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Consequences of elevated CO_2 exposure across multiple life stages in a coastal forage fish

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Ocean acidification may impact the fitness of marine fish, however, studies reporting neutral to moderate effects have mostly performed short-term exposures to elevated CO_2, whereas longer-term studies across life stages are still scarce. We performed a CO_2 exposure experiment, in which a large number ($n > 2200$) of Atlantic silverside *Menidia menidia* offspring from wild spawners were reared for 135 days through their embryonic, larval, and juvenile stages under control (500 µatm) and high CO_2 conditions (2300 µatm). Although survival was high across treatments, subtle but significant differences in length, weight, condition factor and fatty acid (FA) composition were observed. On average, fish from the acidified treatment were 4% shorter and weighed 6% less, but expressed a higher condition factor than control juveniles. In addition, the metrics of length and weight distributions differed significantly, with juveniles from the high CO_2 treatment occupying more extreme size classes and the length distribution shifting to a positive kurtosis. Six of twenty-seven FAs differed significantly between treatments. Our results suggest that high CO_2 conditions alter long-term growth in *M. menidia*, particularly in the absence of excess food. It remains to be shown whether and how these differences will impact fish populations in the wild facing size-selective predation and seasonally varying prey abundance.

Keywords: condition factor, fatty acid, growth distributions, *Menidia menidia*, ocean acidification, survival.

Introduction

Understanding how climate change is affecting the fitness and therefore abundance and distribution of marine organisms constitutes one of the most important, if necessarily complex and challenging tasks of our time (Hoegh-Guldberg and Bruno, 2010; Doney *et al.*, 2012). Marine environments are not only warming, they are also gradually acidifying due to the dissolution of rising atmospheric (Doney *et al.*, 2009) and metabolic carbon dioxide (CO_2), the latter particularly manifesting in coastal regions, where increasing nutrient input often fuels excessive primary production and subsequent microbial respiration (Wallace *et al.*, 2014). In recognition of these processes, exploring the sensitivity of marine organisms to high CO_2 environments has become one of the most eagerly pursued research priorities in biological oceanography during the past two decades (Browman, 2016). Most of this research has taken the form of laboratory experiments, which are a first necessary step to distinguish CO_2-sensitive from CO_2-tolerant traits in marine taxa, species, and life-stages, before issues of evolutionary adaptation, potential trade-offs, indirect effects, and the responses of entire ecosystems can be addressed (Sunday *et al.*, 2014; Malvezzi *et al.*, 2015).

Perhaps unsurprisingly, this recent swell of empirical research has revealed an intriguing complexity of organismal responses to high CO_2 that continues to defy easy answers or generalizations. Although meta-analyses have shown a majority of responses to high CO_2 to be negative (Hendriks *et al.*, 2010; Kroeker *et al.*, 2010), there is abundant evidence for non-linear (Ries *et al.*, 2009), neutral (Hurst *et al.*, 2013), or even positive effects of exposures to elevated CO_2 (Gooding *et al.*, 2009). Calcifying invertebrates and early life stages of marine species are likely most sensitive to the symptoms of ocean acidification (OA) (Kleypas *et al.*, 2006; Waldbusser *et al.*, 2013; Bednaršek *et al.*, 2014);

however, contrasting sensitivities have been documented within every taxon (Ries *et al.*, 2009; Hendriks *et al.*, 2010) or even among populations within the same species (Frommel *et al.*, 2013; Stiasny *et al.*, 2016).

As a group, marine fish have shown similar complexity. Juvenile and adult fish tolerate CO_2 levels far beyond average climate change predictions (>2000 µatm, Ishimatsu *et al.*, 2008). However, fish early life stages, while still developing acid-base competency, might be more vulnerable as some have exhibited reduced survival in response to elevated CO_2 levels (Ishimatsu *et al.*, 2008; Baumann *et al.*, 2012; Chambers *et al.*, 2014; Pimentel *et al.*, 2014; Frommel *et al.*, 2016; Stiasny *et al.*, 2016). On the other hand, studies reporting no adverse survival effects are at least as numerous (Franke and Clemmesen, 2011; Hurst *et al.*, 2012; Frommel *et al.*, 2013; Hurst *et al.*, 2013; Munday *et al.*, 2016). Most studies to date have documented some form of sublethal response to high CO_2 exposure including abnormal behavior, reduced orientation (Munday *et al.*, 2009b), predator avoidance (Dixson *et al.*, 2010), and swimming capacity (Pimentel *et al.*, 2014), elevated or depressed metabolism (Munday *et al.*, 2009a; Rummer *et al.*, 2013), skeletal malformations (Chambers *et al.*, 2014), otolith hypercalcification (Checkley *et al.*, 2009; Bignami *et al.*, 2013), tissue damage (Frommel *et al.*, 2013, 2016), and increased levels of fatty acids (FAs) (Díaz-Gil *et al.*, 2015). Collectively, these findings suggest that high CO_2 environments impact fish early life stages, even if short-term survival under artificial laboratory settings (e.g. no predators) remains statistically unaffected.

How acidification affects fish growth is another surprisingly difficult question. Growth is an important fitness-relevant trait, given that in the wild growth rate is generally inversely related to survival (Anderson, 1988; Hare and Cowen, 1997). However, laboratory studies have reported growth responses to high CO_2 levels that cover the entire spectrum of negative to neutral (Baumann *et al.*, 2012; Hurst *et al.*, 2013) to positive effects (Munday *et al.*, 2009c), which is often simply attributed to species-specific reaction norms. In addition, the diversity of responses may suggest the existence of confounding experimental factors that hinder cross-study comparisons and the development of a unifying framework. First, most acidification studies to date have provided fish larvae with excess food rations, which is practical to rule out feeding related growth differences, but likely disguises the additional metabolic costs of high CO_2 environments, because survivors can simply compensate or overcompensate for those costs by increased consumption. Second, the majority of OA studies on fish have so far employed relatively short-term experimental designs that spanned days to a few weeks post-hatch and measured growth mostly during one ontogenetic stage (i.e. eggs, larvae, or juveniles). For most fish species, particularly those of commercial value, this amounts to a small fraction of their overall life span and could therefore be insensitive to potential carry-over effects from one life stage to the next. Third, logistical constraints in rearing set-ups and analytical throughput often preclude the assessment of large sample sizes; thus, the majority of studies have so far based their conclusions on comparing response means derived from a limited number of individuals (i.e. $n = 5$–50 per treatment). The resultant statistical power suffices to detect strongly divergent responses, whereas potential subtle shifts in trait distributions (e.g. range, skewness, kurtosis of length, weight or condition), which may be as important as central tendencies, remain undetectable.

Here we report on a large laboratory rearing experiment to assess the growth consequences of high CO_2 environments in the Atlantic silverside (*Menidia menidia*), an ecologically important forage fish that is abundant in nearshore habitats along the North American Atlantic coast (Middaugh *et al.*, 1987). From spawners collected in the wild, we reared several thousand offspring under contrasting CO_2 conditions from fertilization to approximately four months post-hatch, thereby not only spanning the embryonic, larval, and juvenile stages but also about one third of the life span of this annual, semelparous species. During the late larval and juvenile stages, food was provided in standardized, non-excess rations, and several growth-related traits including length, weight, and condition factor were assessed for over 2200 survivors at the end of the experiment. We hypothesized that long-term, continuous CO_2 exposure in *M. menidia* incurs additional metabolic costs that result in divergent distribution metrics for length, weight, and condition factor in surviving juveniles. In addition, we measured FA profiles in a smaller subset of individuals to determine if high CO_2 exposure alters the retention of FAs from diet.

Methods

CO_2 treatments and measurements

Following best practices and guidelines for OA research (Riebesell *et al.*, 2010) we used gas proportioners (ColeParmer) to mix air with 100% CO_2 (bone dry grade) that was delivered to the bottom of each replicate rearing container via airstones. Control conditions were achieved by forcing compressed laboratory air through a series of CO_2 stripping units containing granular soda lime (AirGas), a particle filter (1 µm) and then to each replicate via airstone. Two standardized treatment levels were administered; control (CO_2 stripped air only, ~500 µatm CO_2, $pH_{NBS} = 8.05$) and high CO_2 conditions (air:CO_2 mix, ~2300 µatm CO_2, $pH_{NBS} = 7.45$). These treatments represent levels commonly used in OA research and conditions experienced seasonally by *M. menidia* offspring in the wild (Murray *et al.*, 2014). Target pH levels were monitored daily using a handheld pH probe (Orion ROSS Ultra pH/ATC Triode and Orion Star A121 pH Portable Meter, Thermo Scientific) calibrated bi-weekly with two-point pH_{NBS} references. During the course of the experiment, each replicate tank was sampled three times for measurements of total alkalinity (A_T; µmol kg^{-1}). Seawater was siphoned into 300 ml borosilicate bottles and immediately analyzed for A_T at 17 °C using an endpoint titration (Mettler Toledo G20 Potentiometric Titrator). Salinity was measured via refractometer and methodological accuracy of alkalinity titrations were verified using Dr. Andrew Dickson's (University of California San Diego, Scripps Institution of Oceanography) certified reference material for A_T in seawater (Batch 147 = 2231 µmol A_T kg $seawater^{-1}$). The partial pressure (pCO_2; µatm) and fugacity of CO_2 (fCO_2; µatm) as well as dissolved inorganic carbon (C_T; µmol kg^{-1}) and carbonate ion concentration (CO_3^{2-}; µmol kg^{-1}) were calculated in CO2SYS (V2.1, http://cdiac.ornl.gov/ftp/co2sys) based on measured A_T, pH_{NBS}, temperature, and salinity using K1 and K2 constants from Mehrbach *et al.* (1973) refit by Dickson and Millero (1987) and Dickson (1990) for $KHSO_4$. An overview of the carbonate chemistry is given in Table 1.

Table 1. Mean (±SD) pH_{NBS} and temperature (°C) from daily measurements.

Tank	Treatment CO_2	pH_{NBS}	Temp	Salinity	A_T	C_T	pCO_2	fCO_2	CO_3^{2-}
A	High	7.42 ± 0.11	17.5 ± 0.4	31	2102 ± 10	2138 ± 13	2295 ± 65	2287 ± 65	31.3 ± 0.6
B	High	7.43 ± 0.12	17.5 ± 0.4	31	2123 ± 27	2158 ± 24	2283 ± 95	2275 ± 94	32.2 ± 1.8
C	Control	8.06 ± 0.13	17.3 ± 0.3	31	2112 ± 7	1958 ± 7	500 ± 7	498 ± 7	116.8 ± 1.6
D	Control	8.07 ± 0.12	17.2 ± 0.6	31	2110 ± 1	1956 ± 1	499 ± 7	497 ± 7	116.6 ± 1.4

Mean (±SD) salinity, total alkalinity (A_T; μmol kg^{-1}), dissolved inorganic carbon (C_T; μmol kg^{-1}), partial pressure of CO_2 (pCO_2; μatm), fugacity of CO_2 (fCO_2; μatm), and carbonate ion concentration (CO_3^{2-}; μmol kg^{-1}) measured from three seawater samples of each replicate tank. Salinity was measured via refractometer and A_T from endpoint titrations. C_T and pCO_2, fCO_2, and CO_3^{2-} were calculated in CO2SYS.

Field sampling and experimental design

Experiments were performed at University of Connecticut's Avery Point Campus in the Rankin Laboratory, a seawater facility adjacent to eastern Long Island Sound. Ripe adult *M. menidia* were collected on 1 May 2015 from Mumford Cove (41° 19.25′ N 72° 1.09′W), a shallow embayment dominated by eelgrasses (*Zostera marina*) and open to the Long Island Sound. Adults were sampled with a 30 × 2 m beach seine, separated by sex, transported live to our laboratory, and held for 48 h in large aerated tanks (17 °C, ambient CO_2, no food). On the day of fertilization (3 May 2015), ≥20 ripe individuals from each sex were strip-spawned and eggs evenly distributed onto window screens (1 mm fiberglass mesh) submerged in plastic dishes with clear seawater. Strip-spawned adults were measured for total length (TL, to lower 0.5 cm; mean $TL_{females}$ = 9.7 cm, mean TL_{males} = 8.7 cm). Fertilized embryos quickly attach to the screens via chorionic filaments, which facilitates precise enumeration and even allotment to treatments and replicates. Following established protocols for rearing *M. menidia* offspring (Murray *et al.*, 2014), replicate containers (20 l) were filled with filtered (to 1 μm) and UV sterilized seawater (31) from Long Island Sound and placed in water baths (~300 l) controlled for temperature and light conditions [17 °C, 15:9 (L:D) h] throughout the duration of the experiment. Within 2h of fertilization, each of four replicates per treatment received exactly 200 embryos to measure early life survival, while four other replicates per treatment each received ~400 offspring for long-term rearing. Larvae hatched ~14 days post-fertilization (dpf) and were immediately provided with excess rations of newly hatched brine shrimp nauplii *Artemia salina* (San Francisco strain, Brine Shrimp Direct) and a commercial larval powder food (first four days, Otohime Marine Weaning Diet, size A, Reed Mariculture). At 2 days post-hatch (dph), living larvae from survival replicates were counted by gently scooping small groups into replacement containers. Between 1 to 14 dph, all containers were cleaned daily with partial (10%) water exchange.

At 16 dph, larvae from the survival replicates were counted and a sub-sample ($N_{control}$ = 37, N_{high} = 33) was preserved in 10% formaldehyde/seawater solution for later TL measurements (nearest 0.01 mm) via calibrated, digital images (ImagePro Premier V9.1). All surviving larvae were transferred to larger (50 l) tubs and maintained under the previously described protocol. At 33 dph, larvae from the survival replicates were counted and then all larvae transferred to 50 l tubs fitted with screen-covered holes (1 mm mesh) to promote water exchange from a 300 l seawater bath. Due to space constraints, from 33 to 54 dph larvae from the survival replicates were pooled into a single container per CO_2 treatment. Rations of nauplii and commercial powder food (Otohime B1, Reed Mariculture) were standardized to the known number of larvae per replicate. At 54 dph, all juveniles from survival and grow-out replicates were counted and pooled at equal numbers into 300 l circle tanks (two tanks per treatment, ~615 fish per tank). Juveniles were fed standardized rations (to the known number of juveniles per tank) of newly hatched nauplii and B1 commercial powder food. Tanks were siphoned for waste daily and partial water changes completed twice weekly, ensuring that ammonia levels were consistently below 0.25 ppm. Additional sub-samples for length measurements (TL, nearest 0.01 mm) were made at 36 dph ($N_{control}$ = 20, N_{high} = 20), 68 dph ($N_{control}$ = 20, N_{high} = 20) and 100 dph ($N_{control}$ = 28, N_{high} = 28).

At 122 dph, the experiment was terminated and all surviving juveniles were euthanized via an overdose of Tricaine-S (MS 222, Western Chemical) for preservation. While some juveniles from each treatment were immediately frozen at −80 °C for FA analyses; ~75% of the samples were fixed in 10% buffered formaldehyde/seawater solution for TL ($N_{control}$ = 1025; N_{high} = 1100, nearest 0.01 mm) and weight measurements ($N_{control}$ = 720; N_{high} = 786, nearest 0.01g).

FA analysis

Individual FA profiles were assessed for 60 *M. menidia* juveniles 122 dph (n = 15 per tank). Ten individuals per tank were chosen randomly, after which an additional five individuals were chosen among the smallest size classes (20–28 mm) to extend the range of sizes examined. The resultant samples spanned almost the entire size range (TL, 0.1 mm) of the experimental population. These fish along with samples of the early larval and juvenile diets (*A. salina* nauplii, brineshrimpdirect.com and Otohime B1, Reed Mariculture, respectively) were preserved individually at −80 °C and subsequently shipped on dry ice to the Fisheries and Mariculture Laboratory at the University of Texas Marine Science Institute for FA analysis.

Concentrations of 27 FAs (expressed as % of total FAs and mg FA g^{-1} dry weight) were measured using a gas chromatograph (Shimadzu GC-2014 Scientific Instruments; www.ssi.shimadzu.com) set with a Phenomenex ZB-WAX plus capillary column (30 m long; 0.53 mm ID; 1.0 μm thick; www.phenomenex.com) following the methods of Faulk and Holt (2005). For each sample, lipids were cold-extracted from ~50 mg dry mass by homogenizing in a solution of chloroform–methanol (2:1 v/v) plus a measured amount of tricosanoic acid (23:0) as an internal standard for quantification of mg g^{-1} dry mass of FAs. FA methyl esters were prepared by saponification in potassium hydroxide, followed by 14% boron trifluoride in methanol. Individual FAs were identified by comparison to commercial standards (Supelco, Inc).

Statistical analysis

Growth and survival analyses were performed using SPSS (V20, IBM). Percent survival was calculated for both treatments from hatch to 2, 2–14, 14–33, and 33–122 dph. An angular transformation (arcsine of the square root of percentage/100) was applied to percentage data before testing for significance between treatments via independent samples t-test. TL was calculated as treatment means ± SD for each group of sub-sampled offspring. Each group was tested for homogeneity of variance between treatments using Levene's test and for significance between treatments and tanks using independent samples t-test. At 122 dph, TL and wW distributions from both treatments were significantly non-normal (one-sample Kolmogorov-Smirnov test), and TL distributions expressed unequal variances (Levene's test); thus non-parametric Mann-Whitney U tests were used to evaluate significance between treatments, and non-parametric Kruskal-Wallis pair-wise comparisons tested for significant tank effects. A non-parametric Levene's test showed ranked TL data to have equal variances, thus meeting the equality of variance assumption. Condition factor (k) was calculated for 122 dph samples using the model $k = wW/TL^b$, where b was derived from the fitted relationship $wW = a{*}TL^b$ using pooled TL and wW data from both treatments (supplementary Fig. S1). Confidence interval (CI) for 122 dph TL, wW and k medians, as well as skewness and kurtosis were generated from a bias-corrected accelerated bootstrap routine using a sample size of 1000.

To compare FA profiles, we pooled data for each treatment to compare the concentration of each FA both in absolute (mg g dw^{-1}) and in relative terms (% of total FAs). An angular transformation was applied to relative FA values prior to statistical analyses. We used two sample t-tests to compare FA-specific means and calculated relative differences between treatments [$\Delta FA = (FA_{mean\ control} - FA_{mean\ high})/FA_{overall\ mean}$] for visualization. We then performed principal component (PC) analysis on relative FA concentrations (% of total FAs) and extracted all PCs with eigenvalues > 1 (Systat, version 13). We then explored the relationships between PCs and TL and used t-tests to test for differences between CO_2 treatments for each PC. For PCs that varied with mean fish size, we tested for effects of CO_2 treatment using analysis of covariance.

Results

Survival

Survival was generally high across life stages and CO_2 treatments (Fig. 1). Survival (mean ± SD) at hatch (15 dpf) was high across control (83 ± 8%) and high CO_2 (87 ± 5%) treatments. Highest mortality was observed during the early larval stage (hatch to 14 dph) where survival was not significantly different (independent samples t-test, $T_6 = -1.468$, $p = 0.193$) in high CO_2 (50 ± 21%) compared with control treatments (27 ± 20%). Late larval survival (14–33 dph) was similar in control (94%) and high CO_2 treatments (94%). Juvenile survival (33 and 122 dph) was similar in control (84 ± 2%) and high CO_2 (88 ± 1%) treatments.

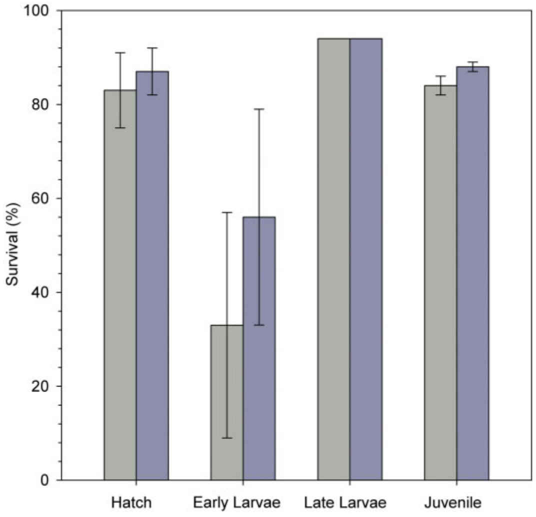

Figure 1. Mean (±SD) survival of *M. menidia* offspring reared at control (grey, 500 µatm) and high CO_2 (dark grey, ∼2300 µatm) for hatchlings (fertilization-2 dph), early larvae (2–14 dph), late larvae (14–33 dph), and juveniles (33–122 dph). No SD calculated for late larval period as larvae from survival experiment were temporarily pooled into a single replicate. This figure is available in black and white in print and in colour at ICES Journal of Marine science online.

Sub-sample lengths

At 16 dph, larvae from the control were not significantly different (8.9 ± 1.2 mm) than high CO_2 individuals (mean ± SD = 8.6 ± 1.1 mm). After 36 dph, larvae from high CO_2 (13.9 ± 2.6 mm) were not significantly different than control samples (13.5 ± 2.1 mm). At 68 dph juveniles from the control (23.3 ± 2.2 mm) were significantly longer ($T_{38} = -2.098$, $p = 0.043$) than samples from high CO_2 (21.3 ± 3.7 mm). By 100 dph, control juveniles had increased their mean size to 33.1 ± 5.0 mm, which was significantly longer ($T_{54} = -3.209$, $p = 0.002$) than high CO_2 juveniles (28.7 ± 5.4 mm). A summary of growth data can be found in Table 2.

122 dph distributions of length, weight and condition

After 122 days of high CO_2 exposure juveniles were significantly shorter (Mann-Whitney $U_{2,125} = 6.605$, $p \leq 0.001$, Fig. 2a, Table 3) and weighed significantly less ($U_{1,506} = 2.963$, $p = 0.003$, Fig. 2b, Table 3) than control fish. Bootstrapped TL and wW results showed no overlap of mean and median 95% CIs (Table 3). However, high CO_2 juveniles exhibited a significantly higher k ($U_{1,506} = -9.719$, $p < 0.001$, Fig. 2c, Table 3) across most TL classes (Fig. 3). In addition, high CO_2 significantly altered the shape of TL (two-sample Kolmogorov-Smirnov, $D_{2,125} = 2.956$, $p \leq 0.001$, Fig. 4a) and wW distributions ($D_{1,506} = 1.389$, $p = 0.042$, Fig. 4b). The effect was most prominent on TL, where the high CO_2 distribution was more variable, exhibited a larger SD and a greater range (Table 3). Skewness of TL distributions was similar; however, we found a sign change to kurtosis, with the statistic shifting to positive in the high CO_2 treatment (Table 3). Bootstrapped 95% CIs of kurtosis showed overlap, but a tendency for high CO_2 distribution to be more positive (Table 3).

Tank effects

Significant within treatment tank effects were found for 122 dph TL and k measurements. Control tanks exhibited a similar wW (Supplementary Fig. S2) but differed significantly in TL (Kruskal-Wallis $H_3 = -2.708$, $p = 0.041$, Supplementary Fig. S2) and k ($H_3 = 4.394$, $p < 0.001$, Supplementary Fig. S2). TL and wW from high CO_2 tanks were similar; however, k differed significantly ($H_3 = -3.872$, $p = 0.001$, Supplementary Fig. S2a–c).

FA composition

As expected, the FA profiles of *M. menidia* closely resembled the FA profile of the juvenile diet (Otohime B1, Fig. 5a). Juveniles from the control treatment tended to have slightly higher amounts of specific FAs by weight (mg g dw^{-1}) than juveniles from the high CO_2 group (Fig. 5b and c); however, significant differences (t-test, $p < 0.05$) were detected for only six of the 27 FAs ($FA_{control} > FA_{high}$: 16:2*n*−4, 18:3*n*−3, 20:3*n*−3; $FA_{high} > FA_{control}$: 12:0, 18:0, 20:4*n*−6) (Fig. 5b). In the analysis of relative FA concentrations, six PCs with eigenvalues > 1 were extracted, explaining a cumulative 82.3% of the total variance (46.6, 10.0, 9.5, 6.9, 5.0, and 4.3%, respectively). PC1 was strongly positively correlated with TL ($p < 0.001$), but neither the slope ($p = 0.16$) nor the elevation of the linear regressions of PC1 on TL differed statistically between treatments ($p = 0.61$, ANCOVA df = 1). The only other PC that was significantly correlated with TL was PC4 for the control treatment. There were significant differences between treatments for scores on PC2 ($p < 0.001$) and PC6 ($p = 0.002$). The high CO_2 treatment had lower scores on PC2 and higher scores on PC6 than the control treatment, resulting in significant separation of these groups (Fig. 6a). Loadings on these two axes (Fig. 6b) suggested that the CO_2 treatment had higher concentrations of 18:3*n*−6, 20:2*n*6, 20:4*n*3, 20:3*n*6, 15:1, and 18:4*n*3 and lower concentrations of 16:2*n*−4, 20:3*n*−3, 20:5*n*3, and 18:3*n*3 than the control treatment. Of these suggested differences, only 18:3*n*6 was significantly higher than the CO_2 treatment and only 16:2*n*4, 20:3*n*3, and 18:3*n*3 were significantly lower in the CO_2 treatment compared with the control treatment (t-test, $p < 0.05$).

Discussion

We reared a large cohort (n > 2200) of *M. menidia* offspring under control (~500 µatm) and high CO_2 (~2300 µatm) conditions for 4.5 months and found small but significant differences between treatments in length, weight, condition factor, and FA profiles. Although survival was not different across treatments, juveniles reared under high CO_2 were on average 4% shorter and weighed 6% less, but expressed a higher condition factor than control juveniles. Furthermore, we detected subtle shifts in the distributions of length and weight. High CO_2 juveniles exhibited a more variable TL distribution (greater SD) with a positive kurtosis, indicating more fish populating extreme size classes. Our findings therefore suggest that high CO_2 induces a small, but detectible growth reduction in developing *M. menidia* offspring.

In this experiment, early post-hatch survival was unaffected by high CO_2 levels, which is consistent with our past experiments demonstrating that offspring from Long Island Sound acquire CO_2 tolerance by early May (Murray *et al.*, 2014). Past studies, however, did not find any influence of CO_2 on early growth of *M. menidia*. In fact, very few studies testing the effects of high CO_2 on fish have reported significant growth reductions (Baumann *et al.*, 2012); while most have found neutral (Franke and Clemmesen, 2011; Hurst *et al.*, 2012; Frommel *et al.*, 2013; Chambers *et al.*, 2014), or even positive effects (Munday *et al.*, 2009c; Hurst *et al.*, 2013). Similarly contradictory findings have been reported for CO_2-induced changes to metabolic scope. For example, high CO_2 increased resting metabolic rates (RMR) in two tropical cardinal fish species, *Ostorhinchus doederleini* and *Ostorhinchus cyanosoma* (Munday *et al.*, 2009a), but reduced RMR in the tropical damselfish *Acanthochromis polyacanthus* (Rummer *et al.*, 2013). Temperate species including Atlantic cod (*Gadus morhua)* (Melzner *et al.*, 2009), Atlantic halibut (*Hippoglossus hippoglossus*) (Gräns *et al.*, 2014) and the Antarctic *Notothenia rossi* (Strobel *et al.*, 2012) showed minor or no effects on aerobic performance after prolonged CO_2 exposure.

Table 2. Summary of TLs (mm) from sub-sampled M. menidia larvae and juveniles from control (500 µatm) and high CO2 (2,300 µatm) treatments at 17 °C.

Age (dph)	CO_2 treatment	N	Mean TL (mm)	SD	Min	Max	df	t	p
16	Control	37	8.9	1.2	6.5	11.3	68	1.281	0.204
	High	33	8.6	1.1	6.2	10.8			
36	Control	20	13.5	2.1	11.0	17.1	38	-0.553	0.584
	High	20	13.9	2.6	7.0	17.8			
68	Control	20	23.3	2.2	19.3	27.4	38	2.098	0.043
	High	20	21.3	3.7	14.1	28.7			
100	Control	28	33.1	5.0	26.0	45.0	54	3.209	0.002
	High	28	28.7	5.4	19.0	39.0			

Samples taken 16, 36, 68, and 100 dph. Significance tests ($p < 0.05$) generated from independent samples t-test.

The conflicting reports of growth and metabolic responses may reflect species-specific reaction norms, or may highlight the limitations of relatively short-term CO_2 exposure experiments to predict complex metabolic consequences. In this study, high CO_2 had a slight positive effect on late larval growth. Only after two months of exposure, covering three distinct ontogenetic stages, did the effect on growth rate produce significant effects on size. At 100 dph, high CO_2 juveniles were 13% shorter than the control, which suggested a substantially larger effect than actually found after 122 dph. This may indicate a biased sub-sample at 100 days or compensatory growth in high CO_2 juveniles during the final three weeks of the experiment. In contrast to the present work, most OA studies on fish have experimented with either embryonic or early larval stages, or examined only juvenile or adult stages. Thus, they potentially missed longer-term consequences to growth and important carry-over effects from early-life exposure to adulthood (Pechenik, 2006; McCormick and Gagliano, 2008). For example, reductions to survival in larval *M. beryllina* occurred only if high CO_2 exposure also covered the embryonic stage (Baumann *et al.*, 2012). Likewise, consistent carry-over effects from larvae to adults have been observed in acidification experiments on the Olympia oyster *Ostrea lurida* (Hettinger *et al.*, 2013). In this study, the effects of high CO_2 on length and weight were negative; however, high CO_2 juveniles expressed a significantly higher condition factor than control fish, because they were slightly heavier per unit of length. Although counter-intuitive, this is consistent with experiments on Atlantic cod, which showed CO_2-induced increases in total lipid content, but not FA composition (Frommel *et al.*, 2012). While it is thus possible that high CO_2 promotes lipid accumulation, perhaps at the expense of increasing length, higher weight over length growth in fish from the high CO_2 group could also have other explanations, including overcalcification (Bignami *et al.*, 2013) or potential subtle changes in shape that may confound condition indices.

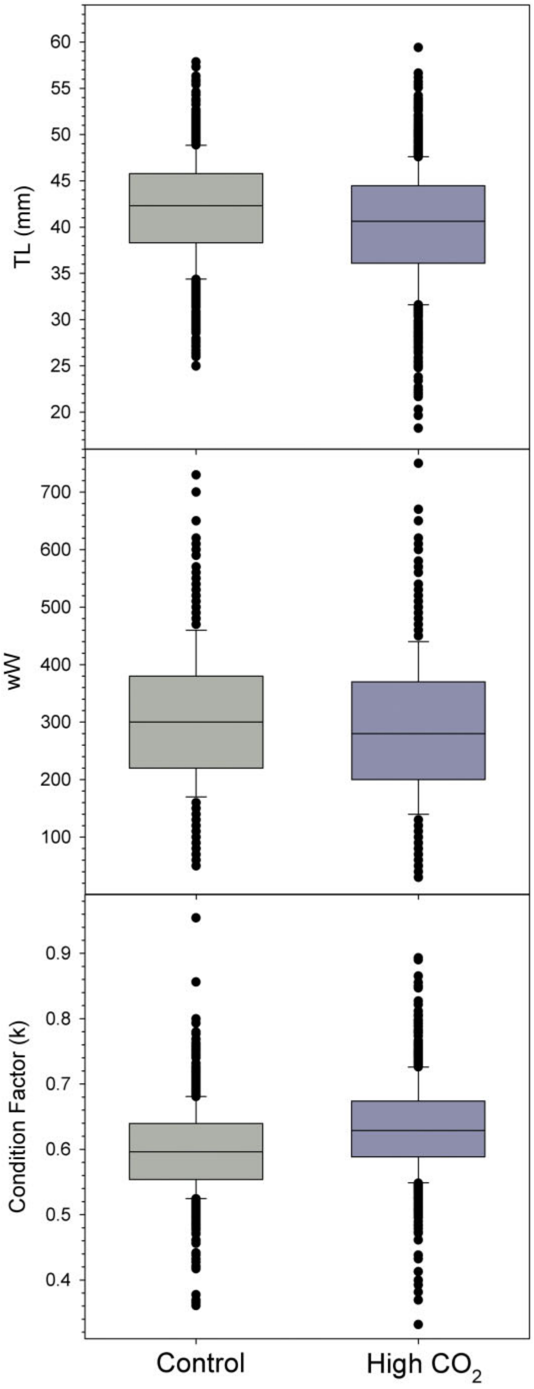

Figure 2. TL (mm), wW (mg), and k of juvenile *M. menidia* reared at control (grey, 500 µatm) and high CO_2 (dark grey, 2300 µatm) for 122 dph. Black lines represent treatment medians. Filled boxes encompass 25th and 75th percentiles. Circles represent individuals outside 10th and 90th percentiles. This figure is available in black and white in print and in colour at ICES Journal of Marine science online.

Quantifying carryover metabolic responses to high CO_2 in fish early life stages is often complicated by *ad libitum* feeding regimes. Typically employed to avoid acute mortality when larval fish transition from endogenous to exogenous feeding (May, 1974), as well as precluding feeding related growth effects, excess feeding allows larvae to increase their food consumption and thus mask any additional metabolic costs associated with high CO_2. Increased food consumption under acidified conditions has been shown in the juvenile anemonefish *Amphiprion melanopus* (Nowicki *et al.*, 2012). It is also consistent with our personal observations that *M. menidia* larvae hatching under high CO_2 often appear to start feeding on nauplii faster than their conspecifics under control CO_2 levels. Perhaps elevated food consumption is driven by a stimulation of gustatory senses (Nowicki *et al.*, 2012), similar to a range of other sensory effects associated with high CO_2 exposure (Munday *et al.*, 2009b; Dixson *et al.*, 2010). Alternatively, increased consumption could be an active response to a CO_2-induced increase in metabolic rates. To date, at least two studies have demonstrated smaller oil globules in fish larvae hatching under high CO_2 conditions (Chambers *et al.*, 2014; Munday *et al.*, 2016), suggesting increased metabolic demands or a shift in the use of nutritional resources by fish embryos. Either way, newly hatched larvae may have a shorter period to initiate first feeding before starvation, a critical factor determining early life survival (May, 1974). Fish larvae in the wild experience dispersed and often ephemeral food levels, which may not afford them the opportunity to simply increase feeding and could thus face a metabolic deficit and ultimately starvation. There is now a need for studies to further explore how food availability may influence the effects of CO_2 exposure.

Juveniles from the high CO_2 treatment exhibited a more variable TL distribution with a larger standard deviation, broader range, and positive kurtosis. Although the kurtosis statistic was only slightly greater than zero, the bootstrapping confirmed the tendency for the high CO_2 TL distribution to be more positive than the control. A positive kurtosis describes a more peaked distribution, produced by a movement of individuals from the 'shoulders' of the distribution to the center and tails (DeCarlo, 1997). This suggests the variability of the high CO_2 distribution is influenced more by a few individuals in extreme size classes, rather than many individuals only slightly different than the mean. Although the effect is modest, the shift in distribution shape suggests that acidified conditions produce more slow-growing, and perhaps fast-growing, *M. menidia* offspring. Given that *M. menidia* is an annual species that faces intense size-selective overwintering mortality (Schultz *et al.*, 1998), even small changes in juvenile length distributions may have important implications for its populations dynamics, particularly in the presence of size-selective predation mortality (Houde and Hoyt, 1987).

Although some individuals from the experimental population were heavily affected by CO_2, the majority only exhibited small or negligible effects. The presence of both tolerant and sensitive phenotypes suggests traits associated with CO_2 tolerance are not universally expressed in *M. mendia.* CO_2 tolerance may be inherited seasonally via transgenerational plasticity (Murray *et al.*, 2014), but there is also a significant genetic component to CO_2 tolerance ($h^2 = 0.20$, Malvezzi *et al.*, 2015). Elevated phenotypic variability is often triggered by environmental stressors and maintained especially if the stressor is of intermittent spatial or temporal frequency (Hoffmann and Hercus, 2000). The preferred spawning and nursery habitat of *M. menidia* are shallow

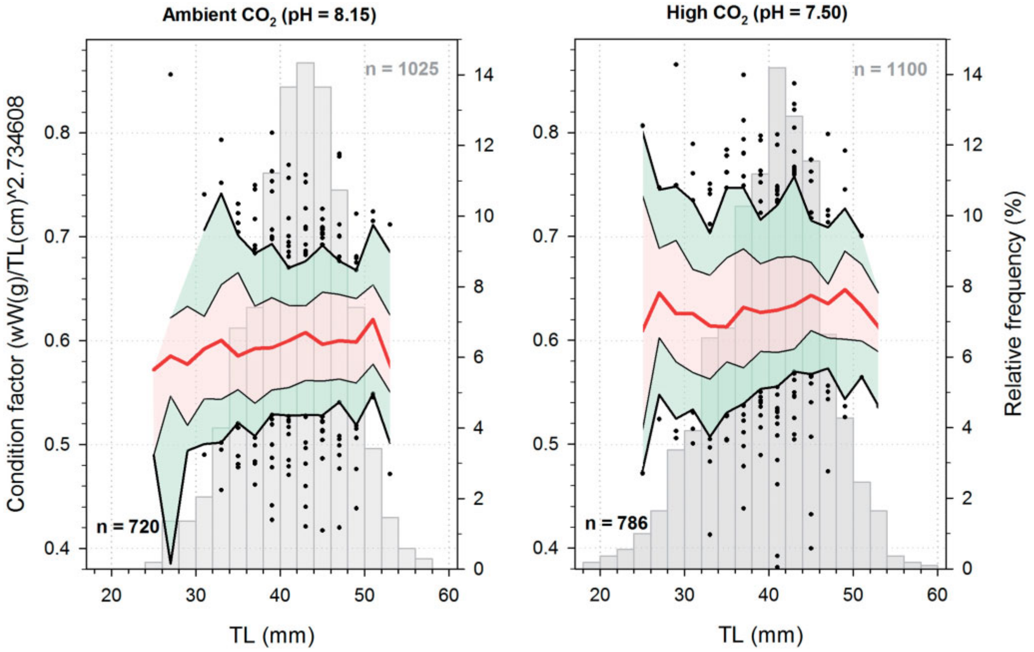

Figure 3. Distributions of condition factor per 2mm TL interval for juvenile *M. menidia* reared for 122 dph at control **(a)** and high CO_2 conditions **(b)**. Thick and thin black lines correspond to the 10th/90th and 25th/75th percentiles, respectively, while the red line depicts the median. Data below the 10th and above the 90th percentiles are depicted by black dots. Underlying grey bars show relative frequencies for each 2 mm TL class. Black and grey numbers correspond to numbers of individuals measured for both TL and wW, or for TL only, respectively. This figure is available in black and white in print and in colour at ICES Journal of Marine science online.

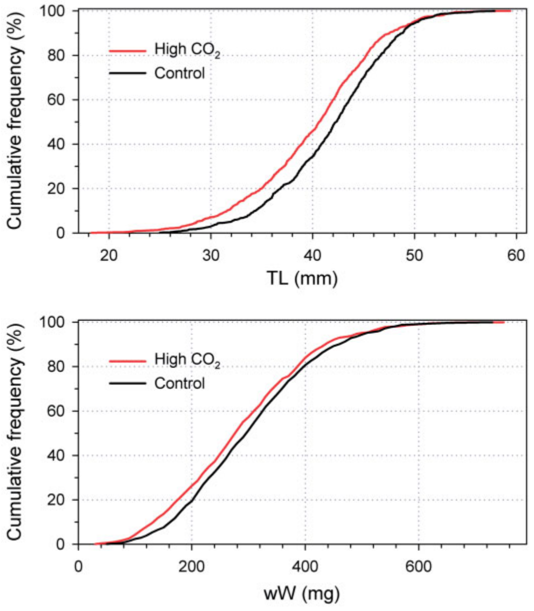

Figure 4. Cumulative frequency distributions of TL **(a)** and wet weight (wW, **b**), in juvenile *M. menidia* reared for 122 dph at control and high CO_2 conditions. This figure is available in black and white in print and in colour at ICES Journal of Marine science online.

subtropical to temperate estuaries (Conover and Ross, 1982); i.e. coastal systems that often exhibit large seasonal pH variability, driven largely by metabolically produced CO_2 coinciding with warming spring-summer temperatures (Baumann *et al.*, 2015). However, some coastal habitats, like those harboring robust seagrass communities, are better buffered against metabolic CO_2 and often maintain higher pH levels than ambient ocean seawater (Hendriks *et al.*, 2014). Local adaptation in *M. menidia* is thought to be maintained by the continuous selection of locally-suited genotypes (Clarke *et al.*, 2010), evidenced by the countergradient variation of traits such as growth rate and energy allocation (Billerbeck *et al.*, 2000). Thus, a genetic basis for variability in CO_2-tolerance is likely driven by selective pressures during early life pH exposure, particularly if expressing CO_2-tolerant traits creates tradeoffs that are detrimental in high pH environments (Kelly and Hofmann, 2013). That is, variable pH environments may select for CO_2-tolerant genes, while well buffered systems do not. Even though the wild adults used to fertilize this experiment were collected from a well buffered system in Mumford Cove (Baumann *et al.* unpublished data) CO_2-tolerant genotypes were probably well represented given the species' significant population connectivity occurring during their offshore overwinter migration (Clarke *et al.*, 2010). As an annual fish, the strategy of continuous selection for local adaptations allows *M. menidia* to thrive across broad thermal gradients, but likely also across fine-scale differences in pH conditions.

Our analyses of FA profiles revealed that most (19 of 27) FAs measured were at somewhat higher levels in fish from the control treatment than the CO_2 treatment. However, significantly higher

Table 3. Summary statistics of TL (mm), wW (mg), and condition factor (k) distributions for *M. menidia* juveniles reared for 122 dph at control and high CO_2 conditions.

		Mean	Median	SD	Range	Skewness (± SE)	Kurtosis (± SE)
TL (mm)	**Control**	41.8 [41.4/42.2]	42.3 [41.9/42.7]	5.6 [5.4/5.9]	25.0-57.9	−0.31 ± 0.08 [−0.44/−0.18]	-0.06±0.15 [-0.25/0.15]
	High CO_2	40.0 [39.7/40.4]	40.6 [40.3/41.1]	6.3 [6.0/6.6]	18.3–59.4	−0.31 ± 0.07 [−0.46/−0.17]	0.13±0.15 [−0.11/0.37]
wW (mg)	**Control**	307 [299-316]	300	115 [109/121]	50–730	0.42 ± 0.09 [0.28/0.55]	−0.03 ± 0.18 [−0.39/0.32]
	High CO_2	289 [281–297]	280	119 [113/125]	30–750	0.42 ± 0.09 [0.27/0.58]	0.03 ± 0.17 [−0.34/0.44]
k	**Control**	0.60 [0.59/0.60]	0.60 [0.59/0.60]	0.07 [0.06/0.07]	0.36–0.95	0.20 ± 0.09 [−0.17/0.61]	1.73 ± 0.18 [0.57/2.89]
	High CO_2	0.63 [0.63/0.64]	0.63 [0.63/0.64]	0.07 [0.07/0.08]	0.33–0.89	0.10 ± 0.09 [−0.20/0.38]	1.17 ± 0.17 [0.55/1.74]

Intervals in brackets represent 95% CI [low/high] based on 1000 bootstraps.

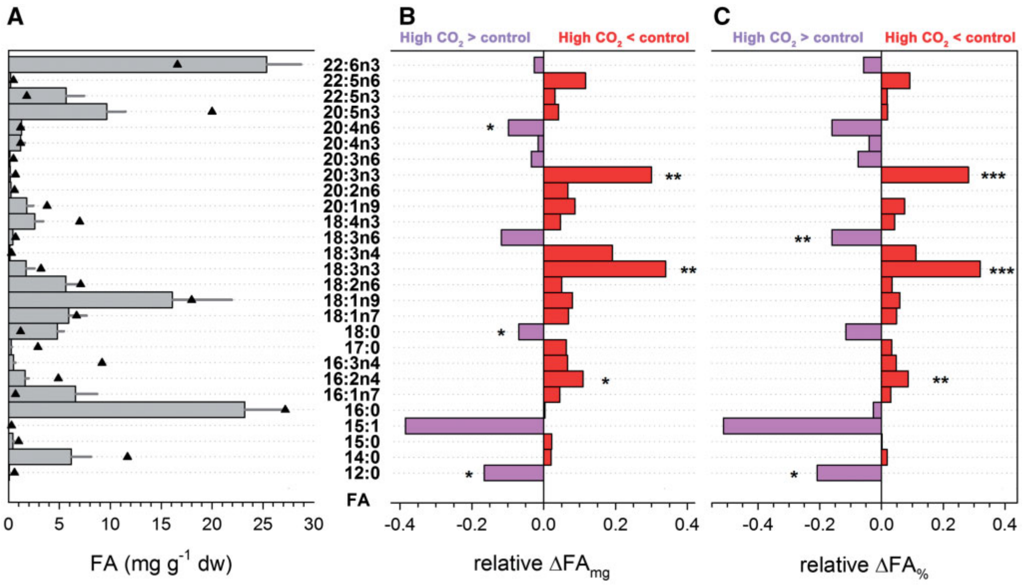

Figure 5. FA profiles of juvenile *M. menidia* reared in the laboratory to 4.5 months post-fertilization under contrasting CO_2 conditions. **(a)** average ± 1 SD concentrations of 27 FAs (mg FA per g dry weight of fish) with black squares depicting FA-concentrations in the juvenile diet (Otohime B1); **(b)** average difference in FA concentrations (mg g^{-1}) between high and control CO_2 treatment scaled by the overall FA-specific mean; **(c)** average difference in FA concentrations (% of total FA content) between high and control CO_2 treatment scaled by the overall FA-specific mean. Purple and red bars correspond to higher and lower FA-content, respectively, in the control relative to the high CO_2 treatment. Asterisks correspond to *p*-values < 0.05 (*), <0.01(**), and < 0.001(***), (t-test, SPSS). This figure is available in black and white in print and in colour at ICES Journal of Marine science online.

values were confined to three FAs on both an absolute (mg g dw^{-1}) and relative basis (% total FA). On the other hand, three FAs were significantly elevated in juveniles from the CO_2 treatment. The subtle differentiation of FA profiles between high and control CO_2 environments in *M. menidia* juveniles contrasts with findings for larval red drum (*Sciaenops ocellatus*) reared at comparable CO_2 levels through 23 dph, which showed significant increases in FA concentration in 19 of 27 FAs measured (Díaz-Gil *et al.*, 2015). The reason for the differences between these two studies is unknown, but might be attributable to species-specific differences in response to CO_2 exposure, or an effect of rearing temperature (27 vs. 17 °C, this study) or developmental stage (larvae vs. juveniles, this study).

The significant effects of high CO_2 conditions on some of the long-chain (18- to 22-carbon) highly unsaturated FAs observed in *M. menidia* may signify the activation of a stress response. Pro-inflammatory eicosanoids are built from the omega-6 FA arachidonic acid (20:4*n*–6), while anti-inflammatory eicosanoids are built from the omega-3 FA eicosapentaenoic acid (20:5*n*–3) (James *et al.*, 2000). The two long-chain FAs (18:3*n*–3 and 20:3*n*–3) that were elevated in juveniles from the control treatment are precursors of 20:5*n*–3. The corresponding

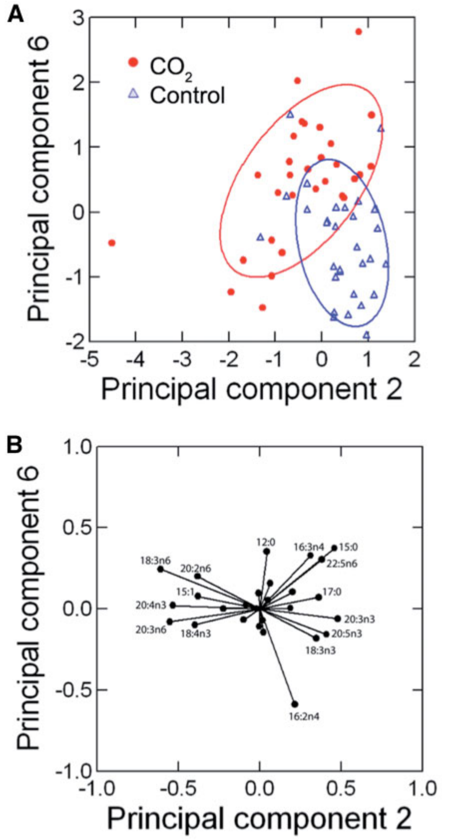

Figure 6. PC analysis of FA profiles (% FA data). **(a)** Scores on PCs 2 and 6 for the high CO_2 and control treatments with confidence ellipsoids (±2 SD). **(b)** Loadings on PCs 2 and 6. This figure is available in black and white in print and in colour at ICES Journal of Marine science online.

lower levels of 18:3$n-3$ and 20:3$n-3$ in juveniles from the high CO_2 treatment may reflect elevated synthesis of anti-inflammatory eicosanoids. Reduced production of pro-inflammatory eicosanoids in fish from the same treatment might account for the accumulation of 20:4$n-6$ in tissues of juveniles, which should be tested by more direct measurements of cortisol and other markers of stress. In addition, the use of *Artemia* nauplii and pellet foods in this experiment likely produced fish with different FA profiles compared with the wild population feeding on natural food. It therefore remains to be demonstrated whether natural foods produce similar physiological responses at contrasting CO_2 levels.

In summary, by rearing a large number of *M. menidia* offspring across multiple life-stages, this study demonstrated the existence of subtle but potentially important effects of OA on the growth of this important coastal forage fish. It further demonstrated the importance of evaluating CO_2 exposure over multiple life-stages to capture long-term changes in metabolic processes. A similar study design would also prove valuable to quantify potential intra- or inter-generational carry-over effects of OA exposure. Last, the potential interaction between restricted food and acidified environments certainly warrants further examination and may be of particular importance to better understand whole ecosystem consequences of OA.

Data

Citable source data of this study are openly available from the BCO-DMO data portal (doi: 10.1575/1912/bco-dmo.652124)

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the article.

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