

RESEARCH ARTICLE

Acidification and hypoxia interactively affect metabolism in embryos, but not larvae, of the coastal forage fish *Menidia menidia*

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ABSTRACT

Ocean acidification is occurring in conjunction with warming and deoxygenation as a result of anthropogenic greenhouse gas emissions. Multistressor experiments are critically needed to better understand the sensitivity of marine organisms to these concurrent changes. Growth and survival responses to acidification have been documented for many marine species, but studies that explore underlying physiological mechanisms of carbon dioxide (CO₂) sensitivity are less common. We investigated oxygen consumption rates as proxies for metabolic responses in embryos and newly hatched larvae of an estuarine forage fish (Atlantic silverside, *Menidia menidia*) to factorial combinations of CO₂ × temperature or CO₂ × oxygen. Metabolic rates of embryos and larvae significantly increased with temperature, but partial pressure of CO₂ (P_{CO₂}) alone did not affect metabolic rates in any experiment. However, there was a significant interaction between P_{CO₂} and partial pressure of oxygen (P_{O₂}) in embryos, because metabolic rates were unaffected by P_{O₂} level at ambient P_{CO₂}, but decreased with declining P_{O₂} under elevated P_{CO₂}. For larvae, however, P_{CO₂} and P_{O₂} had no significant effect on metabolic rates. Our findings suggest high individual variability in metabolic responses to high P_{CO₂}, perhaps owing to parental effects and time of spawning. We conclude that early life metabolism is largely resilient to elevated P_{CO₂} in this species, but that acidification likely influences energetic responses and thus vulnerability to hypoxia.

KEY WORDS: Ocean acidification, Interactive effects, Early life history, Respirometry, *Menidia menidia*, Multi-stressor

INTRODUCTION

Anthropogenic ocean warming and acidification are emerging potential stressors to marine fishes (Hollowed et al., 2013; Heuer and Grosell, 2014), many of which are already threatened by overexploitation (Dulvy et al., 2003). Ocean acidification is the result of carbon dioxide (CO₂) dissolving into seawater and changing the equilibrium of carbonate species, resulting in more hydrogen ions and thus reduced pH. In shallow coastal and estuarine waters, this process is amplified by natural and anthropogenic fluctuations in pH related to ecosystem metabolism (Cai et al., 2011; Wallace et al., 2014; Baumann and Smith, 2018). The fluctuating and often extreme conditions in estuarine habitats require that studies account for the


multiple stressors that coastal organisms simultaneously experience, including extreme temperature, pH and oxygen (Gundersen et al., 2016). In the past 20 years, studies on the biological effects of ocean acidification have rapidly expanded, particularly those focusing on calcifying organisms (Riebesell et al., 2000; Orr et al., 2005; Kroeker et al., 2013; Browman, 2016). More recently, ocean acidification research has expanded to marine fishes (Munday et al., 2009a, 2010; Frommel et al., 2012; Baumann et al., 2012; Chambers et al., 2014; Baumann et al., 2018), and incorporated additional environmental factors such as warming (Munday et al., 2009b; Rosa et al., 2014; Murray and Baumann, 2018; Dahlke et al., 2017) and hypoxia (DePasquale et al., 2015; Gobler and Baumann, 2016; Miller et al., 2016). While adult fishes are expected to be largely resilient to elevated CO₂ levels predicted for the average surface ocean over the next centuries (Ishimatsu et al., 2008), exposure of fish early life stages to acidified water can elicit significant reductions in growth and survival (Baumann et al., 2012; Murray et al., 2014; Chambers et al., 2014; Dahlke et al., 2017). The underlying metabolic changes that contribute to observed growth and survival effects in fish are less well documented, and relatively few studies have quantified effects on oxygen consumption, mitochondrial respiration and acid–base regulation (Heuer and Grosell, 2014; Dahlke et al., 2017). Studies on fish responses to seawater acidification have often revealed large variation among species, populations and even subsequent experiments (Couturier et al., 2013; Munday et al., 2009b; Rummer et al., 2013; Murray and Baumann, 2018; Clark et al., 2020), making mechanistic understanding of long-term CO₂ tolerance challenging.

The Atlantic silverside, *Menidia menidia*, and the closely related inland silverside, *Menidia beryllina*, are widely used fish models for quantifying biological effects of anthropogenic stressors (Conover and Munch, 2002; Baumann et al., 2012; DePasquale et al., 2015). Although silversides are not commercially exploited, these abundant estuarine forage fish represent an ecologically important link between planktonic and higher piscivorous trophic levels in coastal ecosystems (Bayliff, 1950; Cadigan and Fell, 1985). Offspring growth and survival in *M. menidia* and *M. beryllina* have been shown to decrease under elevated CO₂ levels in some (Baumann et al., 2012; Murray et al., 2017), but not all studies (Baumann et al., 2018; Murray and Baumann, 2018).

Acidification has also been applied simultaneously with low partial pressure of oxygen (P_{O₂}) in several *M. menidia* experiments because CO₂-acidified conditions co-occur with hypoxia in eutrophied estuaries (Melzner et al., 2013; Wallace et al., 2014; Gobler and Baumann, 2016; Baumann and Smith, 2018) and both stressors are intensifying globally (Doney et al., 2009; Keeling et al., 2010; Gruber, 2011). Hypoxia alone can cause fish to either expend energy escaping patches of low oxygen (Pihl et al., 1991; Weltzien et al., 1999) or engage mechanisms such as metabolic suppression (Richards, 2009), adjustment of oxygen-binding and transport properties of the blood (Silkin and Silkina, 2005; Wells, 2009), and altered behavior (Pollock et al., 2007). In early life stages, when

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List of symbols and abbreviations

A_T	total alkalinity
DIC	dissolved inorganic carbon
DM	dry mass
DO	dissolved oxygen
f_{CO_2}	fugacity of carbon dioxide
MLR	multiple linear regression
\dot{M}_{O_2}	oxygen consumption rate
P_{CO_2}	partial pressure of carbon dioxide
P_{crit}	critical oxygen partial pressure
P_{O_2}	partial pressure of oxygen
Q_{10}	temperature coefficient of metabolic rate
RMR	routine metabolic rate
SDR	SensorDish reader
TL	total length

compensatory mechanisms are still developing, hypoxia can delay hatching, slow growth, and reduce pre- and post-hatch survival (Rombaugh, 1988; DePasquale et al., 2015). Combined high CO_2 and low oxygen can have synergistic effects on hatching success and larval survival, for example in *M. menidia* (DePasquale et al., 2015; Cross et al., 2019; Morrell and Gobler, 2020). In *M. menidia* adults, exposure to acidified water increased the oxygen levels at which surface respiration, loss of equilibrium and death occurred (Miller et al., 2016). These studies highlight variable sensitivity to acidification and the likelihood of *M. menidia* to respond to multiple interacting stressors differently than they do to the sum of individual stressors. Responses to CO_2 and P_{O_2} treatments have been documented without interacting effects for other fish species and response variables, including cellular metabolic enzyme activity of juvenile rockfish (Davis et al., 2018), survival of juvenile weakfish (Lifavi et al., 2017), and growth and survival of inland silversides (DePasquale et al., 2015). One mechanism by which acidification could interact with hypoxia is by modifying the critical oxygen partial pressure (P_{crit}), or the P_{O_2} threshold below which routine metabolic rate (RMR) becomes oxygen dependent (Richards, 2009). In the case of the intertidal sculpin *Clinocottus analis*, CO_2 acidification not only increased RMR, but also raised P_{crit} , making the fish less hypoxia tolerant (Hancock and Place, 2016).

In order to evaluate which species of fish are most vulnerable to ocean acidification and how fishes may be able to acclimate or adapt to it, it is important to have a mechanistic understanding of how acidification affects fishes. Although many trait responses to high CO_2 have been examined for many species, the mechanistic responses and bioenergetic consequences of elevated CO_2 combined with other stressors are poorly understood. Because most physiological processes, including metabolic rates (Peck and Moyano, 2016), directly depend on temperature, acidification effects are likely temperature dependent, although there is both supporting and contradicting empirical evidence for this in fishes (Pörtner et al., 2017; Jutfelt et al., 2018; Murray and Baumann, 2018). For example, while elevated CO_2 increased survival and development speed in Antarctic dragonfish (*Gymnodraco acuticeps*) embryos, rearing embryos in water 3°C warmer caused CO_2 to have the opposite effect (Flynn et al., 2015). Additive and synergistic interactions between CO_2 and temperature were detected in ventilation and metabolic rates of juvenile emerald rockcod, in which elevated rates under high temperature at 14 days of exposure were compensated in the ambient but not elevated CO_2 treatments by 28 days (Davis et al., 2017).

RMR has been measured in fishes exposed to elevated CO_2 and temperature, and results depend heavily on the species and the

methods used (Heuer and Grosell, 2014). For different species, acclimation to combined temperature and CO_2 treatments may show temperature but not CO_2 effects on RMR (Strobel et al., 2012), separate effects of both variables, or synergistic interactions (Munday et al., 2009b). Negative effects of high CO_2 may manifest mostly at the upper and lower ends of a species' thermal tolerance window because aerobic scope and thus the energetic capacity for acid–base regulation may become limited near thermal extremes (Fry, 1971; Pörtner, 2010; Lefevre, 2016). Responses to stressors at thermal extremes are also useful in understanding how climate change will affect distributions, because thermal extremes are a strong determinant of geographic ranges (Calosi et al., 2010; Lynch et al., 2014). It is therefore critical to quantify responses of multiple endpoints across a range of temperatures (Pörtner, 2012; Twiname et al., 2019).

Over the course of two spawning seasons, we conducted six independent experiments to quantify routine metabolic rates of *M. menidia* embryos and newly hatched larvae reared in elevated CO_2 partial pressure (P_{CO_2}) and across a range of oxygen and thermal conditions that already occur in the species' habitat (Middaugh et al., 1987). We focused on the early life stages because these stages tend to be more sensitive to stressful conditions than adults (Pörtner et al., 2005; Baumann et al., 2012; Harvey et al., 2013; Dahlke et al., 2020), and growth and survival of early life stages influence recruitment and population dynamics (Chambers and Trippel, 1997; Houde, 1987; Pörtner and Peck, 2010). Both synergistic (Munday et al., 2009b) and antagonistic (Lefevre, 2016) interactions between CO_2 and temperature treatments on resting metabolic rate have been quantified in fishes, and the nature of the interaction may depend on whether the species in question increases or decreases metabolism under elevated CO_2 . Our previous results on *M. menidia* show reduced or unchanged growth and survival under high CO_2 (Murray and Baumann, 2018; Baumann et al., 2018), which suggests additional energetic demands of pH regulation and thus greater oxygen demand. Therefore, we hypothesized that embryonic and larval metabolic rates would increase with both P_{CO_2} and temperature, and that P_{CO_2} effects would synergistically interact with temperature. Low P_{O_2} conditions were expected to induce metabolic suppression, thereby potentially counteracting or concealing any elevation in metabolism owing to high P_{CO_2} . An interaction between P_{O_2} and P_{CO_2} was also expected because reduced pH from elevated CO_2 can reduce the oxygen-binding capacity of the blood (Brauner and Randall, 1996), so acidified seawater may differentially affect oxygen consumption depending on the P_{O_2} level. Overall, we expected larvae to be less sensitive to the treatments than embryos based on results of previous studies (Chambers et al., 2014; Murray and Baumann, 2018; Cross et al., 2019) and the more advanced regulatory mechanisms of fish larvae relative to embryos (Rombaugh, 1988).

MATERIALS AND METHODS

Animals and experimental design

Detailed descriptions of animal collection and husbandry methods can be found in Murray and Baumann (2018). Briefly, wild, spawning-ripe adult *Menidia menidia* (Linnaeus 1766) were collected from Mumford Cove (41°19'25"N, 72°1'7"W), Groton, CT, USA, in late spring and early summer of 2016 and 2017 and transported to the Rankin seawater laboratory at University of Connecticut's Avery Point Campus. Adults were strip-spawned, and fertilized eggs were allowed to attach to 1-mm mesh screens, which were randomly distributed into 20-liter rearing containers with mesh-covered (100 µm) holes to allow overflow without loss of embryos or larvae. One container with 100 embryos was placed into

Table 1. Overview of target levels for carbon dioxide partial pressure (P_{CO_2}), temperature and oxygen partial pressure (P_{O_2}) for six experiments for which respirometry was conducted on embryos (E) and newly hatched larvae (L) of *Menidia menidia*

Experiment	Fertilization date (mm/dd/yyyy)	P_{CO_2} (μ atm)	Temperature ($^{\circ}$ C)	P_{O_2} (kPa)	Stage
1	04/22/2016	400, 2200	17, 24	21–23	E, L
2	05/03/2016	400, 2200, 4200	17, 20, 24	21–23	L
3	05/19/2016	400, 2200, 4200	17, 20, 24	21–23	E, L
4	05/26/2017	400, 2200, 4200	24, 28	21–23	E, L
5	05/09/2017	400, 2200, 4200	24	7.5, 12.0, 23.0	E, L*
6	06/09/2017	400, 2200, 4200	24	9.0, 12.0, 23.0	E, L

*In experiment 5, respirometry was only performed on larvae from the 23.0 and 12.0 kPa P_{O_2} treatments owing to low hypoxic hatch survival.

each treatment tank within 2 h post-fertilization, so initial exposure was acute and exposure time (in days) is equivalent to age at sampling. All experimental methods were approved and conducted according to University of Connecticut Institutional Animal Care and Use Committee protocol A14-032.

Of six factorial experiments conducted in 2016 and 2017, experiments 1–4 quantified CO_2 × temperature effects and experiments 5 and 6 quantified CO_2 × oxygen effects (Table 1). Experiment 1 used 400 and 2200 μ atm as target P_{CO_2} levels, crossed with two temperatures: 17 $^{\circ}$ C and 24 $^{\circ}$ C. Experiments 2 and 3 factorially crossed three P_{CO_2} levels (400, 2200 and 4200 μ atm) with three temperatures (17 $^{\circ}$ C, 20 $^{\circ}$ C and 24 $^{\circ}$ C). Experiment 4 used the same three target P_{CO_2} levels crossed with 24 $^{\circ}$ C and 28 $^{\circ}$ C. The target temperature levels represent the range of temperatures typically encountered by *M. menidia* in Long Island Sound during the spawning season of late April to early July, except for 28 $^{\circ}$ C, which is closer to the species' upper thermal extreme (Middaugh et al., 1987). The target P_{CO_2} levels represent the current average oceanic P_{CO_2} (400 μ atm), the approximate level predicted as the average oceanic P_{CO_2} for the next 300 years under a rising CO_2 emissions scenario (RCP8.5; IPCC, 2013) and often experienced by estuarine fishes such as *M. menidia* in the summer (2200 μ atm), and a higher level

(4200 μ atm) that will occur more often in the future as average P_{CO_2} rises in conjunction with coastal eutrophication and hypoxia (Wallace et al., 2014). Experiments 5 and 6 exposed *M. menidia* early life stages to three levels of P_{CO_2} (400, 2200 and 4200 μ atm) crossed factorially with three target levels of P_{O_2} : normoxic (23 kPa), suboxic (12 kPa) and hypoxic (7.5 or 9 kPa). The hypoxic P_{O_2} condition was increased to 9 kPa in experiment 6 to avoid the complete larval mortality observed at 7.5 kPa in experiment 5. The different treatments used across all experiments, each with $n=1$ rearing container per treatment, resulted in one to four treatment replicates for experiments 1–4, and one or two treatment replicates for experiments 5 and 6. The lack of consistent replication is dealt with using measured treatment means as quantitative rather than categorical variables in the analysis (see Data analysis). We sampled at least 10 individuals per treatment for each respirometry trial, so after outlier removal and pooling of experiments, the final dataset contained 8 to 55 embryos and 9 to 68 larvae from each treatment (Table 2). The target treatment levels of all six experiments are summarized in Table 1, and measured water chemistry, temperature and oxygen values are summarized in Tables S1 and S2.

Gas and temperature manipulations and measurements are described in detail by Murray and Baumann (2018) and Cross et al.

Table 2. Age at sampling (equivalent to exposure time) in days post-fertilization (dpf) and mean routine metabolic rates (\pm s.e.m.) of *M. menidia* embryos and larvae across P_{CO_2} , temperature and P_{O_2} treatments

Temperature ($^{\circ}$ C)	P_{O_2} (kPa)	Age at embryonic sampling (dpf)	Age at larval sampling (dpf)	P_{CO_2} (μ atm)	Embryonic sample size	Mean \pm s.e.m. embryonic RMR (μ mol O_2 h $^{-1}$)	Larval sample size	Mean \pm s.e.m. larval RMR (μ mol O_2 mg $^{-1}$ h $^{-1}$)
17	>20	11–12	14–15	400	26	0.016 \pm 0.002	41	0.078 \pm 0.004
				2200	27	0.020 \pm 0.001	35	0.071 \pm 0.005
				4200	12	0.026 \pm 0.002	24	0.066 \pm 0.005
20	>20	8	10–11	400	12	0.027 \pm 0.002	23	0.071 \pm 0.007
				2200	12	0.020 \pm 0.002	26	0.088 \pm 0.006
				4200	13	0.024 \pm 0.002	24	0.087 \pm 0.009
24	>20	5	6–7	400	55	0.024 \pm 0.001	65	0.126 \pm 0.006
				2200	46	0.021 \pm 0.002	68	0.118 \pm 0.006
				4200	16	0.017 \pm 0.002	33	0.118 \pm 0.009
28	>20	4	5	400	11	0.039 \pm 0.004	9	0.196 \pm 0.018
				2200	11	0.035 \pm 0.004	10	0.191 \pm 0.018
				4200	11	0.034 \pm 0.002	10	0.207 \pm 0.022
24	23.0	5	6–7	400	26	0.022 \pm 0.002	21	0.165 \pm 0.013
				2200	28	0.028 \pm 0.003	19	0.148 \pm 0.018
				4200	29	0.034 \pm 0.003	19	0.174 \pm 0.017
24	12.0	5–6	7–8	400	26	0.018 \pm 0.002	15	0.182 \pm 0.019
				2200	31	0.026 \pm 0.002	22	0.179 \pm 0.013
				4200	30	0.025 \pm 0.002	18	0.173 \pm 0.019
24	9.0	7	9	400	13	0.025 \pm 0.004	11	0.126 \pm 0.030
				2200	14	0.022 \pm 0.003	11	0.142 \pm 0.020
				4200	8	0.027 \pm 0.004	10	0.130 \pm 0.024
24	7.5	7	–	400	18	0.025 \pm 0.003	–	–
				2200	17	0.018 \pm 0.003	–	–
				4200	19	0.017 \pm 0.002	–	–

Dashes (–) indicate treatments for which too few embryos survived to hatching for larval respirometry to be performed.

(2019). The actual P_{CO_2} levels were calculated based on measured pH, temperature, salinity and total alkalinity (A_T). A_T samples were collected three times per experiment and measured using an endpoint titration (G20 Potentiometric Titrator, Mettler Toledo, Columbus, OH, USA). Based on these measurements, the P_{CO_2} (μatm), fugacity of CO_2 (f_{CO_2} ; μatm), dissolved inorganic carbon (DIC; $\mu\text{mol kg}^{-1}$) and carbonate ion concentration (CO_3^{2-} ; $\mu\text{mol kg}^{-1}$) were calculated in CO2SYS (V2.1). In the $\text{CO}_2 \times$ temperature experiments, oxygen was maintained at $\sim 100\%$ air saturation (>20 kPa). For experiments 1 and 4, this was achieved with continuous bubbling and validated daily for each tank with a handheld probe (Intellical LDO101 Laboratory Luminescent Dissolved Oxygen Sensor, Hach, Loveland, CO, USA). For experiments 2, 3, 5 and 6, dissolved oxygen (DO, mg l^{-1}) measurements were automatically taken twice hourly in each tank by a DO probe (LDO Model 2, Hach) connected to a LabView (National Instruments, Austin, TX, USA) program, which adjusted bubbling of CO_2 -stripped air or nitrogen gas to maintain target oxygen levels. DO measurements were converted to P_{O_2} using the oxygen solubility at the measured temperature and salinity (García and Gordon, 1992). Mean temperature, P_{O_2} , pH and carbon chemistry parameters measured and calculated over the duration of each experiment can be found in Tables S1 ($\text{CO}_2 \times$ temperature experiments) and S2 ($\text{CO}_2 \times$ oxygen experiments).

Microrespirometry

Closed respirometry measurements were conducted on embryos (901–1227 μm diameter) randomly sampled from each treatment 1–3 days prior to hatch and larvae (4075–6330 μm total length) sampled on the day of hatching. Because temperature and P_{O_2} influenced the development speed, the sampling days correspond to different numbers of days post-fertilization, but similar developmental stages (see Table 2 for exact ages). The P_{CO_2} level did not affect time to hatching, but embryos from higher temperatures and higher P_{O_2} levels hatched up to 7 days earlier than the other treatments (Table 2; Murray and Baumann, 2018; Cross et al., 2019). Individual embryos were gently removed from screening and placed into microrespirometry wells. Newly hatched larvae were removed from the rearing containers with pipettes with at least a 5-mm wide tip to avoid injury and minimize handling stress. All respirometry trials were conducted during daylight hours between 09:00 and 18:00 h, during which time newly hatched larvae exhibited consistent levels of routine activity.

Oxygen consumption rates of embryos and larvae were measured by two 24-channel SensorDish readers (SDRs; PreSens Precision Sensing, GmbH, Regensburg, Germany) and glass well plates equipped with an optical oxygen sensor spot in each well (Loligo Systems, Viborg, Denmark). Each 0.5-ml well received a single embryo or larva, and at least one well contained only treatment water to measure background (control) respiration. We used both 24-well sensor plates and loaded individuals from each P_{CO_2} treatment within a given temperature or P_{O_2} level into the plates simultaneously. The wells were filled completely with water from the treatment tank that each individual came from, cleared of air bubbles, and sealed airtight with parafilm, silicone and acrylic sheets. Well plates were placed in temperature-controlled water baths during the respirometry measurement period, and the system was covered to prevent light from interfering with the sensors and to minimize larval activity. Individuals were allowed to recover from any handling stress and sensors were allowed to equilibrate with the water bath temperature for 10 min before measurements started. DO (mg l^{-1}) was recorded every 15 s by the PreSens SDR software until DO had decreased by 3 mg l^{-1} in at least one of the wells. Trial duration ranged from 15 to

60 min, depending on the temperature. In the case of the suboxic and hypoxic treatments from experiments 5 and 6, however, the trials lasted 5 min regardless of the DO differential, given the already low oxygen in the treatment water. Stirring is recommended to maintain homogeneous oxygen concentrations throughout closed respirometry chambers (Peck and Moyano, 2016), but magnetic stir bars cannot be used with this system. The system can be used atop a shaker, but shaking induces premature hatching of embryos and could disturb the larvae. However, this type of system has been used in the past without shaking or stirring (e.g. Flynn and Todgham, 2018; Zimmer et al., 2020), and calculations of oxygen diffusion rates for our conditions showed that diffusion should maintain homogeneity of oxygen as the fish consumes oxygen. At the end of each measurement period, embryos and larvae were checked for injury or death, and any other factors that might have affected oxygen consumption rates were noted. Between trials, the wells were rinsed with DI water but not dried in order to keep the sensors hydrated, and between experiments they were rinsed and gently swabbed with a 35% ethanol solution.

Data analysis

Calculations of metabolic rates and all statistical tests were conducted in R statistical software (v4.0.0; <https://www.r-project.org/>) using the stats package and the olsrr package (<https://CRAN.R-project.org/package=olsrr>). Because temperature influences oxygen solubility and metabolic rates of fish, we measured temperature simultaneously with DO throughout the measurement period and only used data for periods of time in which the temperature changed by less than $\sim 0.03^\circ\text{C min}^{-1}$. The SDRs measure phase from each optical sensor spot, and the SDR software uses phase and temperature to calculate DO (mg l^{-1}) at each time point. An approximately linear section of data was chosen via visual inspection, and a linear model was fitted to the DO values with respect to time for each well. The slope ($\text{mg O}_2 \text{l}^{-1} \text{s}^{-1}$) of the linear model was used to calculate oxygen consumption rate (\dot{M}_{O_2} ; $\mu\text{mol O}_2 \text{h}^{-1}$) with the following formula: $\dot{M}_{\text{O}_2} = (\text{slope}/0.032) \times 3600 \times 0.0005$, where 0.032 is the molar mass of O_2 (mg mol^{-1}), 3600 converts seconds to hours, and 0.0005 liters is the well volume. The mean \dot{M}_{O_2} from control wells was subtracted from each fish-containing well of the same treatment to account for microbial respiration and obtain fish \dot{M}_{O_2} . Size differences in embryos were negligible and quantifying embryo mass was impractical, so \dot{M}_{O_2} was not normalized to mass and is reported as whole-embryo routine metabolic rate (RMR; $\mu\text{mol O}_2 \text{individual}^{-1} \text{h}^{-1}$). Larval total length (TL, mm) was measured in images (ImageJ) taken by a digital camera (TrueChrome Metrics, Tucson Photonics Co., Fuzhou, Fujian, China) connected to a stereo microscope (Nikon Eclipse E200). TL was then converted to dry mass (DM, mg) using the relationship $\ln(\text{DM}) = 2.997 \times \ln(\text{TL}) - 6.703$ (H. Baumann, personal communication). DM was then used to calculate the larval mass-specific RMR ($\mu\text{mol mg}^{-1} \text{h}^{-1}$) as $\text{RMR} = \dot{M}_{\text{O}_2}/\text{DM}$ (Peck and Moyano, 2016).

As a measure of sensitivity of metabolic rates to rearing temperature, the temperature coefficient (Q_{10}), the change in RMR associated with a 10°C rearing temperature increase, was calculated at each P_{CO_2} . The formula used for this calculation is $Q_{10} = (\text{RMR}_b/\text{RMR}_a)^{10/(T_b - T_a)}$, where T is the mean temperature in 17°C treatments (a) and 28°C treatments (b). Within P_{CO_2} treatments and life stages, Q_{10} was calculated for every possible pairing of individuals from the 17°C and 28°C treatments. Bootstrapping was used to sample with replacement from the pool of Q_{10} values 100,000 times, in order to estimate a mean Q_{10} and 95% confidence intervals.

Multiple linear regression (MLR) was used to determine whether metabolic rate changed as a linear function of P_{CO_2} , temperature,

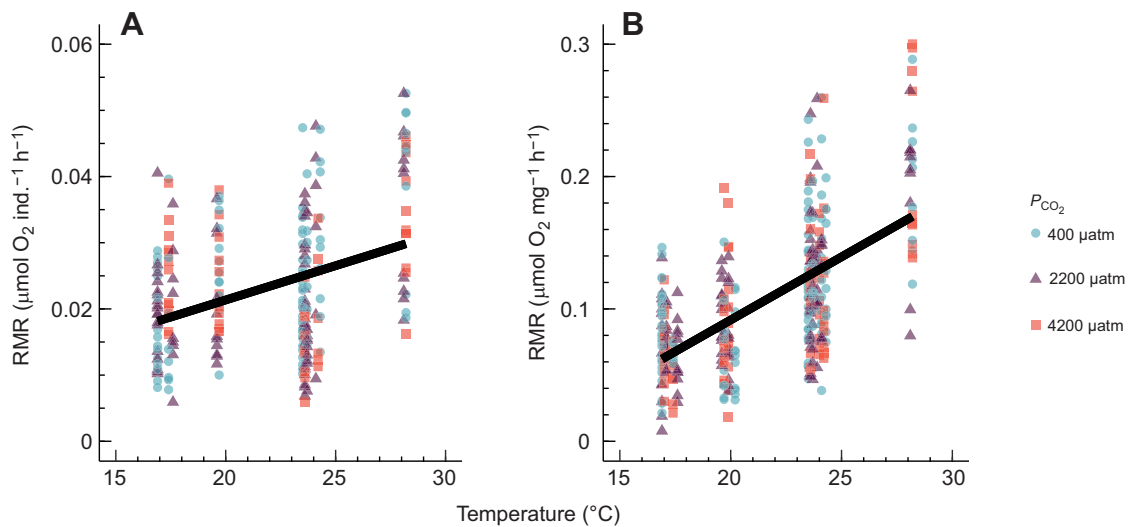


Fig. 1. Temperature- and carbon dioxide partial pressure (P_{CO_2})-dependent routine metabolic rates (RMRs) of *Menidia menidia* embryos and larvae. (A) Whole-body RMR ($\mu\text{mol O}_2 \text{ individual}^{-1} \text{ h}^{-1}$) of embryos and (B) mass-specific RMR ($\mu\text{mol O}_2 \text{ mg}^{-1} \text{ h}^{-1}$) of newly hatched larvae from experiments 1–4. Regression lines are fitted to the metabolic rates as a function of temperature to illustrate the significant effect of temperature (MLR, embryos: $F_{3,247}=10.96$, $P<0.001$; larvae: $F_{3,364}=69.87$, $P<0.001$). Sample sizes for each temperature and P_{CO_2} treatment combination are $n=10$ –46 for embryos and $n=9$ –68 for larvae.

P_{O_2} or the interaction between P_{CO_2} and either of these secondary variables. Our experiments had pseudoreplication, in that individuals were sampled from only one experimental unit (rearing container) per treatment in each experiment. Using the measured predictor variables and treating them as quantitative (continuous) values rather than categorical groups allows the use of linear regression and does not require more than one experimental unit (Hurlbert, 2004). This analysis gave us more statistical power and more informative results than an analysis with categorical predictor variables would have (Cottingham et al., 2005; Havenhand et al., 2010). MLR also has the benefits of accounting for the variability in, order of and distance between experimental treatments. This approach allows us to define quantitative relationships between the predictor and response variables that have predictive capacity beyond the treatment levels used and can be incorporated into ecological models such as dynamic energy budgets (Cottingham et al., 2005; Kooijman, 2009).

Outliers were identified using Cook's distance and standardized residuals and removed from the dataset under the assumption that extreme values represented active or stressed fish, rather than routine metabolism. Data were square-root transformed in the regression in order to satisfy the assumptions of normality and homoscedasticity, as tested using the `ols_test_normality` and `ols_test_breusch_pagan` functions, respectively, in the `olsrr` package (v0.5.3). All statistical tests were interpreted with a significance threshold of $\alpha=0.05$.

RESULTS

$CO_2 \times$ temperature

In experiments 1–4, embryonic metabolic rates increased significantly (MLR, $F_{3,248}=11.94$, $P<0.001$) with temperature, with a 79% increase in mean metabolic rate from the lowest (17°C) to the highest (28°C) temperature across all P_{CO_2} treatments. However, metabolic rates were unaffected by P_{CO_2} treatment (MLR, $F_{3,248}=11.94$, $P=0.07$; Fig. 1A). In larvae, metabolic rates increased significantly (MLR, $F_{3,364}=69.87$, $P<0.001$) with temperature but were also statistically unaffected by P_{CO_2} treatment (MLR, $F_{3,364}=69.87$, $P=0.189$; Fig. 1B). Mean metabolism increased by 173% from the lowest (17°C) to the highest (28°C) temperature across all P_{CO_2} treatments. Mean metabolic rates by treatment and sampling age are reported in Table 2, and

multiple regression model results are summarized in Table S3. Bootstrapped mean Q_{10} values for *M. menidia* embryos decreased as P_{CO_2} increased (Fig. 2). The bootstrapped 95% confidence intervals for embryonic Q_{10} did not overlap between the three P_{CO_2} levels (Table S4). In larvae, however, Q_{10} increased from 400 to 2200 $\mu\text{atm } P_{CO_2}$, and decreased slightly from 2200 to 4200 $\mu\text{atm } P_{CO_2}$ (Fig. 2). The bootstrapped 95% confidence intervals for the elevated P_{CO_2} levels overlapped with each other, but not with that of the 400 $\mu\text{atm } P_{CO_2}$ level (Table S4). Overall, the Q_{10} was higher in larvae than embryos under elevated P_{CO_2} but similar for both life stages under ambient P_{CO_2} .

$CO_2 \times$ oxygen

For experiments 5–6, we detected a significant interaction of $P_{CO_2} \times P_{O_2}$ on embryonic metabolic rates (MLR, $F_{3,255}=8.74$,

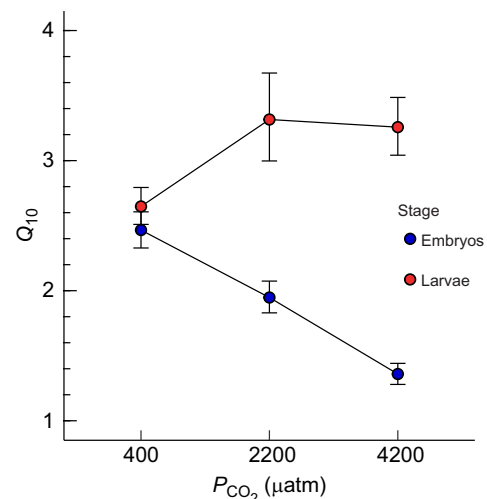


Fig. 2. The effect of P_{CO_2} on Q_{10} of RMRs in *M. menidia* embryos and larvae. Bootstrapped mean Q_{10} values of *M. menidia* embryos and larvae calculated from routine metabolic rates at 17°C and 28°C, from experiments 1–4. Error bars indicate bootstrapped 95% confidence intervals, and sample sizes for each P_{CO_2} level are $n=10$ –27 for embryos and $n=9$ –41 for larvae.

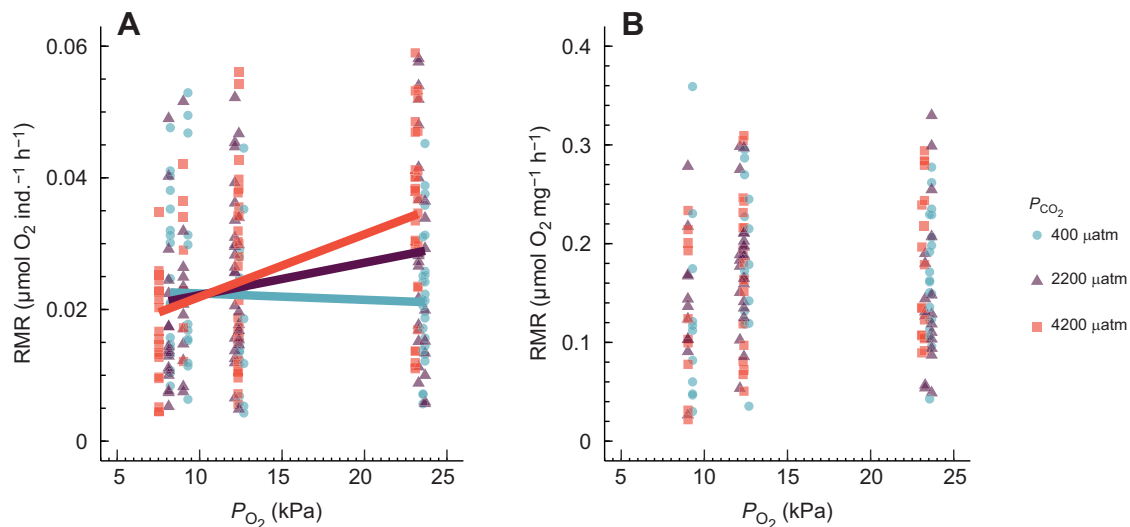


Fig. 3. Oxygen partial pressure (P_{O_2})- and P_{CO_2} -dependent RMRs of *M. menidia* embryos and larvae. (A) Whole-body RMR ($\mu\text{mol O}_2 \text{ individual}^{-1} \text{ h}^{-1}$) of embryos and (B) mass-specific RMR ($\mu\text{mol O}_2 \text{ mg}^{-1} \text{ h}^{-1}$) of newly hatched larvae from experiments 5–6. Regression lines are fitted to embryonic metabolic rates (A) as a function of P_{O_2} within each P_{CO_2} treatment to illustrate the significant P_{CO_2} and P_{O_2} interaction (MLR, $F_{3,258}=7.96$, $P=0.005$). No regression lines are shown for larvae (B) because there were no significant effects (MLR, $F_{3,142}=0.325$, $P>0.05$). Sample sizes in each P_{O_2} and P_{CO_2} combination are $n=8$ –31 for embryos and $n=10$ –22 for larvae.

$P=0.004$; Fig. 3A). For embryos reared under normoxic (23.0 kPa) conditions, exposure to elevated (4200 μatm) P_{CO_2} increased metabolic rates by 55% compared with ambient P_{CO_2} . However, this effect of elevated P_{CO_2} diminished with decreasing P_{O_2} . Additionally, metabolic rates of embryos in ambient P_{CO_2} treatments were similar across all P_{O_2} levels, but they increased with P_{O_2} under elevated P_{CO_2} (Table 2). Metabolic rates of newly hatched larvae were statistically unaffected by P_{CO_2} or P_{O_2} (MLR, $F_{3,142}=0.325$, $P>0.05$; Fig. 3B).

DISCUSSION

Rearing *M. menidia* offspring and measuring their RMRs in combinations of P_{CO_2} and temperature revealed, as expected, that temperature is the dominant determinant of embryonic and larval metabolism at these treatment levels. This is consistent with other studies in which temperature had stronger effects than CO_2 on physiological endpoints in fishes, including larval growth and survival (Murray and Baumann, 2018), time to hatch (Gobler et al., 2018), metabolic rates and aerobic scope (Gräns et al., 2014), and hatching success and ionocyte abundance (Dahlke et al., 2017). In other cases, sensitivity of fishes to CO_2 has depended on temperature through interacting effects (Munday et al., 2009b; Pimentel et al., 2014; Murray et al., 2019). Seawater acidification is thought to affect fish by limiting functional capacity of tissues, particularly at thermal extremes (Pörtner, 2012). If such a CO_2 response exists in *M. menidia*, perhaps our temperature treatments were not extreme enough to elicit a limitation of functional capacity that is detectable through routine metabolism.

Thermal sensitivity (Q_{10}) of RMR was similar for embryos and larvae at ambient (400 μatm) P_{CO_2} but declined with elevated P_{CO_2} in embryos and increased with P_{CO_2} in larvae (Fig. 2; Table S4). A study on larval flatfish (*Solea senegalensis*) exposed to elevated CO_2 and temperature found a trend similar to that of our embryos, that increasing CO_2 reduced Q_{10} , and also found that Q_{10} increased with fish age (Pimentel et al., 2014). Fish embryos and larvae are generally more stenothermal than juveniles and young adults, and in some species these early life stages are incapable of thermal acclimation

(Rombaugh, 1988; Rijnsdorp et al., 2009). However, *M. menidia* nursery habitats have rapidly fluctuating conditions (Conover and Ross, 1982), hence some capacity for acclimation to temperature and CO_2 is expected (Baumann, 2019). Reduced thermal sensitivity of embryonic RMR under elevated P_{CO_2} may be a beneficial adaptation that helps embryos with limited mobility survive periods of high temperature and acidity. In contrast, larvae are free swimming and active, with a higher metabolic rate overall and more well-developed mechanisms for gas and ion exchange (Nilsson and Östlund-Nilsson, 2008). The increase in larval Q_{10} with elevated P_{CO_2} indicates that larvae are slightly more sensitive to combined high P_{CO_2} and temperature, but their more advanced ability to regulate their activity and physiology may make this sensitivity less detrimental than it would be for embryos.

The combined P_{CO_2} and P_{O_2} treatments had a significant interactive effect on embryonic, but not larval, metabolic rates. In normoxia (23.0 kPa), RMR increased with P_{CO_2} , consistent with our hypothesis that seawater acidification increases metabolic costs of acid–base balance. However, the RMR of high P_{CO_2} embryos decreased with declining P_{O_2} , suggesting RMR became oxygen dependent and the embryos from these treatments had reached their P_{crit} , the P_{O_2} level below which aerobic metabolism depends directly on available oxygen (Richards, 2009). This oxygen dependence was not detected in the ambient P_{CO_2} treatments, indicating that P_{crit} was higher in acidified water. Increased P_{crit} can be expected as a result of elevated P_{CO_2} because the Bohr effect of reduced blood pH leads to lower hemoglobin– O_2 binding efficiency, and thus inhibits oxygen uptake (Brauner and Randall, 1996; Wells, 2009; Gobler and Baumann, 2016). Severely reduced oxygen uptake inhibits mitochondrial production of ATP and forces the fish to activate anaerobic pathways, which are much less efficient than aerobic metabolism and thus unsustainable as a long-term response (Richards, 2009). Although we did not quantify anaerobic metabolism in this study, doing so in future work would improve understanding of energetic costs of chronic acidification and hypoxia. Nevertheless, the synergistic effect we detected on embryos suggests that P_{CO_2} influences both P_{crit} and the oxygen-independent RMR

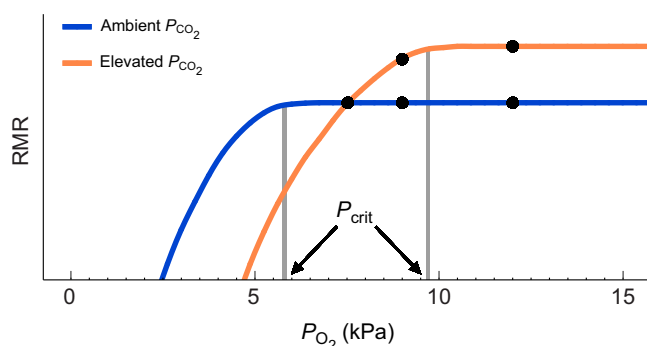


Fig. 4. Conceptual diagram of the relationship between P_{O_2} and RMR of *M. menidia* embryos in ambient and elevated P_{CO_2} . Hypothesized shifts in the relationship between embryonic RMR and P_{O_2} are shown for elevated (orange) versus ambient (blue) P_{CO_2} . Our results (measured at the P_{O_2} levels marked by black dots) suggest that P_{CO_2} can influence both the critical oxygen partial pressure (P_{crit} , gray lines) and the oxygen-independent RMR. At higher P_{O_2} levels, RMR increases with P_{CO_2} , potentially owing to increased metabolic demand. As P_{O_2} decreases, embryonic RMR reaches P_{crit} and becomes oxygen dependent at a higher P_{O_2} level in acidified than in ambient P_{CO_2} conditions. Low intracellular red blood cell pH caused by high P_{CO_2} can be expected to reduce hemoglobin– O_2 affinity (Bohr effect) and make embryonic RMR less hypoxia resistant, which could manifest as an increase in P_{crit} for embryos in elevated P_{CO_2} . See Discussion for more information.

(Fig. 4). This further highlights the importance of multistressor studies, as hypoxia sensitivity could be underestimated when co-occurring effects of acidification are not considered. Such a principle has important implications for informing policy initiatives aimed at maintaining ecologically sound oxygen levels in coastal marine systems (Paerl, 2006).

Embryos were more sensitive than larvae to the P_{CO_2} and P_{O_2} treatments, with larvae having no significant response to either variable. However, survival to hatching was so low in the 7.5 kPa P_{O_2} treatments that we did not have enough larvae left to measure larval metabolic rates for that treatment. This lowest P_{O_2} level yielded the lowest growth and survival at both stages (Cross et al., 2019), so exclusion of this level from larval metabolic measurements likely precluded detection of any P_{O_2} effect, such as oxygen dependence indicative of P_{crit} in larvae. Future work should investigate whether acidification increases P_{crit} of *M. menidia* larvae as might be expected owing to reduced oxygen affinity of hemoglobin under ambient P_{CO_2} (Brauner and Randall, 1996; Wells, 2009). Surprisingly, recent work on juvenile European sea bass found a decrease in P_{crit} accompanied by increased hemoglobin– O_2 affinity under acutely increasing P_{CO_2} (Montgomery et al., 2019), so it is also possible that *M. menidia* are capable of reducing P_{crit} under short-term acidification. In contrast, woolly sculpin exposed to long-term elevated P_{CO_2} had higher RMR and P_{crit} relative to ambient treated fish (Hancock and Place, 2016), which suggests that a response similar to that of our embryos may occur in later stage fishes under a long enough acidified duration. Nonetheless, larvae exposed to high P_{CO_2} may be more hypoxia resistant than embryos owing to increased metabolic control upon hatching, given that embryos often become less hypoxia tolerant closer to hatching as oxygen demand increases but egg surface area for diffusion remains constant (Rombaugh, 1988; Kamler, 1992).

The embryos' metabolic response to elevated P_{CO_2} in the $CO_2 \times$ oxygen experiments is consistent with previous work that has shown the highest sensitivity to seawater acidification in the early life stages of marine animals (Pörtner et al., 2005; Baumann et al., 2012, 2018; Harvey et al., 2013; Dahlke et al., 2020). Early-life mortality

has the potential to impact fish population sizes through reduced recruitment (Chambers and Trippel, 1997; Houde, 1987; Hurst and Conover, 1998), and our results indicate a metabolic mechanism for increased *M. menidia* embryo mortality measured in combined high P_{CO_2} and hypoxia treatments (Cross et al., 2019). With these experiments, we have shown that it is critical to conduct experiments that expose animals to treatment conditions directly after fertilization to imitate natural exposure and allow detection of stage-specific, early-life effects.

Aside from the interactive effect of P_{CO_2} and P_{O_2} on embryos, our experiments showed no other responses of *M. menidia* early life stage RMR to seawater acidification. A review of fish responses to CO_2 indicated that routine metabolism, the response that we measured, might not be as sensitive to CO_2 as other measures, such as standard or active metabolism (Heuer and Grosell, 2014). Additionally, previous experiments exposing *M. menidia* to elevated P_{CO_2} have yielded highly variable results for growth and survival of embryos and larvae, potentially owing to genetic variability or maternal egg provisioning (Baumann et al., 2018; Snyder et al., 2018). The high inherent variability in *M. menidia* responses to high P_{CO_2} suggests that populations will be resilient and able to adapt to acidified conditions. Based on our experiments, this species is metabolically tolerant of isolated high P_{CO_2} , but the negative synergistic response of embryos to high P_{CO_2} and low P_{O_2} conditions that co-occur in estuaries, demonstrates the potential for stage-specific responses and the importance of considering multiple co-occurring stressors.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: H.B., J.A.N.; Methodology: T.G.S., H.B., C.S.M., A.I.M., J.A.N.; Formal analysis: T.G.S.; Investigation: T.G.S., H.B., C.S.M., A.I.M., J.A.N.; Resources: H.B., J.A.N.; Writing - original draft: T.G.S.; Writing - review & editing: T.G.S., H.B., C.S.M., A.I.M., J.A.N.; Visualization: T.G.S.; Funding acquisition: H.B., J.A.N.

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Data availability

The data supporting this paper are available through the Biological and Chemical Oceanography Data Management Office database (Baumann et al., 2020): <http://lod.bco-dmo.org/id/dataset/827774>.

Supplementary information

Supplementary information available online at <https://jeb.biologists.org/lookup/doi/10.1242/jeb.228015.supplemental>

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