). The temperature of the samples and hydrochloric acid was maintained at 25 °C during analysis. 25 mL samples were analysed in triplicate and a mean TA value reported. Phosphate and silicate concentrations were measured using a continuous flow injection autoanalyzer (Bran Luebbe, SEAL Analytical Ltd, Fareham, Hampshire, UK). TA, phosphate and silicate results were converted to µmol $kg^{-1}$ using the density calculated from salinity and temperature. The $pCO_2$ of the system was then calculated using CO2SYS (Lewis and Wallace, 1998) with equilibrium constants from Dickson and Millero (1987) and Dickson (1990) for $KHSO_4$. Input parameters into the software were TA, $pH_{NBS}$, temperature, salinity, phosphate and silicate.

## 2.3 Animals

Fertilised *D. labrax* eggs from a mixed spawn (multiple males and females) were purchased from Écloserie Marine de Gravelines, France, and transferred to 12 x 10 L incubators, each held within one of 12 x 150 L experimental tanks (3 tanks per system, see Fig. 1) at 13 °C and

ambient $pCO_2$. Upon hatch, 1,000 larvae were transferred from the incubators into each experimental tank and the incubators removed. Experimental conditions in each system were then ramped up to the required $pCO_2$ over 24 h and temperature at the rate of 1 °C $d^{-1}$. Animals were maintained for an experimental period of 42 d, fed *ad libitum* on rotifers (*Brachionus plicatilis*, over the period $d_{2\text{-}26}$, attaining 10 individual $mL^{-1}$) and enriched brine shrimp (*Artemia salina*, from $d_9$ onwards increasing to 1 individual $mL^{-1}$) twice daily and sampled on a regular basis.

## 2.4 Sampling

At each sampling time point post-hatch ($d_7$, $d_{14}$, $d_{28}$ and $d_{42}$) 10 *D. labrax* larvae were removed from each tank, killed by an overdose of anaesthetic (MS222; Acros Organics) and photographed with a Leica DFC 290 camera attached to a Nikon SMZ 800 stereo microscope. These larvae were then stored at -20 °C for subsequent freeze drying and determination of dry weight and elemental analysis. Mortality combined with the regular sampling meant that the final sampling on $d_{42}$ fully depleted one of the experimental tanks. The other tanks were emptied on $d_{46}$ and all the larvae counted before being returned. For comparative purposes, the number of larvae in each tank on $d_{42}$ was calculated from the $d_{46}$ values and any mortality recorded between $d_{42}$ and $d_{46}$. Larvae were maintained under experimental conditions for a further 35 days (to $d_{80}$).

The daily mortality rate, Z ($d^{-1}$), was calculated using Eq. 1.

$$N_t = N_0 e^{-Zt} - \sum_{i=1}^{n} r_i e^{-Z(t-t_i)} \quad (1)$$

Here, $N_0$ is the number of animals stocked into the tank, $N_t$ is the number of larvae in the tank at time $t$ and a number ($r_i$) of larvae were sampled at each sampling time ($t_i$).

Larval development was evaluated from the micrographs, with larval morphometric analysis and gut contents quantified using Leica Application Suite software, v3.8. Yolk sac volume was estimated from the length (L) and height (W) of the sac using the formula for a spheroid, $V = LH^2$ (Blaxter and Hempel, 1963) and the volume of oil droplets calculated from the formula for a sphere ($\pi r^3$ where $r$ = droplet radius).

Specific growth rate ($\mu$, $d^{-1}$) was calculated from Eq. 2, where $W_i$ = dry weight at $t_i$ and $W_j$ was the dry weight at $t_j$.

$$\mu = \frac{Ln\,(W_i) - Ln\,(W_j)}{t_i - t_j} \quad (2)$$

## 2.5 Weaning trial

The period when cultured fish are weaned onto dry food is typified by higher mortality. To investigate possible effects of experimental ocean acidification conditions in combination with this additional stressor, 6 glass aquaria were connected to the 2 systems set to 19 °C (ambient and 1,000 µatm $pCO_2$, 3 aquaria each). Fifty larvae ($d_{49}$ post-hatch) were then transferred from each 19 °C tank into a corresponding glass aquarium. Only the 19°C systems were used for this work. The larvae were then gradually weaned onto dry food over 7 d and maintained for a total of 26 d (larvae were $d_{75}$ post-hatch at the end of the weaning trial) with mortality recorded daily.

## 2.6 Respirometry

On $d_{67\text{-}69}$, post-metamorphic, juvenile *D. labrax* were taken from two tanks per treatment to determine their individual routine metabolic rate (RMR) and maximal metabolic rate (MMR). Custom built, closed re-circulating respirometers (75 mL) were used to measure water oxygen concentration. Water was pumped through each respirometer *via* a small, closed, external circuit using a peristaltic pump (75 ± 0.5 mL $min^{-1}$; model 2058, Watson Marlow Pumps, Falmouth, UK) and tubing with low oxygen permeability (Masterflex tygon tubing ). This ensured sufficient water movement throughout the chamber and even distribution of oxygen within the respirometer. Preliminary experiments were conducted using hypoxic seawater (*ca.* 20 % oxygen saturation) over a 4 h period to confirm no oxygen diffused into the respirometer during an experimental run. (Note: experimental oxygen consumption measurements were run for a maximum period of 20 min). Respirometers were housed in re-circulating waterbaths set to either 17 °C (± 0.1 °C) or 19 °C (± 0.1 °C) during oxygen consumption measurements.

Water oxygen concentration was measured using a 4-channel Firesting $O_2$ fibre-optic oxygen meter (Pyro-Science, Germany), fitted with retractable needle-type fibre-optic oxygen probes (Model OXYR50, Pyro-Science, Germany) and an integrated temperature sensor (Model TDIP15, Pyro-science, Germany). Juvenile fish were fasted overnight before being placed in the respirometers and allowed to acclimate (4 h) prior to measuring RMR (defined as allowing low levels of spontaneous activity; Burton et al., 2011). Fish from each experimental

tank (weight range: 52-521 mg wet weight; WW) were individually placed into separate respirometers. By this time ($d_{67\text{-}69}$), some of the experimental tanks were empty so only two experimental tanks were used from each treatment. Eight fish were taken from each experimental tank, except for Tank 8 (17 °C, ambient $pCO_2$), which supplied only four animals. Each respirometer was connected to the fish's tank of origin during the following 4 h acclimation to ensure conditions were maintained under the correct temperature and $pCO_2$ levels, and also that oxygen did not drop below 95 % oxygen saturation. The respirometers were covered in foil when they contained an animal to reduce light levels and disturbance. After acclimation, the respirometers were disconnected from these tanks so that measurements could be taken, with water circulation maintained using the peristaltic pump. The initial oxygen reading was recorded no longer than 2 min after the respirometer was disconnected from the acclimation set-up. Measurements of routine oxygen consumption were made over the course of 20 min, with water oxygen content measured within each individual respirometer every 2 min. Following the completion of oxygen consumption measurements, the animals were removed from the respirometer, euthanised as described earlier, rinsed briefly in fresh water to remove external salts, blotted dry and weighed (WW).

To measure MMR in juvenile sea bass, individuals were exhaustively exercised using a burst swimming protocol similar to that described by Killen et al. (2007), using small, open, circular swim chambers similar to those designed by Nilsson et al. (2007). Briefly, 8 fish (weight range: 58 – 649 mg WW) were collected from two experimental tanks per treatment and placed individually into swim chambers; only 4 fish were available from Tank 8. Swim chambers were filled with water from the experimental tank from which the fish originated to ensure the correct temperature, $pCO_2$ and oxygen levels. Swim chambers were placed on a magnetic stirrer, with water speed regulated by a stirring magnet in the bottom of each chamber. No attempt was made to calibrate the speed of the water current during the experiment because the small size and circular shape of swim chambers meant flow rate would have varied between the inner and outer edges of the chamber (Nilsson et al., 2007). However, as a burst swimming protocol was used rather than measuring critical swimming speed ($U_{crit}$), absolute speed is of little importance as fish were swam to exhaustion. Water motion was set in place once the fish were placed in the swim chambers and the fish began to swim against the current. The speed was set to a point at which the fish began to perform burst type swimming and this speed was maintained until the fish reached exhaustion (when they were unable to maintain their position in the water column, either resting on the bottom

or the side of the swim chamber; this was usually achieved within 7-10 min). Fish were removed from swim chambers immediately after reaching exhaustion, briefly exposed to air (30 s; Roche et al., 2013), and then placed in respirometers with oxygen consumption recorded each minute over the first 10 min of recovery. This method of measuring MMR uses the excess post-exercise oxygen consumption (EPOC) principle (Gaesser and Brooks, 1984). Oxygen concentration was shown to decrease at a constant linear rate during this recovery period, and therefore maximal oxygen consumption was calculated using the data across the entire 10 min recovery period.

Rates of oxygen consumption (mg $O_2$ $h^{-1}$) were calculated during each trial using a linear regression of the data. Data were then normalised against WW to account for metabolic scaling. Whilst much uncertainty surrounds the effects of body size on metabolic rate in teleosts, and specifically the precise value of the metabolic scaling coefficient, we used a metabolic scaling exponent of 0.8, as proposed for juvenile fish (Clarke and Johnston, 1999) using Eq. (3):

$$LnY = Lna + bLnM \quad (3)$$

where *LnY* is the natural log of the metabolic rate (RMR or MMR), *LnM* is natural log of body mass (WW, g), *b* is the scaling exponent and *Lna* is the natural log of measured $MO_2$ (mg $O_2$ $h^{-1}$). This value was then normalised to a 300 mg fish. The factorial aerobic scope (FAS) was calculated as mean MMR/RMR.

## 2.7 Carbon-nitrogen analysis

Freeze-dried samples were used for elemental analysis to investigate the carbon and nitrogen content of animals during the trial. Samples were homogenised using a pestle and mortar and then placed overnight in a dessicator. Samples (weight range: 0.255 – 0.330 mg) were then weighed into tin capsules (Elemental Microanalysis, Okehampton, UK), sealed, and analysed using an ANCA GSL elemental analyser interfaced with a PDZ Europa 20/20 isotope ratio mass spectrometer. Sample run time was typically 12 min; 8 standards (isoleucine: 1.5 – 50 µg N, 5 – 250 µg C) were run at the beginning of the run and 4 standards were run every 12 samples to enable correction for any drift.

## 2.8 Statistical analysis

Data were analysed using GraphPad Prism 6 for Windows (GraphPad Software, San Diego, USA). Data were analysed using one or two-way ANOVA, matched by tank where appropriate.

# 3 Results

Full data have been logged with BODC (doi will be supplied before publication). In the text that follows, tables and figures located in the Supplementary Information PDF file are labelled with the prefix "S".

## 3.1 Main incubation ($d_{0\text{-}42}$)

Table 1 shows experimental conditions and mean measurements of pH, temperature and TA, and calculated $pCO_2$ values over the duration of the experiment. Hatching occurred under ambient conditions, with 81 ± 15.3 % (mean ± 1 SD) of larvae hatching successfully. Mean yolk sac volume at hatch was 0.360 ± 0.075 $mm^3$ and yolk sacs were fully absorbed in all larvae by the next sampling time ($d_7$ post-hatch). Oil droplets, which are used after yolk reserves, were visible at $d_7$ but there was no significant $pCO_2$ or temperature effect on their residual volumes (two-way ANOVA, Table S1, Fig. S1).

All tanks still contained larvae at $d_{42}$ (Fig. 2a shows $N_{42}$ values for all tanks), although this final sampling fully depleted tank 7. Two-way ANOVA showed a significant temperature effect ($F_{1,8} = 21.29$, $p < 0.01$) on final number, with warmer tanks showing higher numbers, but no $pCO_2$ effect (Table S2). Daily mortality rate ($Z$) was significantly affected by both temperature ($F_{1,8} = 22.79$, $p < 0.01$) and $pCO_2$ ($F_{1,8} = 9.099$, $p < 0.1$) and there was a significant interaction ($F_{1,8} = 6.207$, $p < 0.05$) between these two factors (two-way ANOVA, Table S3, Fig. 2b) with both increased temperature and elevated $pCO_2$ associated with lower daily mortality.

There was no significant treatment effect on larval dry weight (matched two-way ANOVA, Table S4, Fig. 3a), $\mu$, when calculated between each sampling period (two-way ANOVA, Table S5, Fig. 3b) or larval total length (matched two-way ANOVA, Table S6) during the course of the study. Other measurements, such as standard length (Table S7, Fig. S2), pre-anal length (Table S8, Fig. S3) and head height (Table S9, Fig. S4) showed significant ($p <$

0.05) treatment effects (matched two-way ANOVA) but post-test comparisons (Bonferroni) revealed that these effects were not attributable to the $pCO_2$ treatments and were also inconsistent across sample times. At the later sample times ($d_{28}$ and $d_{42}$ post-hatch) larvae reared at 19 °C had significantly larger eyes (measured as eye diameter) than those reared under 17 °C (matched two-way ANOVA, Fig. 4a, Table S10), consistent with them being developmentally more advanced. Similarly, *D. labrax* larvae reared at 19 °C had significantly lower C:N ratios at $d_{42}$ post-hatch than those reared at 17 °C (Fig. 4c, Table S11), indicating a more complete consumption of lipid originating from the yolk sac and oil droplets.

At later sample times it was possible to count the number of *A. salina* prey in the larval gut, although not with all animals. There was no significant difference in the number of *A. salina* larva$^{-1}$ between sample times or treatments (two-way ANOVA, Table S12, Fig. 4b). As there was no significant difference in final number between the treatments at 19 °C (Fig. 2a), grazing rates could be calculated for tanks at this temperature from counts of residual feed conducted in the morning before the larvae were fed, the known amount of food added, and the count of residual feed conducted in the afternoon before the second feed (5 h after the morning feed). There was no significant difference in mean grazing rate between the tanks incubated under ambient or 1,000 µatm $pCO_2$ on supply of either prey organism (*B. plicatilis* $d_{2\text{-}26}$; *A. salina* $d_{9\text{-}42}$; Table S13, Fig. S5).

## 3.2 Weaning trial

The use of glass aquaria during the weaning trial allowed individual mortality to be accurately recorded, coupled with the use of survival analysis (Mantel-Cox log rank test) to compare treatments. Survival analyses were performed between each replicate aquarium within each treatment, with no significant differences found between aquaria ($p = 0.6085$ and 0.2677 for ambient and 1,000 µatm $pCO_2$ respectively). Replicates were then pooled for survival analysis between treatments, with no significant difference found between fish reared under ambient or 1,000 µatm $pCO_2$ ($p = 0.7039$, Fig. S6). There was no significant difference in larval dry weight between treatments at the end of the trial (unpaired t-test, $F_{2,2} = 8.7156$, $p = 0.2058$, Fig. S7).

### 3.3 Respirometry

Juvenile fish ($d_{67\text{-}69}$) were used for respirometry experiments. By this time, two-way ANOVA showed a significant effect on WW for both temperature ($F_{1,56} = 57.20$, $p < 0.0001$) and $pCO_2$ ($F_{1,56} = 7.356$, $p < 0.01$) and a significant interaction between the two factors ($F_{1,56} = 5.301$, $p < 0.05$). Fish raised at 19 °C were significantly heavier than those at 17 °C, and fish raised under 19 °C / 1,000 µatm $pCO_2$ were also significantly heavier than those raised under 19 °C / ambient $pCO_2$ (two-way ANOVA, Table S14, Fig. 4a). There was also a significant temperature effect on length (total length; $F_{1,56} = 64.86$, $p < 0.0001$), with fish raised at 19 °C significantly longer than those raised at 17 °C, but no $pCO_2$ effect (two-way ANOVA, Table S15, Fig. 4b).

RMR and MMR were analysed initially for differences between the two tank replicates, with no significant differences found within each treatment (two-way ANOVA, tank and RMR/MMR as factors, P range = 0.0807 – 0.7524). RMR or MMR values were then pooled and analysed using two-way ANOVA for differences between treatments. Neither temperature nor $pCO_2$ had a significant effect on RMR and there was no interaction between these two factors (table S16, Fig. 4c) for this parameter. MMR showed a significant temperature effect ($F_{1,56} = 4.896$, $p < 0.05$), with fish under warmer temperatures exhibiting increased MMRs compared to those at colder temperature but there was no significant $pCO_2$ effect or interaction (Table S17, Fig, 5c). Juvenile *D. labrax* exposed to ambient $pCO_2$ showed a substantial increase in FAS between 17 and 19°C (from 1.59 to 2.14, Fig 5c) that was not evident in fish raised under 1,000 µatm $pCO_2$ (1.77 to 1.64; Fig. 5c).

## 4 Discussion

Larvae of European sea bass, *Dicentrarchus labrax*, appear to be resilient to near-future ocean acidification, showing decreased mortality under a near-future temperature and atmospheric carbon dioxide concentration. Post-metamorphic (juvenile) sea bass raised since hatch under warmer conditions also showed significantly higher maximal metabolic rates (MMR) than those raised under cooler conditions. Juvenile *D. labrax* raised under a combination of increased atmospheric $pCO_2$ and temperature were significantly heavier and, interestingly, showed a lower aerobic scope than those raised under the increased temperature but ambient $pCO_2$. These findings may have important implications for both sea bass in a changing ocean

and also for the interpretation of results from other studies that have shown resiliency in marine teleosts exposed to higher $pCO_2$s.

Daily mortality, *Z*, corrects for the periodic removal of experimental animals through sampling, enabling comparisons to be drawn between treatments. It should be noted, however, that *Z* assumes mortality is constant over the experimental period. It is more likely that mortality rates varied during the course of this trial but direct measurements of survival are always problematic in these sort of studies. Volumetric sampling requires the distribution of the larvae to be homogenised (e.g. through agitation) before the samples are taken, which has predictable deleterious effects on survival. Similarly, counting all of the larvae in a tank at specified times requires emptying the tank, incurring a substantial level of mortality. The most effective way to record mortality would be to sacrifice tanks for counting at specified times, which was beyond the scope of this trial. *Z* is therefore a convenient statistic for estimating average mortality over the course of this study.

The majority of studies that have investigated the effect of near-future ocean conditions on the early life stages of marine fish species have advocated some form of resiliency. Most of these studies have concentrated on eggs and post-hatch larvae raised for relatively short durations. Incubating eggs of Atlantic herring (*Clupea harengus*) under a $pCO_2$ range (480 – 4635 µatm) did not affect embryogenesis or hatch-rate or the total length, dry weight, yolk sac area and otolith area of newly hatched larvae, and whilst there was a significant decrease in the RNA:DNA ratio with increasing $pCO_2$, it was only significant when the highest treatment $pCO_2$ (4,635 µatm) was included in the analysis (Franke and Clemmesen, 2011). Similarly, Frommel et al. (2013) did not see any $pCO_2$ effect on the survival, hatch rate, growth or biochemical composition of eggs and non-feeding larvae (max. 11 d post-hatch) of Baltic cod, *Gadus morhua*, at a range of $pCO_2$s (380 - 4,000 µatm). Raising larval cobia (*R. canadum*) for 22 d under 800 and 2,100 µatm $pCO_2$ had no effect on somatic growth, development, swimming ability or swimming activity, although larvae raised under elevated $pCO_2$s did possess significantly larger otoliths than control animals (Bignami et al., 2012). Finally, Hurst et al. (2013) raised walleye pollock (*Theragra chalcogramma*) embryos and larvae under a range of $pCO_2$s (287 – 1,933 µatm) to ca. 30 d post-hatch and saw only "minor responses".

Incubations for longer time periods may be required for more subtle effects of near-future conditions to emerge. Unfortunately, fish larvae are prone to considerable levels of mortality under even the most stringent culture conditions so longer studies are challenging and require

more sophisticated facilities. Signals of differential survival or growth can easily be hidden in this background noise of larval mortality. Our study used 12,000 larvae, distributed across 12 experimental tanks in a state-of-the-art aquaculture system, with calculated daily mortalities (0.02 – 0.07 $d^{-1}$) substantially lower than those in similar study using larval cobia (*Rachycentron canadum*) and 800 µatm $pCO_2$ (0.13 – 0.18 $d^{-1}$; Bignami et al., 2012), yet our final sampling, coupled with mortality, fully depleted one of the tanks. In fact, larval mortality is very likely the reason for the short durations of many of the other studies that have investigated the effects of ocean acidification on larval fish. Suitable culturing facilities, such as mesocosms, allow more substantial incubation times. Frommel et al. (2011), who incubated newly-fertilised *G. morhua* (Norwegian coastal cod) eggs in mesocosms with flow-through of fresh seawater and natural zooplankton prey for 7 weeks, saw an apparent (but not significant) increase in survival under a substantially higher $pCO_2$ of 1,800 µatm compared to control animals at 380 µatm $pCO_2$ (324 ± 513 larvae after 7 weeks vs. 153 ± 134, mean ± 1 SD), although they also recorded some organ damage, especially under extreme hypercapnic conditions of 4,200 µatm $pCO_2$.

Whilst a substantial body of work has investigated metabolic rate in fish, those studies have used either larger (fingerlings through to adults) or very small (eggs or young larvae) life stages so appropriate values for comparison to the metabolic rates of the recently metamorphosed fish used in this study are rare in published work. The values for RMR, MMR and FAS in our study compare well with the only study we are aware of that measured the metabolic rate of marine fish over their entire life histories (Killen et al., 2007). Killen et al. (2007) measured the standard and maximal metabolism in three marine fish species (ocean pout, *Macrozoarces americanus*; lumpsucker, *Cyclopterus lumpus*; and short-spined sea scorpion, *Myoxocephalus scorpius*) and showed that metabolic rate and aerobic scope were highly dependent upon the size of the animals. Hence comparisons of our values with other life stages of even the same species are not appropriate. Killen et al. (2007) produced biphasic (pre- and post-metamorphosis) regressions of standard metabolic rate (SMR), MMR and FAS for the entire size range of each species (incorporating a change in mass of over 6 orders of magnitude for some species) enabling direct comparisons with the values calculated for recently metamorphosed juveniles in our study. The metabolic rates in our study agree with those recorded by Killen et al. (2007), although they are slightly higher than for the three species in Killen et al. (2007) because *D. labrax* is an active species, unlike the relatively sedentary benthic and semi-pelagic species used by these workers. This also means that RMR

will be considerably higher than SMR in *D. labrax*, unlike in Killen et al. (2007), because the sea bass continued to swim whilst the RMR was measured. The aerobic scopes calculated in this study are therefore probably underestimates.

When the incubation was continued past metamorphosis, juvenile seabass held at 19 °C and 1,000 µatm $pCO_2$ were significantly heavier than any other treatment group, including fish incubated at 19 °C but ambient $pCO_2$. Rapid growth is especially advantageous to young fish as it decreases the length of time an individual is vulnerable to a particular predator, decreasing size-specific mortality (Glazier, 2005), and has also been seen in a tropical reef species raised under elevated atmospheric $CO_2$ concentration. Orange clownfish (*Amphiprion percula*) grown at 1030 ppm $CO_2$ until they were settlement-stage juveniles were significantly longer and heavier than control fish (390 ppm $CO_2$), although it should be noted that these tropical reef fish show different developmental times and life history to the temperate species used in our study and were 11 d post-hatch when measured (Munday et al., 2009a).

It is interesting that the increased growth in *D. labrax* was not supported by an increased RMR. Similarly, there was no observed effect of $pCO_2$ or temperature on feeding in (pre-metamorphic) *D. labrax* larvae, although it should be noted that often it was not to possible to count the prey in the stomachs of individual larvae, reducing our ability to identify differences. In addition, the larvae were fed *ad libitum* so a snapshot of their stomach contents may not be the most appropriate method for determining feeding rate. We were unable to determine gut transit time and heavier larvae may have processed food more rapidly and so consumed more. On the other hand, the lack of difference in grazing rates between the two $pCO_2$s in the 19°C tanks supports support the finding that $pCO_2$ does not increase feeding rate. Increased growth may not therefore come at a cost, unless the aerobic scope of the fish is considered.

Larvae at 19 °C were an average of 72 degree days older than those at 17 °C (788 ± 4 vs. 716 ± 1, mean ± 1 SD; Note: a degree day is a value used in aquaculture to predict the stage of development of early life stages, it is calculated by multiplying the mean temperature in °C by the incubation time in d) by $d_{42}$ and whilst they did not show any difference in weight or length at this time, their lower C:N ratio suggests a greater degree of oil consumption and concomitant protein deposition, which would be expected to be mainly in the form of muscle (Rosenlund et al., 1983). Coupled with the fact that these animals also possessed larger eyes, it would appear that larvae raised at 19 °C were showing signs of being developmentally more

advanced by $d_{42}$ and that this was evident in length and weight by $d_{67\text{-}69}$. The increased FAS of the juveniles raised at 19 °C and ambient $pCO_2$ (2.14) compared to those raised at 17 °C and the same $pCO_2$ (1.59) is therefore consistent with the paradigm of Killen et al. (2007) that aerobic scope gradually increases through ontogeny. It is notable that fish raised under higher $pCO_2$ conditions did not follow this pattern; the calculated FAS was actually lower for fish raised at 19 °C (1.64) than those raised at 17 °C (1.77) under 1,000 µatm $pCO_2$. Aerobic scope describes an organism's capacity to perform any energetic activity above basal metabolism and a decreased aerobic scope could have severe implications for young fish, limiting the availability of energy for physiological activity or behaviours, such as escape responses. Munday et al. (2009b) saw a similar effect in experiments using adult coral reef fish; aerobic scope was decreased with both increasing temperature and $CO_2$ (to produce a pH of 7.8, equivalent of ca. 1,000 ppm; Munday et al., 2009b). Unlike Munday et al. (2009b), who acutely exposed wild animals (albeit with an acclimation period of 1 week), we measured decreased aerobic scope in fish raised under chronically elevated $pCO_2$.

The animals for this study came from aquaculture, which may, in part explain the results. Animals in aquaculture will routinely experience relatively high $pCO_2$s as a result of large numbers of animals respiring within a relatively small, enclosed environment. Indeed, the ambient $pCO_2$ in our system was observed to be *ca.* 200 µatm higher than would be expected from atmospheric $CO_2$ concentration alone. It is also worth noting that marine aquaculture facilities in the future will use water from a higher $pCO_2$ ocean with an already decreased buffering capacity that will only contribute to the resultant systemic decline in pH. In addition, fish are also often raised artificially under warmer temperatures than in the wild to promote growth. It could therefore be argued that animals reared through multiple generations in this environment may be more tolerant of warmer, higher $pCO_2$ conditions. Then again, fish in an industrial setting exist in an inherently unnatural environment with an abundance of food and absence of predators and interspecific competitors. Any energetic cost incurred by fish under near-future oceanic conditions, for example, will not be a strong selection pressure in an energy replete environment. Similarly, a reduced juvenile aerobic scope, such as observed in this study, will be of little importance to a cultured animal whereas it would be disadvantageous to a wild fish, especially if it persisted to adulthood. The experimental design used in this study, with *ad libitum* feeding, may therefore have limited ability to discover energetic costs for fish living in a near-future ocean. On the other hand, whilst a restricted diet regimen may reveal subtle effects on larval growth and development, larviculture is

challenging and such a diet may also result in high levels of mortality which, as discussed earlier, could conceal effects.

Further studies are required that raise other teleosts under near-future ocean conditions, for longer durations, to ascertain whether "resiliency" is maintained throughout ontogeny under natural conditions. The results of this study are cautiously optimistic for *D. labrax*, although it is important to discover whether the differences in juvenile physiology observed in this study manifest in adult fish.

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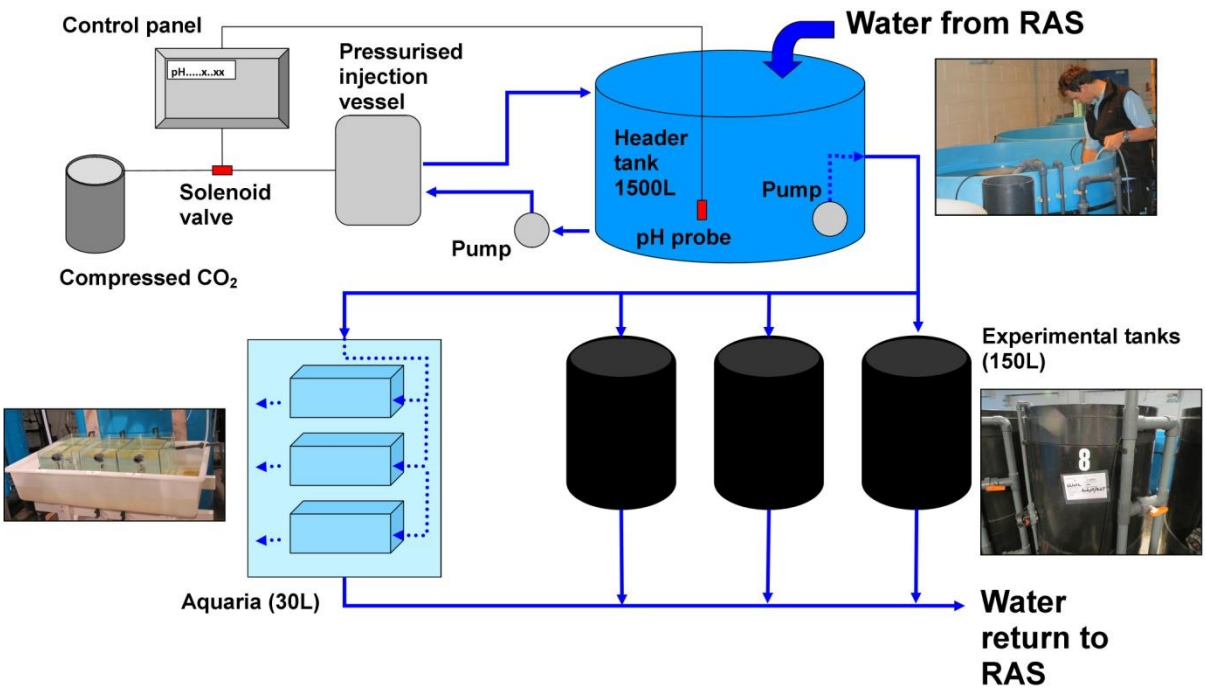
Table 1. Experimental conditions for the duration of the experiment (75 d). Temperature and $pH_{NBS}$ are calculated from daily measurements in the experimental tanks had they had ramped to the desired conditions and as long as they contained animals ($N$ = 41 - 67). Total alkalinity (TA), which was used to calculate the $pCO_2$ values, was measured in the header tanks less frequently (typically twice a week but less frequently as the experiment progressed, $N$ = 9 - 11). Mean values ± 1 SD.

| System | Tank | Predicted conditions | | Measured parameters | | | |
|---|---|---|---|---|---|---|---|
| | | $pCO_2$ (µatm) | Temperature | $pH_{NBS}$ | T (°C) | TA (µmol $kg^{-1}$) | $pCO_2$ (µatm) |
| 1 | 1 | 1000 | 19°C | 7.82 ± 0.05 | 18.70 ± 0.62 | 2250 ± 34 | 1029 ± 90 |
| | 2 | 1000 | 19°C | 7.82 ± 0.05 | 18.67 ± 0.61 | | 1019 ± 85 |
| | 3 | 1000 | 19°C | 7.82 ± 0.05 | 18.78 ± 0.63 | | 1042 ± 91 |
| 2 | 4 | 1000 | 17°C | 7.82 ± 0.05 | 17.05 ± 0.34 | 2252 ± 34 | 988 ± 140 |
| | 5 | 1000 | 17°C | 7.83 ± 0.05 | 17.06 ± 0.30 | | 988 ± 90 |
| | 6 | 1000 | 17°C | 7.82 ± 0.05 | 17.03 ± 0.38 | | 1036 ± 47 |
| 3 | 7 | Ambient | 17°C | 8.03 ± 0.02 | 17.06 ± 0.40 | 2255 ± 33 | 597 ± 37 |
| | 8 | Ambient | 17°C | 8.02 ± 0.03 | 17.03 ± 0.31 | | 602 ± 42 |
| | 9 | Ambient | 17°C | 8.03 ± 0.03 | 17.02 ± 0.30 | | 596 ± 36 |
| 4 | 10 | Ambient | 19°C | 8.03 ± 0.03 | 18.88 ± 0.62 | 2251 ± 33 | 592 ± 42 |
| | 11 | Ambient | 19°C | 8.03 ± 0.03 | 18.80 ± 0.70 | | 587 ± 37 |
| | 12 | Ambient | 19°C | 8.03 ± 0.03 | 18.87 ± 0.60 | | 579 ± 59 |




1

2

3 Figure 1. Schematic of one of the four identical experimental systems used for the study; each
4 maintained a different temperature / $pCO_2$ combination. RAS = Recirculating Aquaculture
5 System.

6

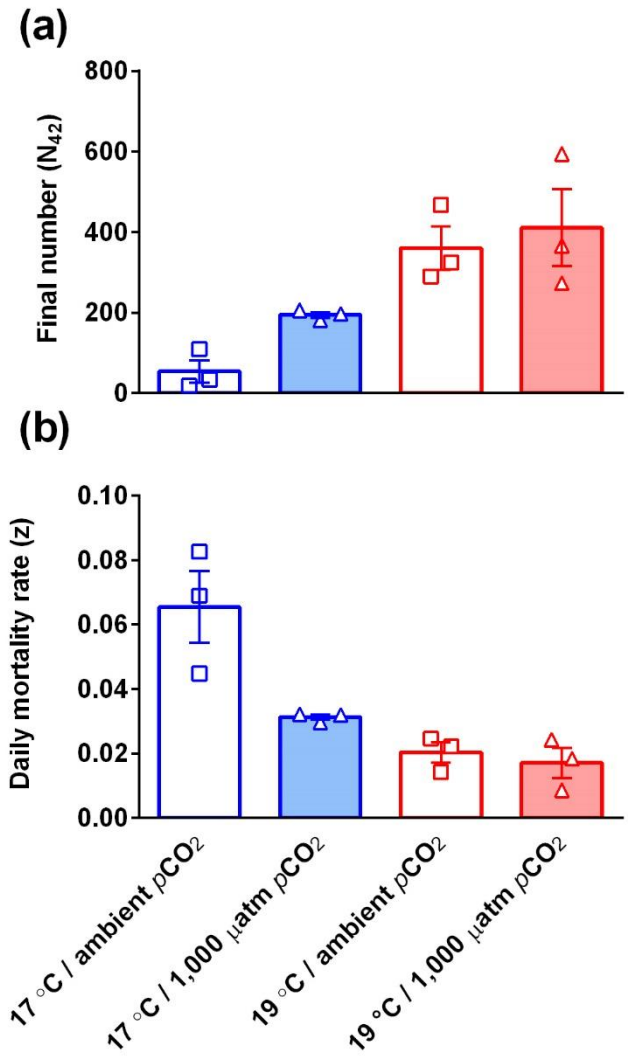



Figure 2. (a) Final numbers and (b) daily mortality of *D. labrax* larvae after incubation for 42 days under each experimental condition. Data points are mean values for each experimental tank, column heights are means and error bars are ± 1 Standard Error of the Mean, SEM. See Table 1 for details on each experimental condition.

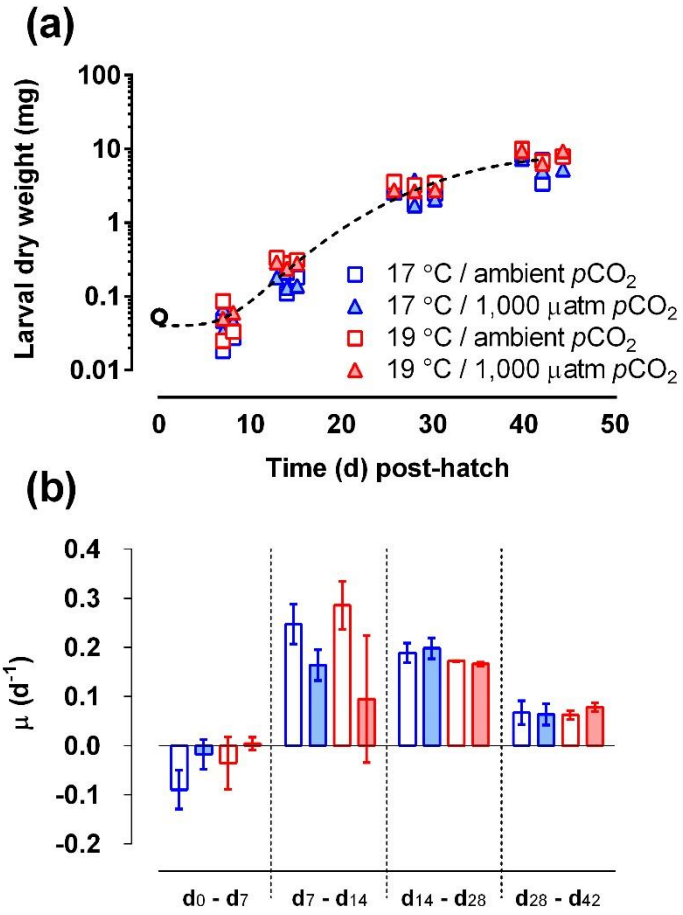



Figure 3. (a) Dry weight of sea bass larvae incubated for 42 days under each experimental condition, data points are mean values for each experimental tank, open circle = $d_0$ data, line = nonlinear regression (Weibull model using shared parameters across treatments). (b) Specific growth rate, $\mu$, of *D. labrax* larvae after incubation for 42 days under each experimental condition calculated between each sampling point. Data points are mean values for each experimental tank, column heights are means and error bars are ± 1 SEM. See Table 1 for details on each experimental condition.

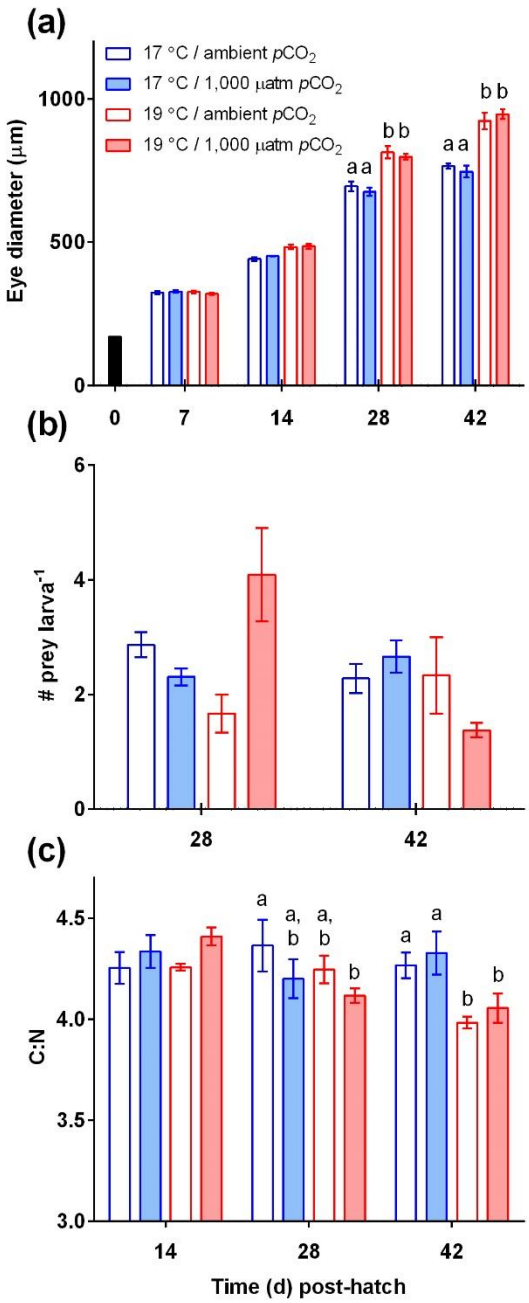



Figure 4. (a) Eye diameter of *D. labrax* larvae incubated for 42 days under each experimental condition. (b) The number of *Artemia salina* prey in the gut of *D. labrax* larvae at $d_{28}$ and $d_{42}$ under each experimental condition. (c) C:N ratios of *D. labrax* larvae at $d_{14}$, $d_{28}$ and $d_{42}$ under each experimental condition. Columns that do not share a letter are significantly different (two-way ANOVA with Bonferroni post-test, $P<0.05$). Mean values ± 1 SEM, $N = 3$. See Table 1 for details on each experimental condition.

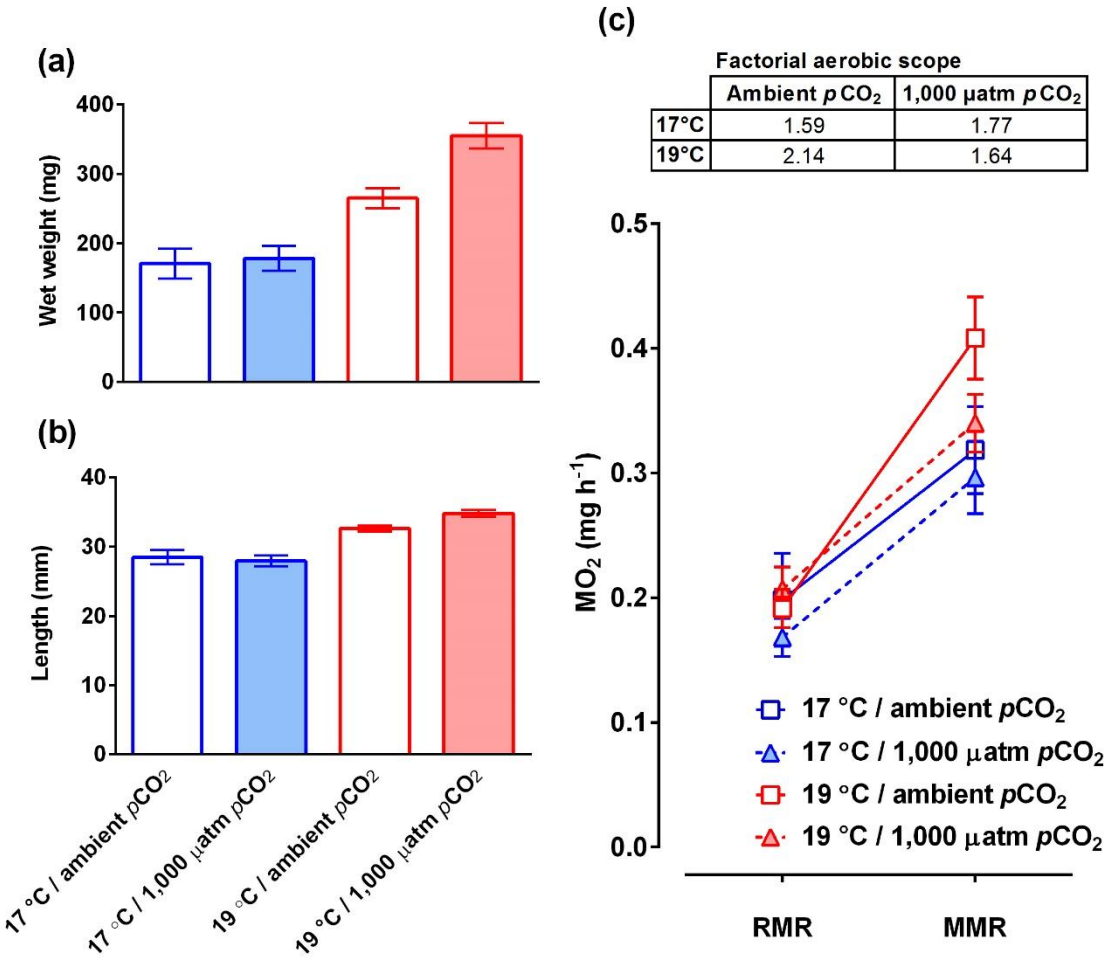



Figure 5. (a) Wet weight and (b) total length of juvenile ($d_{67\text{-}69}$ post-hatch) *D. labrax* used in the metabolic rate study. Mean values ± 1 SEM, *N* = 12-16. (c) Routine metabolic rates (RMR) and maximal metabolic rates (MMR) of juvenile *D. labrax* ($d_{67\text{-}69}$ post-hatch) grown under each experimental condition. Mean values ± 1 SEM, *N* = 12-16. See Table 1 for details on each experimental condition.