**Chapter 2:** Metabolic response of the coastal fish *Menidia menidia* reared in elevated CO2 to progressive hypoxia

**Abstract**

Although rising carbon dioxide (CO2) levels are a threat to many marine organisms and ecosystems, the responses in fish have often been subtle and vary between and within species. The combined effects of acidification with other forms of global change, such as hypoxia, may be more important to document and predict than acidification alone. Even in largely tolerant species it is important to understand the mechanisms behind responses to elevated CO2 and hypoxia so that we can predict population-level impacts of low-magnitude responses and use knowledge about tolerant compared to sensitive species in management contexts. In order to learn more about metabolic responses of Atlantic silverside (*Menidia menidia*) early life stages to combined acidification and hypoxia, we exposed offspring to CO2 treatments (~450, 2000, and 4000 µatm) and measured their oxygen consumption during progressive hypoxia exposure. We found stage-specific responses of routine metabolism (MO2) to elevated CO2 in which embryonic MO2 increased with CO2 but 5 day-post-hatch larvae had reduced MO2 at high CO2. We calculated the critical oxygen level (Pcrit) at which MO2 transitions from oxygen-independent to oxygen-dependent as a measure of hypoxia tolerance. High CO2 made embryos less hypoxia-tolerant with significantly higher Pcrit, but larvae exhibited a decrease in Pcrit that signifies enhanced hypoxia-tolerance after hatching. The observed changes to metabolism shed light on how energy is allocated differently in embryos and larvae experiencing current and near-future high CO2 levels as well as how differences between stages can cause one stressor, CO2, to either exacerbate or alleviate another, hypoxia. This species may be one case in which its adaptations to natural severe fluctuations of their estuarine environment have prepared them for global change.

**Introduction**

Acidification of coastal waters and estuaries is both gradually increasing as the oceans absorb atmospheric carbon dioxide (CO2) emissions (IPCC, 2021; Doney et al., 2009) and eutrophication intensifies globally (Diaz and Rosenberg, 2008) and fluctuating on diel and seasonal time scales due to anthropogenic and oceanographic factors that increase community respiration (Cai et al., 2011). The latter also depletes oxygen, and consequentially hypoxia and acidification often co-occur (Wallace and Gobler, 2021). In Long Island Sound and the smaller estuaries connected to it, for example, nutrient loading due to dense human populations on land combines with restricted circulation and high temperatures to create extreme temperature, oxygen, and pH fluctuations in the summer (Baumann et al., 2015) as well as persistent acidification and hypoxia due to stratification and accumulation of organic matter (Wallace and Gobler, 2021). This makes environmental conditions stressful to marine organisms, many of which use estuaries , as critical nursery habitat (Beck et al., 2001). For the species and life stages that do or will experience such conditions, it is critical to understand the physiological responses to current and future combinations of acidification and hypoxia, which are intensifying over time (Keeling et al., 2010; Gruber, 2011).

The responses of fishes to seawater acidification by carbon dioxide (CO2) are typically subtle and variable, particularly in species adapted to estuarine waters that have fluctuating CO2 and pH levels (Kroeker et al., 2010; Kroeker et al., 2013; Heuer and Grosell, 2014; Cattano et al., 2018; Baumann, 2019). Unlike the relatively strong and straightforward mode by which moderately elevated CO2 can impact growth and survival through calcification in shellfish (Orr et al., 2005), fish responses tend to be low in magnitude (Murray et al., 2017; Cattano et al., 2018), life stage-dependent (Harvey et al., 2013; Ishimatsu et al., 2008), and facilitated by uncertain and variable mechanisms (Kroeker et al., 2010; Esbaugh, 2018). Often the presence of an additional stressor, such as temperature or hypoxia, can elicit a response when there otherwise was none or interactively enhance an existing response (Pörtner and Langenbuch, 2005; Crain et al., 2008; Kroeker et al., 2013; Melzner et al., 2013). In such cases the underlying physiological mechanisms can shed light on the meaning of whole-organism responses, or lack thereof, and explain what makes some species more tolerant of global change than others (Melzner et al., 2009). Quantifying physiological processes by which fish respond to multiple stressors can also aid understanding of whether the responses will facilitate tolerance and adaptation over time or lead to population decline (Wittmann and Pörtner, 2013). Estimates of the energetic costs of responding to stressors, such as metabolic rates and enzyme activity, can be useful for predicting or explaining changes to other aspects of the energy budget that have population-level implications, such as reproduction, growth, and survival to recruitment stage (National Research Council, 2005; Watson et al., 2020).

In recent years, increasing discussions and developments have gone into quantitative descriptors of hypoxia tolerance in ectotherms, particularly in the context of metabolic responses to environmental change (Wood, 2018; Regan et al., 2019; Seibel and Deutsch, 2020; Zhang et al., 2022). A commonly used measure of hypoxia sensitivity is the critical oxygen partial pressure (*P*crit) below which a given measure of metabolic rate becomes oxygen-dependent, or oxyregulation switches to oxyconformity (Richards, 2011). Above Pcrit, it is typically assumed that most oxygen demands are being met with the ambient environmental O2. Although Pcrit does not strictly mark the onset of anaerobic metabolism (Wood, 2018), below Pcrit there are demands that must be met with anaerobic metabolism or simply go unmet (Pörtner and Grieshaber, 1993). Pcrit can be a useful way to examine the relationship between hypoxia and ocean acidification and understand the underlying mechanisms of their interactive effects. Ocean acidification may affect the ability to tolerate another stressor such as hypoxia, despite having small or no effects on biological responses when all other conditions are optimal, through physiological processes that go undetected when stressors are not combined. Measurement of embryonic routine metabolism (MO2) in the Atlantic silverside, *Menidia menidia*, demonstrated that while pCO2 alone has little effect, MO2 becomes increasingly oxygen-dependent in elevated pCO2 levels (Schwemmer et al., 2020). This suggests a shift from oxyregulation to oxyconformity indicative of Pcrit being reached at higher oxygen levels than in ambient pCO2. Effects of acidification on hypoxia response may be stage- and species-dependent, as larvae from Schwemmer et al. (2020) did not exhibit the response embryos did, and studies on juvenile European sea bass (Montgomery et al., 2019) and adult wooly sculpin (Hancock and Place, 2016) showed reduced and increased Pcrit, respectively, under elevated pCO2. Impacts of high pCO2 on Pcrit and other measures of hypoxia response may result from a reduction in blood pH, which reduces affinity and capacity of oxygen to bind to hemoglobin via the Bohr and Root effects, respectively (Brauner and Randall, 1996; Wells, 2009). Pcrit can also be useful in setting hypoxia thresholds or indicators used by marine resource managers. In a management and policy context, hypoxia is often somewhat arbitrarily set as 2 or 3 mg L-1 dissolved oxygen but a more ecologically and biologically relevant threshold could be set using knowledge about thresholds of hypoxia response in local organisms, and further enhanced with information about how coinciding stressors affect this threshold.

*M. menidia* is an extensively studied estuarine species that has shown variable, but broadly tolerant, responses to ocean acidification (Baumann et al., 2018). The early life stages, which reside in estuaries during summer, have had reduced growth and survival in elevated pCO2 in some (Murray et al., 2014; Murray et al., 2017) but not all experiments (Baumann et al., 2018). Multistressor experiments have shown interactive effects of pCO2 and hypoxia on hatching and metabolism (Cross et al., 2019; Schwemmer et al., 2020) and of pCO2 and temperature on growth and survival (Murray and Baumann, 2018). *M. menidia* lives in estuarine habitats with great fluctuations in CO2, oxygen, and temperature, and because of their spring and summer spawning season, the early life stages experience the most severe of these conditions while having less mobility than adults (Cadigan and Fell, 1985). The mechanistic responses behind these interactive effects can explain their tolerance and help establish its limits.

Here we use respirometry to quantify the metabolic response of *M. menidia* early life stages reared in elevated CO2 to acute progressive hypoxia. Over the course of two experiments, we reared *M. menidia* offspring of wild-caught adults in various pCO2 levels and measured the metabolic rates of embryos, newly hatched larvae, and 5-day post-hatch (dph) larvae in closed respirometers as hypoxia intensified due to oxygen consumption. The objective was to quantify the relationship between routine metabolic rate (MO2) and ambient pO2­­­, then calculate Pcrit and analyze how the MO2-oxygen relationship changes under elevated pCO­2. We hypothesized that at high oxygen levels the metabolic rates would be elevated under high pCO2, but only in embryos. We also hypothesized that high pCO2 would increase Pcrit based on the previously recorded increase in oxygen-dependence at high pCO2 (Schwemmer et al., 2020), work on other species that exhibit this response (Cruz-Neto and Steffensen, 1997; Hancock and Place, 2016), and the changes to hemoglobin-oxygen binding affinity when blood pH is low (Wells, 2009).

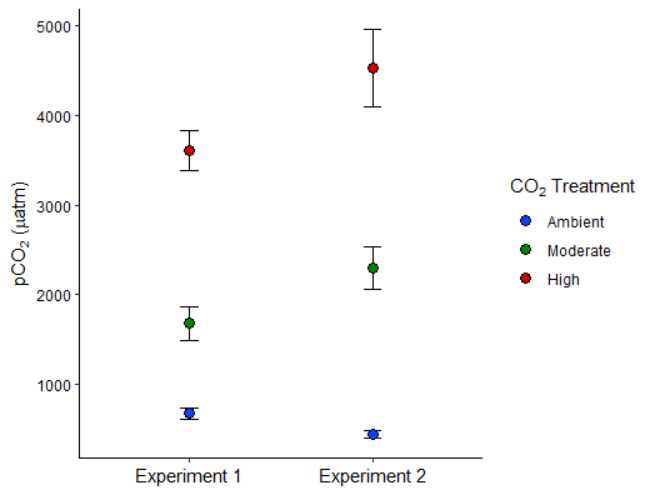
**Methods**

*Experimental design and treatments*

Two experiments were conducted simultaneously in June 2021. In each experiment, three pCO2 levels were used and replicated across three rearing tanks each (n=3). Target pCO2 levels were ambient (450 µatm), moderately elevated (2000 µatm), and high (4000 µatm) to easily compare with previous work on this species using similar treatment levels. To accommodate logistical constraints on respirometry equipment availability, Experiments 1 and 2 were maintained at slightly different temperatures (24 and 22°C, respectively) which allowed us to stagger developmental timepoints for respirometry and measure treatment effects at two different temperatures common in local estuaries in June. Measured mean treatment and water quality parameters for all tanks are reported in Tables 1 and S1.

Both experiments were conducted in closed, recirculating systems at two separate Stony Brook University laboratory facilities: Experiment 1 in Dana Hall, Stony Brook University, Stony Brook, NY, and Experiment 2 in Flax Pond Marine Laboratory, Old Field, NY. Water equilibration baths were filled with seawater that was pumped from a nearby estuary and filtered to 5 µm. All equilibration baths were bubbled with laboratory air using a 50W pump (Air Pump 1110 GPH, Vivosun, Ontario, CA) and air stones. In each elevated CO2 water bath CO2 was added using a gas proportioner in Experiment 1 (Masterflex® Variable-Area Flowmeter, Cole-Parmer, Vernon Hills, IL, USA) and solenoid valve controlled by an Apex AquaController unit in Experiment 2 (Neptune Systems, Morgan Hill, CA, USA). The pH in each water bath was monitored continuously through the Apex Fusion online interface with pH probes (Double Junction pH Probe, Neptune Systems, Morgan Hill, CA, USA). CO2 input was adjusted based on pH readings, manually in Experiment 1 and automatically using Apex Fusion web interface in Experiment 2. Water was kept at target temperatures using various aquarium heaters with integrated thermostats for Experiment 1 or heaters and chillers controlled by the Apex units and the Apex Fusion software in Experiment 2. Water was pumped slowly into rearing containers then allowed to drain back into water baths through 120-µm mesh-covered holes, to mix the water and keep conditions in the rearing containers the same as those in the water equilibration baths.

We manually measured and recorded temperature, pH, salinity, dissolved oxygen, ammonia, nitrate, and nitrite to validate treatment levels and ensure consistent, optimal rearing conditions. One partial water change was done 2 days after larvae hatched to keep ammonia levels down after feeding began. In both laboratories, lights were set on timers for a 14h:10h light to dark cycle to simulate the approximate daylight hours of June in New York. To quantify the carbonate system in each treatment tank, water samples were taken at the beginning and end of each experiment, with an additional sample collected in the middle of Experiment 2, in 250 mL borosilicate bottles, sealed with vacuum grease, and stored at 4°C. The water samples were analyzed for dissolved inorganic carbon (DIC) and total alkalinity (TA) using a VINDTA 3C system (Marianda, Kiel, Germany). DIC and TA were used to calculate pCO2, fugacity of CO2 (*f*CO2), bicarbonate (HCO3-), and carbonate (CO32-) for each treatment. The samples from the third sampling date for Experiment 2 were not sealed well and gas exchange affected DIC but not TA, so the carbonate chemistry parameters were calculated using TA and pH from a handheld probe for this date. Water quality measurements for both experiments can be found in Tables S1 and S2.



**Figure 1.** Experiment 1 and 2 mean pCO2 levels for each treatment with error bars showing standard error. In Experiment 1, each rearing tank was sampled twice. In Experiment 2, each equilibration tank was sampled three times.

**Table 1.** Target and measured mean pH, pCO2, and temperature levels in Experiments 1 and 2.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | pH | | | pCO2 (µatm) | | | Temperature (°C) |
|  | Amb | Mod | High | Amb | Mod | High |  |
| Target levels | 8.1 | 7.5 | 7.2 | 450 | 2000 | 4000 | 24 (Exp. 1),  22 (Exp. 2) |
| Exp. 1 | 7.94 | 7.41 | 7.13 | 680.0 | 1683.0 | 3609.1 | 23.8 |
| Exp. 2 | 8.08 | 7.39 | 7.09 | 441.7 | 2299.2 | 4530.9 | 22.3 |

**Table S1.** Mean and standard error of measured salinity, temperature (°C), pH, total alkalinity (TA, µmol kg-1), and dissolved inorganic carbon (DIC, µmol kg-1) , and calculated partial pressure of CO2 (pCO2, µatm), fugacity of CO2 (fCO2, µatm), concentration of bicarbonate ([HCO3-], µmol kg-1), concentration of carbonate ([CO3-], µmol kg-1), aragonite saturation state (Ωarag), and calcite saturation state (Ωcalc) for each treatment within Experiments 1 and 2. Means and standard errors were calculated across repeat samples and replicate tanks. One measured DIC sample within Experiment 2, High CO2 treatment was sealed improperly and could not be used, so measured pH was instead used to calculate DIC and the other calculated carbonate chemistry parameters.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Experiment | Treatment | Salinity | Temperature | pCO2 | pH | TA | DIC | fCO2 | [HCO3-] | [CO32-] | Ωarag | Ωcalc |
| 1 | Ambient | 27.6 (±1.0) | 23.8 (±0.1) | 680.0 (±62.3) | 7.94 (±0.07) | 2135.3 (±59.0) | 1989.0 (±36.8) | 677.8 (±62.1) | 1850.5 (±24.8) | 117.9 (±15.1) | 1.92 (±0.24) | 2.98 (±0.36) |
| Moderate | 28.0 (±1.0) | 23.8 (±0.1) | 1683.0 (±186.4) | 7.41 (±0.06) | 2177.6 (±67.7) | 2145.7 (±60.4) | 1677.5 (±185.8) | 2035.9 (±56.9) | 58.8 (±7.6) | 0.96 (±0.12) | 1.49 (±0.19) |
| High | 27.7 (±1.0) | 23.8 (±0.1) | 3609.1 (±219.4) | 7.13 (±0.08) | 2161.9 (±63.5) | 2232.2 (±59.1) | 3597.4 (±218.7) | 2095.0 (±57.9) | 27.7 (±2.9) | 0.45 (±0.05) | 0.70 (±0.07) |
| 2 | Ambient | 27.9 (±0.8) | 22.4 (±0.1) | 441.7 (±40.9) | 8.08 (±0.01) | 2097.0 (±32.0) | 1920.8 (±27.37) | 440.2 (±40.8) | 1742.0 (±15.6) | 146.0 (±14.3) | 2.37 (±0.23) | 3.69 (±0.34) |
| Moderate | 28.0 (±0.8) | 22.3 (±0.1) | 2299.2 (±243.0) | 7.39 (±0.06) | 2129.6 (±40.2) | 2137.0 (±43.1) | 2291.6 (±242.2) | 2037.1 (±44.7) | 38.1 (±2.0) | 0.62 (±0.03) | 0.96 (±0.06) |
| High | 28.1 (±0.8) | 22.2 (±0.3) | 4530.9 (±431.0) | 7.09 (±0.04) | 2139.8 (±46.7) | 2217.1 (±44.5) | 4515.8 (±429.5) | 2090.7 (±48.3) | 20.2 (±0.7) | 0.33 (±0.01) | 0.51 (±0.02) |

*Animal acquisition and rearing*

Wild *M. menidia* adults were caught on June 8, 2021, with a 30-meter beach seine in Stony Brook Harbor, New York, USA. Individuals were separated by sex and acclimated to 20°C for 48 hours in two tanks in a dry lab at Stony Brook University. On June 10 gonads were ripe and females’ ovaries released hydrated eggs under gentle pressure. Eggs of 5 females were stripped into trays of seawater containing pieces of 1-mm mesh for chorionic filaments to adhere to. Milt of 8 males was stripped into beakers of seawater, which were then poured over the eggs. Trays were gently stirred and allowed to rest for 15 minutes. Then pieces of mesh were cut to make several small pieces containing ~15 viable eggs, and two pieces of mesh were hung with fishing line in each rearing container so that at least 30 viable eggs were randomly distributed into each container in both experimental systems within 3 hours of spawning. Upon hatching, larvae were fed *ad libitum* with newly hatched brine shrimp nauplii, and excess or dead nauplii were siphoned from rearing containers daily. Brine shrimp cysts were chemically decapsulated prior to hatching to enhance hatch success and ensure no hard shells would be consumed by larvae.

*Respirometry and analysis*

On the day before hatching, the day after hatching (2dph), and 5 days after hatching (5dph), *M. menidia* offspring were sampled for respirometry. Microrespirometry protocols and equipment are described in detail in Schwemmer et al. (2020). Briefly, several fish were removed from each rearing container and sealed in individual microrespirometers, or well plates equipped with optical oxygen sensors (Loligo Systems). These plates were sealed with parafilm and a silicone membrane and compressed with several acrylic sheets in a temperature control chamber to create an air- and water-proof seal. Oxygen sensors were read with a PreSens sensor dish reader and SDR software v4.0.0. The respirometers were shaded with a box to help minimize stress and activity and because the oxygen sensors require darkness. Respirometry trials were conducted until oxygen fell below 0.1 mg L-1 so that metabolic rates could be calculated for a range of ambient oxygen levels including hypoxic ones.

All calculations and statistical analyses were conducted with R software (v4.2.1, R Core Team, 2022), including the packages ‘respirometry’, ‘car’, ‘segmented’, and ‘zoo’ (Birk, 2021; Fox and Weisberg, 2019; Muggeo, 2008; Zeileis and Grothendieck, 2005). Although room temperature was fairly constant, formulas provided by Loligo Systems were used to convert phase data from the microrespirometers into oxygen concentration with data from the integrated temperature sensor, rather than assuming a constant temperature. Oxygen data were subset to exclude changes in temperature at rates greater than 0.5°C h-1 and extreme changes in oxygen that were likely due to temperature change or air bubbles dissolving or degassing in the wells. Any individuals that died, were injured, escaped, or for which the well was not fully sealed were excluded from analysis so that the final sample size for each treatment and time point combination ranged from 8 to 15. For each treatment there were at least two control (animal free) wells used to estimate microbial respiration.

For each well, the make\_bins() function from the ‘respirometry’ package was used to set variable bin widths that were widest at high O2 to produce low-noise routine MO2 estimates and narrowest at low O2 to produce high-resolution Pcrit estimates (Birk, 2021). The maximum bin width was 1/10 of the total trial duration and the minimum bin width was 1/30 of the total trial duration. Then calc\_MO2() from ‘respirometry’ was used, along with the well volume, to calculate the metabolic rate as the slope of O2~Time for each bin within each individual’s dataset, in µmol O2 h-1). Then the average oxygen depletion rate from the control wells were subtracted from the oxygen consumption rates for each individual from the corresponding rearing tank to account for microbial respiration. For larvae, individuals were preserved then measured so that total length could be converted to dry weight using a formula based on previous *M. menidia* data (H. Baumann, personal communication). Each larval oxygen consumption rate was divided by the calculated dry weight of the individual to obtain mass-specific metabolic rates for each bin (µmol O2 mg dw-1 h-1).

First, Pcrit was determined using segmented regression with the function selgmented() in the ‘segmented’ package in R (Muggeo, 2008). This function allows for multiple breakpoints, which was necessary due to some fluctuations in metabolic rates in high O2 from random activity and in low O2 from temporary sharp increases in MO2 likely related to anaerobic byproducts (Pörtner and Grieshaber, 1993). The segmented regression found the best fitting model and when multiple breakpoints were identified, we visually inspected the model fit with the observed data to identify which breakpoint corresponded to a marked shift from oxygen-independence to relative oxygen-dependence. We also recorded presence or absence of a sharp temporary increase in MO2 below Pcrit. If no Pcrit was identified, either because it was outside of the range of oxygen levels measured or didn’t exist, this was also recorded. For embryos, many individuals did not have a Pcrit but rather displayed oxyconformity throughout the respirometry trial. Percentages of individuals within each experiment and treatment exhibiting such oxyconformity or a sharp temporary increase in MO2 below Pcrit were calculated. To test for significant differences in proportion of individuals displaying either of these patterns across pCO2 treatments, a test of equal proportions was conducted. Routine metabolism was calculated as the mean of all MO2 values above Pcrit or, when no Pcrit was found, the mean of all MO2 values above 3 mg l- O2. This means that the routine metabolism reported here encompassed all levels of non-hypoxia-influenced oxygen consumption for the individual, including variable routine swimming activity. Observing the fish during respirometry to identify periods of rest and activity was not possible with this equipment.

Statistical analyses were performed separately for each experiment and statistical comparisons were not made between temperatures because of the methodological differences in implementing treatment conditions, as described above. Routine MO2 and Pcrit data were analyzed for significant differences across treatments and tank effects using a nested ANOVA with the aov() function in R, with the formula: Response ~ pCO2 / factor(Tank). Outliers were identified using residuals and Cook’s distance with a threshold of 4/(N – K – 1) where N is the sample size and K is the number of parameters in the model, and these outliers were removed. The assumptions of ANOVA, normality of data and homogeneity of variances, were tested with the Shapiro-Wilk test and Levene test, respectively. A square root transformation was applied to embryonic MO2 data, and a reciprocal transformation was applied to 2dph larval MO2 data to correct for significant heterogeneity of variances. In cases where the ANOVA detected significant pCO2 effects, a Tukey test was used to test for pairwise significant differences between treatment levels. All statistical tests were interpreted with a significance level of α=0.05.

**Results**

*Routine metabolism*

*M. menidia* routine MO2 was generally higher in Experiment 1, which was performed at a higher temperature than Experiment 2. Metabolic rates of *M. menidia* embryos in Experiment 2, but not Experiment 1, were significantly affected by pCO2 (Figure 2A; ANOVA, p<0.0001, *F*2,26 = 23.2). In Experiment 2, which was conducted at 22°C, embryonic MO2 was significantly greater in the highest pCO2 treatment relative to both the ambient and moderately elevated pCO2 levels (Tukey test, p<0.0001 and p=0.0003, respectively). The moderately elevated pCO2 embryos did not have significantly higher routine MO2 than those in ambient pCO2 (Tukey test, p=0.47).

At 2 dph there was no significant effect of pCO2 on routine metabolic rates in either experiment (Figure 2B; ANOVA, p>0.05). Mass-specific metabolic rates of larvae generally decreased slightly between 2 and 5 dph. Increasing pCO2 significantly affected the mass-specific metabolic rates of 5 dph larvae in both the 24°C (ANOVA, *F*2,14=3.78, p=0.049) and 22°C experiment (ANOVA, p<0.0001, *F*2,22 = 14.8). At 24°C no significant pairwise differences were found (Tukey test, p>0.05) but the mean MO2 at moderate pCO2 lower than those at ambient and high pCO2 (Figure 2C). The 5 dph larvae reared under high pCO2 in Experiment 2 had significantly lower MO2 than those at both ambient pCO2 (Tukey test, p=0.003) and moderate pCO2 (Tukey test, p<0.0001). Experiment 2 also had a significant tank effect for MO2 (ANOVA, p=0.009, *F*4,22=4.4), but there were no other significant tank effects found in nested ANOVAs for any life stage or experiment (ANOVA, p>0.05).

Diagram

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**Figure 2.** Routine metabolic rates of whole *M. menidia* embryos (A, µmol O2 individual-1 h-1) and mass-specific routine metabolic rates (µmol O2 mg-1 h-1) of 2-dph larvae (B) and 5-dph larvae (C) as a function of pCO2 (µatm). Error bars display standard error about the mean. Closed circles represent means from Experiment 1 (24°C) and open circles represent means from Experiment 2 (22°C). Letters denote significant differences between pCO2 levels within an experiment (temperature level), as detected with a Tukey test, with points that do not share a letter being significantly different. Points that lack significance letters had no significant pCO2 effect according to ANOVA.

*Relationship between MO2 and O2*

A slight decrease in Pcrit of *M. menidia* offspring was observed over time across experiments and treatments. A substantial proportion of embryos displayed a steady decline in MO­2 as oxygen decreased such that no breakpoint could be identified at which a relatively oxygen-independent portion of the data shifted to become oxygen-dependent (Figure 2). In these cases, Pcrit could not be estimated and embryos appeared to be oxyconformers regardless of ambient O2 levels. In Experiment 1 the frequency of this phenomenon was not significantly different across CO2 treatments (Proportion Test, *X*-squared=0.695, *p*=0.7), with 40% of ambient embryos, 22.2% of moderate pCO2 embryos, and 33.3% of high pCO2 embryos displaying oxyconformity (Table 3). In contrast, embryos from Experiment 2 had significant differences in proportion with oxyconformity (Proportion Test, *X*-squared=18.936, *p*<0.001), as the highest pCO2 level had 71.4% oxyconformers compared to 0% in ambient and 8.3% of moderate pCO2 and 71.4% of high pCO2 embryos showing the pattern and no embryos showing this in ambient pCO2 (Table 3).

Diagram, engineering drawing

Description automatically generated

**Figure 2.** Examples of a typical MO2-O2 curve with a fitted breakpoint regression (A), an MO2-O2 curve with a sudden transient increase in MO2 at low oxygen with a fitted breakpoint regression (B), and an oxyconforming MO2-O2 curve that frequently occurred in embryos with a fitted linear regression (C). In (A) and (B) the dashed line shows Pcrit, the breakpoint identified by the regression and the point at which relatively oxygen-independent metabolism shifts to highly oxygen-dependent. In (C) no such breakpoint exists because metabolism is consistently oxygen-dependent throughout the trial. The proportions of individuals exhibiting the low-oxygen increase in metabolism in (B) and the oxyconformity shown in (C) for each treatment group are listed in Table 3.

There were no significant effects of pCO2 on Pcrit at the embryo stage in either experiment (Figure 3A, ANOVA, p>0.05). The pCO2 significantly affected larval Pcrit at 2dph in Experiment 1 (Figure 3B; ANOVA, p=0.049, F2,20=3.53). The Pcrit in high pCO2 treated larvae was significantly lower than that in ambient pCO2 (Tukey test, p=0.04), while the moderate pCO2 level was not significantly different from either of the other levels (Tukey test, p>0.05). However, no similar effects were detected in Experiment 2 (ANOVA, p>0.05). At 5 dph, Pcrit declined significantly with increasing pCO2 in both Experiment 1 (Figure 3C, ANOVA, *F*2,14=4.25, p=0.036) and Experiment 2 (ANOVA, *F*2,20=4.97, p=0.018). Pairwise comparisons showed that high pCO2 was significantly lower than at ambient pCO2 in the 24°C experiment (Figure 3C, ANOVA, p>0.05) and the 22°C experiment (Tukey test, p=0.015) but that moderate pCO2 groups were not significantly different from the other treatments. There was a significant decrease in Pcrit with elevated pCO2 in 5dph larvae from Experiment 2 (Figure 3C, ANOVA, p=0.018, F2,20=4.97). There were no significant tank effects on Pcrit (nested ANOVA, p>0.05).

The percentages of offspring with a low-oxygen increase in MO2 below Pcrit (Figure 2) are reported in Table 3. The prevalence of this pattern was lowest among embryos and highest at 2dph. In Experiment 1 larvae (2 and 5 dph) the phenomenon occurs slightly less frequently in elevated pCO2 levels. There were no statistically significant differences in proportion of individuals across treatment groups displaying this pattern (Proportion Test, p>0.05).

Diagram

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**Figure 3.** Critical oxygen levels of *M. menidia* offspring in response to pCO2 treatments at the embryo (A), 2dph larval (B), and 5dph larval (C) stages. Error bars display standard error about the mean. Closed circles represent means from Experiment 1 (24°C) and open circles represent means from Experiment 2 (22°C). Letters denote significant differences between pCO2 levels within an experiment (temperature level), as detected with a Tukey test, with points that do not share a letter being significantly different. Points that lack significance letters had no significant pCO2 effect according to ANOVA.

**Table 2.** Mean routine MO2 and Pcrit (±standard error) for *M. menidia* embryos and larvae reared in three pCO2 treatments in two experiments (Experiment 1 at 24°C and Experiment 2 at 22°C). Routine MO2 for embryos is µmol O2 individual-1 h-1 and for larvae is µmol O2 mg dw-1 h-1. Pcrit is in mg l-1.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | | Routine MO2 | | | Pcrit | | |
| Ambient pCO2 | Moderate pCO2 | High pCO2 | Ambient pCO2 | Moderate pCO2 | High pCO2 |
| Embryos | Exp. 1 | 0.0044(±0.00066) | 0.0036(±0.00075) | 0.0030(±0.00043) | 2.44(±0.54) | 3.01(±0.44) | 2.80(±0.32) |
| Exp. 2 | 0.0016(±0.00030) | 0.0023(±0.00020) | 0.0047(±0.00054) | 1.90(±0.21) | 1.90(±0.32) | 2.51(±0.23) |
| 2dph Larvae | Exp. 1 | 0.29(±0.041) | 0.28(±0.033) | 0.25(±0.047) | 2.04(±0.25) | 1.56(±0.21) | 1.21(±0.26) |
| Exp. 2 | 0.19(±0.017) | 0.15(±0.0071) | 0.21(±0.024) | 1.23(±0.29) | 1.42(±0.23) | 1.34(±0.31) |
| 5dph Larvae | Exp. 1 | 0.23(±0.016) | 0.17(±0.014) | 0.23(±0.022) | 1.23(±0.18) | 0.94(±0.11) | 0.72(±0.17) |
| Exp. 2 | 0.18(±0.024) | 0.20(±0.011) | 0.12(±0.0092) | 1.99(±0.29) | 1.65(±0.16) | 1.17(±0.16) |

**Table 3.** The percentages of *M. menidia* offspring in which a low-oxygen increase in MO2 following a decline in MO2 below Pcrit, for each Experiment, pCO2 treatment, and life stage.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  |  | Ambient pCO2 | Moderate pCO2 | High pCO2 | *p*-value |
| Oxyconformity | Embryos | Exp. 1 (24°C) | 40.0% | 22.2% | 33.3% | 0.7065 |
| Exp. 2 (22°C) | 0.00% | 8.33% | 71.4% | **7.7e-5** |
| Low-DO increase | Embryos | Exp. 1 (24°C) | 10.0% | 22.2% | 11.1% | 0.71 |
| Exp. 2 (22°C) | 18.2% | 16.7% | 35.7% | 0.45 |
| 2dph Larvae | Exp. 1 (24°C) | 100% | 90.0% | 77.8% | 0.28 |
| Exp. 2 (22°C) | 66.7% | 83.3% | 53.8% | 0.29 |
| 5dph Larvae | Exp. 1 (24°C) | 55.6% | 37.5% | 33.3% | 0.60 |
| Exp. 2 (22°C) | 85.7% | 75.0% | 40.0% | 0.10 |

**Discussion**

By rearing *M. menidia* offspring in different pCO2 treatments and quantitatively analyzing their MO2-oxygen relationship we showed that high pCO2 effects on MO2 depend on rearing temperature and occur primarily at pCO2 levels above 4000 µatm, yet high temperatures and high pCO2 are expected as anthropogenic effects intensify. Routine MO2 of embryos and 5 dph larvae at 22°C were significantly affected by pCO2, while their counterparts at 24°C were unaffected. Because of differences in equipment used to control experimental conditions, the 22°C experiment had higher mean moderate and high pCO2 than the 24°C one had. The highest pCO2 treatment in the 24°C experiment was 3609.8 µatm, while the highest treatment at 22°C, and the only treatment that elicited changes in routine MO2, was 4324.5 µatm. Past studies have pointed out that responses to acidification are not always linear but may instead have a bell-curve or threshold-type response (Ries et al., 2009; Bednaršek et al., 2019). The difference we observed between experiments may be indicative of a threshold existing between ~3600-4300 µatm pCO2 for early life stages of this species. This parallels the upper limits of recorded diel pCO2 fluctuations in a Long Island estuary near where the adults were collected for this experiment (Baumann et al., 2015). It is possible that the metabolic impacts of high CO2 are negligible until levels approach the limits of what naturally occurs in the range of the populations these individuals are from.

Because fertilized eggs were gathered from the same batch spawned by several parents and randomly divided between Experiments 1 and 2, genetic variability likely did not cause the different responses between experiments. However, the 2°C temperature difference between experiments may have influenced responses. Temperature-dependent effects of pCO2 have been found in the past for *M. menidia*, with temperature altering how embryo survival and hatch length were impacted by pCO2 but not consistently increasing CO2-sensitivity (Murray and Baumann, 2018). Additionally, past experiments in which significant effects of CO2 were detected mainly took place early or late in the spawning season (Baumann et al., 2018), and the present study utilized one of the last spawning events of the season. In contrast, a past study in which pCO2 did not affect routine metabolism used experiments on offspring from early- and mid-season spawning events (Schwemmer et al., 2020). The sensitivity of *M. menidia* to pCO2 is thought to vary based on the timing of spawning, potentially due to the environment adults are experiencing and their condition or energetic contribution to eggs (Murray et al., 2014; Snyder et al., 2017). Temperature also influences developmental rate, yolk consumption rate, and hatch timing (Blaxter, 1988). This means that even though embryos and larvae from each experiment were sampled at the same time points relative to hatching, they may have been at slightly different points in their development of homeostatic and metabolic mechanisms.

The response shown in the 22°C experiment of embryonic MO2 increasing with pCO2 is similar to the results of Schwemmer et al. (2020) in which mean MO2 increased by over 30% from the lowest to highest pCO2 treatment under normoxia. Increased oxygen consumption suggests that additional ATP is being expended on acid-base balance at the cellular level, as embryos do not yet have the ability to remove CO2 from the blood by increasing ventilation (Rombough, 1988). By 5 dph the pCO2 effect was reversed and the highest pCO2 treatment led to a significantly lower MO2 than the lower pCO2 levels, again only at 22°C. Reduced routine MO2 has been observed in other marine fish species exposed to high CO2, including embryos (Rosa et al., 2014), larvae (Pimentel et al., 2014; Pimentel et al., 2015) and juveniles (Stiller et al., 2015). Reduced MO2 indicates that high pCO2 is not causing additional energy to be expended on homeostasis at this stage (Stiller et al., 2015), which could help explain why *M. menidia* persists in regularly elevated pCO2 levels. Th evidence that little, if any, additional energy is being expended on pumping ions for acid-base balance leaves the possibility that routine ventilation is enough to keep blood pH at acceptable levels. The shift in response from embryos to 5 dph could also be a result of differential mortality with increasing pCO2 of only the most sensitive embryos, leaving tolerant larvae with lower MO2 by 5 dph. Turtle cells incubated in an experiment used the majority of their ATP on the ion exchanger Na+/K+-ATPase and protein synthesis, so if this also holds for fish and metabolism is suppressed under high CO2 both of these functions may be reduced to the detriment of the fish under longer term exposure (Storey and Storey, 2004). Currently, such high pCO2 levels only occur for a few hours at a time certain times of year but as they become more common due to anthropogenic CO2 emissions (Gledhill et al., 2015; IPCC, 2022) chronic exposure to these conditions could slow down growth and development (Hochachka and Somero, 2002). Even if natural variability results in only some fish being sensitive, this could have population-level consequences through recruitment, reproductive output, and predation rates (Houde, 1997).

As we hypothesized, Pcrit increased with seawater acidification, but only in embryos in the 22°C experiment. These results are consistent with previous findings that routine MO2 became more oxygen-dependent in *M. menidia* reared in CO2 and O2 treatments (Schwemmer et al., 2020). We expanded upon this past work by quantifying the MO2-O2 relationship directly in individuals as oxygen decreases, rather than inferring it from separate individuals kept in separate static DO levels. Increased oxygen-sensitivity may result from incomplete development of gills and internal buffering capacity and, consequentially, greater declines in internal pH under high pCO2 conditions (Melzner et al., 2009; Marshall et al., 2016). Low blood pH can reduce the oxygen that fish can take up into the blood through the Root effect, reduced hemoglobin-O2 binding capacity, and Bohr effect, reduced hemoglobin-O2 binding affinity (Brauner and Randall, 1996; Wells, 2009). The elevated embryonic Pcrit coincided with increased routine MO2 under high pCO2, similar to the pCO2 and DO interaction in Chapter 1 (Schwemmer et al., 2020). When metabolism increases and approaches maximum metabolic rate due to activity and other metabolic demands, Pcrit generally increases as well (Pörtner, 2010). Therefore, the elevated Pcrit may be a direct result of increased metabolic demand under high pCO­2. A study on intertidal sculpin found increased Pcrit after acclimation to high pCO2 coincided with not only elevated MO2 but also greater activity rates of Na+/K+-ATPase, an ion exchange enzyme indirectly involved in acid-base regulation (Hancock and Place, 2016; Claiborne et al., 2002; Catches et al., 2006). Further measurements, such as ionoregulatory enzyme activity and gene expression, could elucidate whether changes in *M. menidia* Pcrit under high pCO2 similarly reflect additional energy being spent on acid-base regulation, reductions in oxygen uptake ability due to poorly regulated blood pH, or a combination of these factors.

The enhanced hypoxia-sensitivity caused by acidification in embryos from the 22°C experiment is also reflected in the prevalence of individuals displaying full oxyconformity. In these embryos, MO2 declined steadily as oxygen decreased such that a breakpoint indicative of Pcrit could not be detected. Although this phenomenon occurred similarly across pCO2 levels in the 24°C experiment, it significantly increased with high pCO2 and was absent from the ambient treatment in the 22°C experiment. The starting DO concentration of a respirometry trial was around 5 mg L-1 after a sensor equilibration and warmup period, so this pattern may indicate a Pcrit above that level. If we had been able to start at a higher DO level we may have detected such a high Pcrit, which would have drawn the high pCO2 effect on Pcrit up even more, as these embryos were excluded from our analysis due to inability to detect Pcrit. Embryos of most fish species oxyregulate to some extent, although exceptions exist, but the Pcrit typically increases as embryos near the time of hatching (Rombough, 1988). Therefore, it is likely a higher Pcrit exists in these individuals rather than high pCO2 inducing full oxyconformity. Regardless of this distinction, this pattern suggests that oxygen demand is high but the ability to meet it across a wide range of DO levels is low. This could contribute to immediate mortality or later reductions in growth and survival, as seen in Cross et al. (2019), from reduced ability to use energy and damage from anaerobic byproducts (Richards, 2011; Thomas et al., 2019).

In contrast to 22°C embryos, Pcrit decreased under high pCO2 in 2 dph larvae in the 24°C, as did 5 dph Pcrit in both temperatures. Other studies have measured Pcrit in fish exposed to seawater acidification and found no effects, as we did in embryos at 24°C and 2 dph larvae at 22°C (McKenzie et al., 2003; Couturier et al., 2013; Heinrich et al., 2014). Multiple studies link species’ tolerance of future global change conditions to the degree of fluctuating severe conditions they currently experience (Pörtner and Farrell, 2008; Baumann, 2019). A study on two damselfish species found that Pcrit and resting metabolism were both unaffected by four days of high-CO2 exposure, potentially owing to the periodic hypoxia these species encounter on coral reefs (Couturier et al., 2013). Much like *M. menidia*’s estuarine habitat, the naturally fluctuating conditions on the reef appear to have resulted in fish that tolerate a wide range of conditions without disruption to metabolic processes. Similarly, an elasmobranch that is regularly exposed to diurnal CO2 and O2 fluctuations on shallow coral reef habitats was also displayed no effects of long term high CO2 exposure on its resting metabolism or Pcrit (Heinrich et al., 2014). Although low blood pH is known to reduce the oxygen-binding capacity and affinity of hemoglobin (Brauner and Randall, 1996) the effect may not be strong enough at our pCO2 levels to detectably affect oxygen uptake rates or the fish may have the ability to prevent CO2 from affecting blood pH.

A recent study that took measurements of European sea bass under constant low pCO2 to acutely increasing pCO2 found that Pcrit decreased and hemoglobin affinity for oxygen increased, both of which indicate an increase in hypoxia tolerance with acidification (Montgomery et al., 2019). Furthermore, the authors found no difference in European sea bass blood pH during the high-CO2 exposure, which supports the idea that environmentally typical pCO2 levels may not alter blood pH to the extent necessary for the Bohr and Root effects to inhibit hypoxia tolerance. Montgomery et al. (2019) propose several mechanisms to account for the increase in hemoglobin-O2 affinity at high pCO2, such as the possibility that increasing pCO2 increases the rate of β-adrenergic stimulated increase in intracellular erythrocyte pH that occurs in acute hypoxia or that erythrocyte chloride levels decrease following increases in plasma bicarbonate resulting from respiratory acidosis compensation (Montgomery et al., 2019). The present study did not include the measurements needed to confirm such mechanisms, and future work doing so would help determine if such processes aid tolerance in *M. menidia.*

Interestingly, the impacts most suggestive of sensitivity to high pCO2 only occurred in the 22°C experiment: stage specific changes in MO2 and increased Pcrit in embryos. Meanwhile, the only high pCO2 response in the 24°C experiment was reduced Pcrit in larvae. This suggests the offspring reared at the lower temperature (22°C, Experiment 2) were more sensitive to acidification. The higher temperature, which is not considered stressfully high for this species (Middaugh et al., 1987), may lead to not only earlier hatching but also faster development of the metabolic and ionoregulatory mechanisms that help *M. menidia* withstand fluctuating conditions, which intensify as summer goes on and temperatures rise. The lower prevalence of embryo oxyconformity in the 24°C experiment is further evidence that developmental rate may have played a role, as Pcrit is highly dependent on developmental stage before hatching and some species have reduced ability to oxyregulate as embryos (Rombough, 1988). *M. menidia* also has a distribution extending south to Florida where summer temperatures are even higher, so it makes sense that the species may thrive under warmer temperatures.

Differences in prevalence of a temporary, sharp increase in oxygen consumption at low oxygen were not significant among experiments and pCO2 treatments. However, there was a large difference between embryos and larvae, with the percentage of embryos with this pattern ranging from 10-35.71% while in larvae it ranged from 33.33-100%. This is consistent with the explanation that stress and accumulation of anaerobic byproducts stimulates activity (Pörtner and Grieshaber, 1993) because embryos are less capable of activity than larvae (although at the stage sampled their bodies are developed enough to move around in the chorion). Older *M. menidia* are known to exhibit aquatic surface respiration during hypoxia (Miller et al., 2016) and larvae have also been observed selectively occupying surface water in hypoxic conditions (Cross et al., 2019), showing that the instinct to swim to escape hypoxia starts early. This activity is unconfirmed in our fish, however, because we were not able to observe the respirometry wells during trials. The highest occurrence of this pattern was at the 2dph larval stage in the 24°C experiment, the same group in which Pcrit was reduced by high pCO2. If this low-oxygen sharp increase in MO2 is indeed indicative of greater rates of anaerobiosis, this suggests that the fish with increased hypoxia-tolerance under high pCO2 may have also had a greater capacity for anaerobic metabolism to meet their metabolic needs despite the lack of oxygen.

It is recommended that the MO2-oxygen relationship be assessed in combination with another measure that can provide more physiological information to explain the relationship (Ultsch and Regan, 2019). Future work can uncover more about *M. menidia* hypoxia tolerance under multiple stressors by measuring anaerobic metabolism, behavioral responses (activity, aquatic surface respiration, loss of equilibrium), and enzyme activity as oxygen is depleted. Measuring and observing activity of larvae during respiration was not possible in this experiment because the inside of respirometers was not visible when they are sealed, but future experiments that record video of larvae during respirometry would allow experimenters to separate out periods of rest and activity and better characterize what happens to the fish as oxygen approaches zero, physiological needs are no longer met, and anaerobic metabolites build up that may stimulate activity (Pörtner and Grieshaber, 1993; Pörtner, 2010). Multiple replication studies should be done to encompass the range of natural genetic variation that likely confers population persistence amid extreme environmental fluctuations (Baumann et al., 2018). This study describes two experiments that, for logistical reasons, were done simultaneously at different temperatures and with different methods of acidifying the water. Despite the methodological differences that prevent statistical comparison of the different temperatures, the resulting dataset captures responses across two typical temperatures and a range of slightly different pCO2 treatments to give a more complete picture of how the metabolic response to hypoxia can vary in these conditions.

**Conclusions**

We quantified life-stage dependent responses of routine metabolism and Pcrit to seawater acidification. As we hypothesized, acidification increased the hypoxia-sensitivity of routine metabolism in embryos which, combined with their elevated MO2, suggests increased metabolic demands associated with acid-base balance and possibly inadequate blood pH regulation. Our results support and explain the mechanism of previous findings that acidification interactions with DO stem from high pCO2 increasing hypoxia sensitivity (Schwemmer et al., 2020). This could have important implications for the energy budget influencing when offspring hatch and at what size. Evidence of metabolic suppression under high pCO2 at 5 dph shows important changes in the energy budget after hatching and potentially explains previously observed reductions in larval growth under high pCO2 (Murray et al., 2017; Baumann et al., 2018). Surprisingly, decreased Pcrit in the highest pCO2 level of 2 dph larvae in one experiment indicates acidification may increase hypoxia tolerance. However, the lack of significant effects in some life stages within each experiment highlights natural variability in sensitivity and the effects slight differences in temperature, and thus developmental rate, can have. Hypoxia and acidification are concurrently increasing gradually around the globe and already co-occur in coastal waters due to periodic fluctuations in community respiration (Cai et al., 2011; Gruber, 2011). Understanding the combined effects of these stressors on fish metabolism will aid in predicting and managing population-level impacts of global change and provide insight into the optimal mitigation actions. It is important to understand the responses of tolerant species, which *M. menidia* appears to be, as well as sensitive species, because the mechanisms that lead to tolerance can shed light on potential for future adaptation in a variety of species.

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