**Early life physiological and energetic responses of Atlantic silversides (*Menidia menidia*) to ocean acidification, warming, and hypoxia**

A Dissertation Presented

by

**Teresa G. Schwemmer**

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**Early Life Mechanistic Responses of Atlantic Silversides (*Menidia menidia*) to Ocean Acidification, Temperature, and Hypoxia**

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in

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Global environmental change caused by human actions is making the oceans warmer, deoxygenating coastal waters, and causing acidification through dissolution of atmospheric carbon dioxide (CO2). Understanding physiological mechanisms of fish responses to multiple co-occurring stressors is critical to conservation of marine ecosystems and the fish populations they support. In this dissertation I quantified physiological impacts of near-future levels of multiple stressors in the early life stages of the Atlantic silverside, *Menidia menidia*. In Chapter 1, I measured routine metabolic rates of embryos and larvae reared in combinations of temperature, CO2, and oxygen levels. An interactive effect of acidification and hypoxia in embryos prompted closer examination in Chapter 2, in which I characterized the relationship between metabolism and acute hypoxia in *M. menidia* offspring reared in different CO2 levels. In Chapter 3 I examined the density of skin surface ionocytes, cells used for acid-base balance, as an early life mechanism of high CO2 tolerance. The first three chapters highlighted how different CO2 effects could be depending on temperature, oxygen levels, and life stage. They also showed variable, but often high, tolerance of CO2 with stronger effects of temperature and hypoxia on physiology. Finally, in Chapter 4 I used a Dynamic Energy Budget model to identify the processes of energetic allocation responsible for previously observed experimental hypoxia effects on *M. menidia* hatching, growth, and survival. Energy budget modeling can enhance knowledge about stressor responses by providing the information to link organismal traits to life history and populations, making it more readily applicable to conservation and management. The findings presented here provide a foundation for a more comprehensive understanding of the highly variable effects of global change on *M. menidia* and should be applied to quantifying impacts on fitness and population growth in this ecologically important species.

**Dedication Page**

This dissertation is dedicated to my partner and best friend, Max Grabinski. You have been by my side and supported me in more ways than I could have imagined throughout my time working on this degree. I am so unbelievably fortunate and grateful to have you in my life and I can’t wait to see where life takes us next.

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# **Acknowledgments**

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**Vita, Publications and/or Fields of Study**

This dissertation includes one original manuscript that has been published. All chapters will be submitted for peer review and publication in a scientific journal.

**Chapter 1**

**Schwemmer, T. G.**, Baumann, H., Murray, C. S., Molina, A. I., Nye, J. A. 2020. Acidification and hypoxia interactively affect metabolism in embryos, but not larvae, of the coastal forage fish *Menidia menidia*. *J. Exp. Biol.*, 223: jeb228015. doi: 10.1242/jeb.228015

# **Introduction**

## **Background and Motivation**

Ocean warming, acidification, and deoxygenation are concurrently affecting the coastal waters globally and especially in the Northeast United States (Wallace et al., 2014; Gledhill et al., 2015; Saba et al., 2016; Breitburg et al., 2018). The Earth’s atmosphere is warming as greenhouse gases from anthropogenic emissions accumulate and increasingly trap heat from solar radiation near the Earth’s surface (IPCC, 2022). The oceans take up most of the heat from the atmosphere, resulting in gradually rising temperatures in addition to increasing frequency and intensity of marine heatwaves (Scannell et al., 2016). Similarly, the carbon dioxide (CO2) from the atmosphere dissolves into seawater and changes ocean chemistry including the gradual decline in ocean pH, a process called ocean acidification (Feely et al., 2004; Doney et al., 2009). While anthropogenic CO2 emissions are driving global trends, community respiration in shallow coastal waters and estuaries leads to coastal acidification, more extreme high CO2 levels that fluctuate on diel to seasonal time scales (Cai et al., 2011). This results from a combination of natural stratification and upwelling patterns, freshwater input, and densely aggregated marine life in shallower waters relative to the open ocean, but is also caused by eutrophication from high nutrient inputs by humans (Cai et al., 2021).

Reduced levels of dissolved oxygen (DO) occur in combination with both warming and acidification. Warmer water can hold less DO and hence global warming is thought to be the primary cause of ocean deoxygenation worldwide (Diaz and Rosenberg, 2008; Breitburg et al., 2018). Climate change is also causing increased precipitation in many regions, and the freshwater input along with nutrient pollution causes eutrophication. During the day, algal blooms caused by the excess nutrients can take up CO2 and release oxygen but at night or in shaded subsurface waters, respiration overtakes photosynthesis and depletes oxygen – particularly when stratification, which is also intensified by climate change, traps layers of water from oxygen at the surface (Breitburg et al., 2018).

Assessing species’ biological sensitivity is necessary to determine vulnerability to these concurrent stressors and to conserve marine resources, ecosystems, and biodiversity. A challenge to quantifying biological sensitivity is the potential for non-additive interactions between multiple stressors that can be experienced simultaneously in nature, which include warming, acidification, hypoxia, contaminants, food availability, fishing pressure, and noise (Crain et al., 2008). Multistressor studies of biological responses provide information that can help predict not only a species’ sensitivity but also its ability to acclimate or adapt (Foo and Byrne, 2016; Orr et al., 2020) and even, when steps are taken to bridge individuals to the population and ecosystem levels, the secondary impacts on humans and other species.

Warming and hypoxia have well-studied, straightforward effects on fishes (Fry, 1947; Claireaux and Lagardère, 1999; Pörtner and Knust, 2007; Chabot and Claireaux, 2008; Richards et al., 2009; Claireaux et al., 2016). Rates of physiological processes depend directly on temperature and fishes have ranges of temperatures between which they can function at optimal capacity as well as upper and lower thermal limits beyond which they cannot survive for long (Pörtner, 2010). Oxygen is required for conversion of stored energy for activity, homeostasis, and growth, and hypoxia occurs when the supply of oxygen in the water is exceeded by organismal or community demand (Diaz and Breitburg, 2009). Hypoxia elicits temporary metabolic suppression to reduce oxygen demand, increased ventilation and heart rate to maximize oxygen uptake, and swimming to search for more oxygenated water (Kramer, 1987; Taylor and Miller, 2001; Chapman and McKenzie, 2009; Gamperl and Driedzic, 2009; Stierhoff et al., 2009). Larvae are generally the most sensitive stage, with oxygen demand very low in embryos and coping mechanisms better developed in juveniles and adults (Rombough, 1988).

Ocean acidification, however, has only recently been studied in fishes at relevant levels for global change (Fabry et al., 2008; Ishimatsu et al., 2008), with the number of studies rapidly increasing in the past 20 years and no widely applicable pattern of negatively or positively affecting fish (Kroeker et al., 2010; Kroeker et al., 2013; Heuer and Grosell, 2014; Clements et al., 2022). High CO2 reduces blood pH, and fish can remove CO2 from their blood to regulate internal pH by increasing ventilation as long as external partial pressure of CO2 (pCO2) does not exceed internal. Fish can rapidly correct small changes to internal pH with buffers in the blood and use active and passive transport in the gill and kidney cells to remove hydrogen ions and increase pH (Deigweiher et al., 2008; Brauner et al., 2019). What is less well-understood in fishes is whether there are widely applicable patterns in how the energetic and biochemical costs of acid-base regulation impact traits that relate to fitness and population status, such as growth, survival, behavior, and reproduction. Experimental exposure to near-future levels of acidification has shown effects such as detrimental sensory behavior (Munday et al., 2014; Porteus et al., 2021), reduced growth and survival (Baumann et al., 2012; Stiasny et al., 2016), and reproductive impacts (Welch and Munday, 2016; Concannon et al., 2021), but effects vary within and among species (Kroeker et al., 2010; Baumann et al., 2018; Cattano et al., 2018; Esbaugh, 2018). Measuring multiple physiological mechanisms underlying whole-organism responses is important for understanding the capacity for acclimation (Melzner et al., 2009), even when effects are not detected, because compensation by one physiological mechanism could allow other variables to remain unchanged (Sunday et al., 2014). In this way a lack of effects in experiments do not necessarily mean there are no energetic costs or tradeoffs that may affect fitness and population growth. Comprehensive assessment of responses related to fitness and adaptation is a research priority for anticipating acidification impacts on fished populations and ecosystem health.

The Atlantic silverside, *Menidia menidia*, is an ecologically important forage fish that has frequently been used as a model species to study impacts of environmental stressors (Bengtson et al., 1987; Schultz et al., 1998; Dixon et al., 2017; Baumann et al., 2018). Its annual life cycle, small size, and nearshore abundance make it an ideal species for laboratory experiments (Middaugh et al., 1987). *M. menidia* lives in the coastal and estuarine waters of eastern North America, including in Long Island Sound, NY, USA, and the smaller bays and estuaries attached to it. Long Island Sound is located within one of the most rapidly warming regions of the global oceans and near densely populated urban areas, but the coastal waters have improved in water quality over the last few decades, with nutrient input and hypoxic extent steadily decreasing (Gledhill et al., 2015; Whitney and Vlahos, 2021). The smaller more enclosed bays where *M. menidia* spawn have more extreme conditions, and the spawning season from April to early July results in larvae experiencing some of the most severe fluctuations in temperature, DO, and pCO2 (Murray et al., 2014; Baumann et al., 2015).

Natural variability in sensitivity to three co-occurring stressors makes predicting broader consequences of *M. menidia* responses challenging, but revealing the underlying physiological and energetic mechanisms can improve utility of results. Experiments on *M. menidia* offspring reared in different levels of pCO2 have found reduced early life growth and survival (Murray et al., 2014; Murray et al., 2017; Baumann et al., 2018), but *M. menidia* has remarkably high natural variability in sensitivity to acidification and co-occurring stressors (Murray and Baumann, 2018; Baumann et al., 2018; Cross et al., 2019; Morrell and Gobler, 2020). This species is often tolerant of high pCO2 alone but displays interactive effects when temperature or hypoxia treatments are simultaneously applied (Murray and Baumann, 2018; Cross et al., 2019; Morrell and Gobler, 2020; Concannon et al., 2021). Even small changes in growth and survival can have strong effects on recruitment (Houde, 1989). Quantifying the mechanisms underlying individual-level responses can improve understanding of ways in which stressors interact (Boyd et al., 2018; Baumann, 2019). Establishing mechanisms of response throughout the life cycle can also help connect individual responses to population-level consequences (Nisbet et al., 2000; National Research Council, 2005; Watson et al., 2020). Characterizing the full distribution of responses to global change stressors through multiple replicated studies can reveal whether a great enough proportion of a given species is substantially impacted enough to affect population size and, subsequently, the species that consume or are preyed upon by that species (Wittman and Pörtner, 2013; Baumann, 2019). This dissertation addresses these needs by quantifying responses of *M. menidia* to acidification, temperature, and hypoxia and determining the mechanisms responsible for whole-organism hypoxia effects.

* 1. Objectives

1. Chapter 1 quantifies the routine metabolic rates of *M. menidia* embryos and larvae reared in factorial combinations of CO2 and temperature, and CO2 and oxygen. This work was driven by previously observed declines in growth and survival under high CO2, and the desire to understand the physiological mechanisms responsible as well as how these may vary across temperature and DO levels periodically experienced in the early life environment of this species.
2. Chapter 2 characterizes the relationship between ambient oxygen and routine metabolism in *M. menidia* embryos and larvae, and how that relationship is impacted by high CO2. The objective was to refine understanding of metabolic interactions between DO and CO2 observed in Chapter 1 by recording responses to acute hypoxia.
3. In Chapter 3, I aimed to quantify the relationship between exposure to temperature and CO2 on the structures that largely control acid-base balance in fishes, the as a function of age in *M. menidia*. This chapter sought to elucidate a cellular mechanism underlying other responses to acidification and the role developmental stage, which is influenced by temperature, plays in ionoregulatory capacity.
4. Chapter 4 used hypoxia as a case study in enhancing utility and understanding of experimental effects by identifying the energetic mechanisms responsible for impacts in *M. menidia* early life stages. Using Dynamic Energy Budget (DEB) theory, which can ultimately connect physiology and life history to populations, I changed bioenergetic parameters based on hypothesized hypoxia effects and evaluated which parameters best accounted for differences in three response variables: total length over time, time to hatching, and survival rates.
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1. **Acidification and hypoxia interactively affect metabolism in embryos, but not larvae, of the coastal forage fish *Menidia menidia***
   1. **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 (Gunderson et al., 2016). In the past twenty 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; Munday et al., 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., 2012; 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 compensatory mechanisms are still developing, hypoxia can delay hatching, slow growth, and reduce pre- and post-hatch survival (Rombough, 1988; DePasquale et al. 2015). Combined high CO2 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 CO2 and *P*O2 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*O2 threshold below which routine metabolic rate (RMR) becomes oxygen-dependent (Richards, 2009). In the case of the intertidal sculpin *Clinocottus analis*, CO2-acidification not only increased RMR, but also raised *P*crit, making the fish less hypoxia-tolerant.

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 CO2 have been examined for many species, the mechanistic responses and bioenergetic consequences of elevated CO2 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 CO2 increased survival and development speed in Antarctic dragonfish (*Gymnodraco acuticeps*) embryos, rearing embryos in water 3°C warmer caused CO2 to have the opposite effect (Flynn et al., 2015). Additive and synergistic interactions between CO2 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 CO2 treatments by 28 days (Davis et al., 2017).

RMR has been measured in fishes exposed to elevated CO2 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 CO2 treatments may show temperature but not CO2 effects on RMR (Strobel et al., 2012), separate effects of both variables, or synergistic interactions (Munday et al., 2009b). Negative effects of high CO2 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 CO2 partial pressure (*P*CO2) 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 CO2 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 CO2. Our previous results on *M. menidia* show reduced or unchanged growth and survival under high CO2 (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*CO2 and temperature, and that *P*CO2 effects would synergistically interact with temperature. Low *P*O2 conditions were expected to induce metabolic suppression, thereby potentially counteracting or concealing any elevation in metabolism due to high *P*CO2. An interaction between *P*O2 and *P*CO2 was also expected because reduced pH from elevated CO2 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*O2 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 (Rombough, 1988).

* 1. **Materials and Methods**
     1. *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, 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-L 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 each treatment tank within 2h 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 CO2 × temperature effects and experiments 5-6 quantified CO2 × oxygen effects (Table 1). Experiment 1 used 400 and 2200 μatm as target *P*CO2 levels, crossed with two temperatures: 17°C and 24°C. Experiments 2 and 3 factorially crossed three *P*CO2 levels (400, 2200, and 4200 μatm) with three temperatures (17°C, 20°C, and 24°C). Experiment 4 used the same three target *P*CO2 levels crossed with 24°C and 28°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°C which is closer to the species’ upper thermal extreme (Middaugh et al., 1987). The target *P*CO2 levels represent the current average oceanic *P*CO2 (400 μatm), the approximate level predicted as the average oceanic *P*CO2 for the next 300 years under a rising CO2 emissions scenario (RCP8.5; IPCC, 2013) and often experienced by estuarine fishes such as *M. menidia* in the summer (2200 μatm), and a higher level (4200 μatm) that will occur more often in the future as average *P*CO2 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*CO2 (400, 2200, and 4200 μatm) crossed factorially with three target levels of oxygen partial pressure (*P*O2): normoxic (23 kPa), suboxic (12 kPa) and hypoxic (7.5 or 9 kPa). The hypoxic *P*O2 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, result in one to four treatment replicates for Experiment 1-4, and one or two treatment replicates for Experiments 5 and 6. The lack of consistent replication is dealt with by 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. (2019). The actual *P*CO2 levels were calculated based on measured pH, temperature, salinity, and total alkalinity (*A*T). AT 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*CO2 (μatm), fugacity of CO2 (*f*CO2; utam), dissolved inorganic carbon (DIC; μmol kg-1), and carbonate ion concentration (CO32-; μmol kg-1) were calculated in CO2SYS (V2.1). In the CO2 × temperature experiments, oxygen was maintained at ~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®, Loveland, CO, USA) connected to a LabView (National Instruments®, Austin, TX, USA) program, which adjusted bubbling of CO2-stripped air or nitrogen gas to maintain target oxygen levels. DO measurements were converted to *P*O2 using the oxygen solubility at the measured temperature and salinity (García and Gordon, 1992). Mean temperature, *P*O2, pH and carbon chemistry parameters measured and calculated over the duration of each experiment can be found in Tables S1 (CO2 × temperature experiments) and S2 (CO2 × oxygen experiments).

* + 1. *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*O2 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*CO2 level did not affect time to hatching, but embryos from higher temperatures and higher *P*O2 levels hatched up to seven 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, 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 (SDR; 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*CO2 treatment within a given temperature or *P*O2 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 minutes before measurements started. Dissolved oxygen (DO, mg L-1) was recorded every fifteen seconds 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 minutes, depending on the temperature. In the case of the suboxic and hypoxic treatments from experiments 5 and 6, however, the trials lasted five minutes 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.

* + 1. *Data Analysis*

Calculations of metabolic rates and all statistical tests were conducted in R statistical software (v4.0.0; R Core Team, 2020) using the stats package and olsrr package (Hebbali, 2020). 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 ~0.03°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 fit to the DO values with respect to time for each well. The slope (mg O2 L-1 s-1) of the linear model was used to calculate oxygen consumption rate (*R*O2; μmol O2 h-1) with the following formula: , where 0.032 is the molar mass of O2 (mg mol-1), 3600 converts seconds to hours, and 0.0005 L is the well volume. The mean *R*O2 from control wells was subtracted from each fish-containing well of the same treatment to account for microbial respiration and obtain fish *R*O2. Size differences in embryos were negligible and quantifying embryo mass was impractical, so *R*O2 was not normalized to mass and is reported as whole-embryo routine metabolic rate (RMR). Larval total length (TL, mm) was measured in images (Image J) taken by digital camera (TrueChrome Metrics, Tucsen Photonics Co., Fuzhou, Fujian, China) connected to a stereo microscope (Nikon Eclipse E200). TL was then converted to dry weight (DW, mg) using the relationship (H. Baumann, personal communication, June 23, 2017). DW was then used to calculate the larval mass-specific RMR (μmol mg-1 h-1) as (Peck and Moyano, 2016).

As a measure of sensitivity of metabolic rates to rearing temperature, the temperature coefficient (*Q*10), the change in metabolic rate associated with a 10°C rearing temperature increase, was calculated at each *P*CO2. The formula used for this calculation is , where *T* is the mean temperature in 17°C treatments (*a*) and 28°C treatments (*b*). Within *P*CO2 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 if metabolic rate changed as a linear function of *P*CO2, temperature, *P*O2, or the interaction between *P*CO2 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; Hebbali, 2020). All statistical tests were interpreted with a significance threshold of α = 0.05.

* 1. **Results**
     1. *CO2 × 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*CO2 treatments. However, metabolic rates were unaffected by *P*CO2 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*CO2 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*CO2 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*CO2 increased (Fig. 2). The bootstrapped 95% confidence intervals for embryonic *Q*10 did not overlap between the three *P*CO2 levels (Table S4). In larvae, however, *Q*10 increased from 400 μatm to 2200 and 4200 μatm *P*CO2 (Fig. 2). The bootstrapped 95% confidence intervals for the elevated *P*CO2 levels overlapped with each other, but not with that of the 400 μatm *P*CO2 level (Table S4). Overall, the *Q*10 was higher in larvae than embryos under elevated *P*CO2 but similar for both life stages under ambient *P*CO2.

* + 1. *CO2 × Oxygen*

For experiments 5-6, we detected a significant interaction of *P*CO2 × *P*O2 on embryonic metabolic rates (MLR, *F*3,255 = 8.74, *p* = 0.004; Fig. 3A). For embryos reared under normoxic (23.0 kPa) conditions, exposure to elevated (4200 μatm) *P*CO2 increased metabolic rates by 55% compared to ambient *P*CO2. However, this effect of elevated *P*CO2 diminished with decreasing *P*O2. Additionally, metabolic rates of embryos in ambient *P*CO2 treatments were similar across all *P*O2 levels, but they increased with *P*O2 under elevated *P*CO2 (Table 2). Metabolic rates of newly hatched larvae were statistically unaffected by *P*CO2 or *P*O2 (MLR, *F*3,142 = 0.325, *p* > 0.05; Fig. 3B).

* 1. **Discussion**

Rearing *M. menidia* offspring and measuring their routine metabolic rates in combinations of *P*CO2 and temperature expectedly revealed 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 CO2 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 CO2 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 CO2 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 RMRwas similar for embryos and larvae at ambient (400 μatm) *P*CO2 but declined with elevated *P*CO2 in embryos and increased with *P*CO2 in larvae (Fig. 2; Table 3). A study on larval flatfish (*Solea senegalensis*) exposed to elevated CO2 and temperature found a similar trend to our embryos, that increasing CO2 reduced *Q*10, but 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 (Rombough, 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 CO2 is expected (Baumann, 2019). Reduced thermal sensitivity of embryonic RMR under elevated *P*CO2 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*CO2 indicates that larvae are slightly more sensitive to combined high *P*CO2 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*CO2 and *P*O2 treatments had a significant interactive effect on embryonic, but not larval, metabolic rates. In normoxia (23.0 kPa), RMR increased with *P*CO2, consistent with our hypothesis that seawater acidification increases metabolic costs of acid-base balance. However, the RMR of high *P*CO2 embryos decreased with declining *P*O2, suggesting RMR became oxygen-dependent and the embryos from these treatments had reached their critical oxygen partial pressure (*P*crit), the *P*O2 level below which aerobic metabolism depends directly on available oxygen (Richards, 2009). This oxygen-dependence was not detected in the ambient *P*CO2 treatments, indicating that *P*crit was higher in acidified water. Increased *P*crit can be expected as a result of elevated *P*CO2 because the Bohr effect of reduced blood pH leads to lower hemoglobin-O2 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*CO2 influences both *P*crit and the oxygen-independent RMR (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*CO2 and *P*O2 treatments, with larvae having no significant response to either variable. However, survival to hatching was so low in the 7.5 kPa *P*O2 treatments that we did not have enough larvae left to measure larval metabolic rates for that treatment. This lowest *P*O2 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*O2 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 due to reduced oxygen affinity of hemoglobin under ambient *P*CO2 (Brauner and Randall, 1996; Wells, 2009). Surprisingly, recent work on juvenile European sea bass found a decrease in *P*crit accompanied by increased hemoglobin-oxygen affinity under acutely increasing *P*CO2 (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*CO2 had higher RMR and *P*crit relative to ambient treated fish (Hancock and Place, 2016), which suggests a similar response to our embryos may occur in later stage fishes under long enough acidified duration. Nonetheless, high *P*CO2 exposed larvae may be more hypoxia-resistant than embryos due 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 (Rombough, 1988; Kamler, 1992).

The embryos’ metabolic response to elevated *P*CO2 in the CO2 × oxygen experiments is consistent with previous work that has shown highest sensitivity to seawater acidification in the early life stages of marine animals (Pörtner et al., 2005; Baumann et al., 2012; Harvey et al., 2013; Baumann et al., 2018; 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*CO2 and hypoxia (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*CO2 and *P*O2 on embryos, our experiments showed no other metabolic responses of *M. menidia* early life stages to seawater acidification. A review of fish responses to CO2 indicated that routine metabolism, the response that we measured, might not be as sensitive to CO2 as other measures, such as standard or active metabolism (Heuer and Grosell, 2014). Additionally, previous experiments exposing *M. menidia* to elevated *P*CO2 have yielded highly variable results for growth and survival of embryos and larvae, potentially due 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*CO2 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*CO2, but the negative synergistic response of embryos to high *P*CO2 and low *P*O2, conditions that co-occur in estuaries, demonstrates the potential for stage-specific responses and importance of considering multiple co-occurring stressors.

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* 1. **Figures**

**Chart

Description automatically generated**

**Figure 1.1.** Temperature- and *P*CO2-dependent routine metabolic rates (RMR) of *M. menidia* embryos and larvae.(A) Whole-body RMR (μmol O2 individual-1 h-1) of embryos and (B) mass-specific RMR (μmol O2 mg-1 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*CO2 treatment combination are *n*=10-46 for embryos and *n*=9-68 for larvae.

Chart, line chart

Description automatically generated

**Figure 1.2.** The effect of *P*CO2 on *Q*10 of routine metabolic rates 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*CO2 level are *n*=10-27 for embryos and *n*=9-41 for larvae.

Chart, line chart

Description automatically generated

**Figure 1.3.** *P*O2- and *P*CO2-dependent routine metabolic rates of *M. menidia* embryos and larvae.(A) Whole-body RMR (μmol O2 individual-1 h-1) of embryos and (B) mass-specific RMR (μmol O2 mg-1 h-1) of newly hatched larvae from experiments 5-6. Regression lines are fitted to embryonic metabolic rates (A) as a function of *P*O2 within each *P*CO2 treatment to illustrate the significant *P*CO2 and *P*O2 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*O2 and *P*CO2 combination are *n*=8-31 for embryos and *n*=10-22 for larvae.

Chart, line chart

Description automatically generated

**Figure 1.4.** Conceptual diagram of the relationship between *P*O2 and routine metabolic rate of *M. menidia* embryos in ambient and elevated *P*CO2.Hypothesized shifts in the relationship between embryonic RMR and *P*O2 are shown for elevated (orange) versus ambient (blue) *P*CO2. Our results (measured at the *P*O2 levels marked by black dots) suggest that *P*CO2 can influence both the critical oxygen partial pressure (*P*crit, gray lines) and the oxygen-independent RMR. At higher *P*O2 levels, RMR increases with *P*CO2, potentially due to increased metabolic demand. As *P*O2 decreases, embryonic RMR reaches *P*crit and becomes oxygen-dependent at a higher *P*O2 level in acidified than in ambient *P*CO2 conditions. Low intracellular red blood cell pH caused by high *P*CO2 can be expected to reduce hemoglobin-O2 affinity (Bohr effect) and make embryonic RMR less hypoxia-resistant, which could manifest as an increase in *P*crit for embryos in elevated *P*CO2. See text for more information.

* 1. **Tables**

**Table 1.1**. Overview of target levels for *P*CO2, temperature, and oxygen partial pressure (*P*O2) for six experiments for which respirometry was conducted on embryos (E) and newly hatched larvae (L) of *Menidia menidia*.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Experiment** | **Fertilization Date** | | ***P*CO2 (μatm)** | **Temperature (°C)** | ***P*O2**  **(kPa)** | **Stage** |
| 1 | | 4/22/2016 | 400, 2200 | 17, 24 | 21-23 | E, L |
| 2 | | 5/3/2016 | 400, 2200, 4200 | 17, 20, 24 | 21-23 | L |
| 3 | | 5/19/2016 | 400, 2200, 4200 | 17, 20, 24 | 21-23 | E, L |
| 4 | | 5/26/2017 | 400, 2200, 4200 | 24, 28 | 21-23 | E, L |
| 5 | | 5/9/2017 | 400, 2200, 4200 | 24 | 7.5, 12.0, 23.0 | E, L\* |
| 6 | | 6/9/2017 | 400, 2200, 4200 | 24 | 9.0, 12.0, 23.0 | E, L |

\*In Experiment 5, respirometry was only done on larvae from the 23.0 and 12.0 kPa *P*O2 treatments due to low hypoxic hatch survival.

**Table 1.2.** Age at sampling (equivalent to exposure time) in days-post-fertilization (dpf) and mean routine metabolic rates (±s.e.m.) of *M. menidia* embryos and larvae across *P*CO2, temperature, and *P*O2 treatments.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Temp (°C)** | ***P*O2**  **(kPa)** | **Age at Embryonic Sampling (dpf)** | **Age at Larval Sampling (dpf)** | ***P*CO2 (μatm)** | **Embryonic Sample Size** | **Mean Embryonic RMR (±s.e.m.)**  **(μmol O2 h-1)** | **Larval Sample Size** | **Mean Larval RMR (±s.e.m.) (μmol O2 mg-1 h-1)** |
| 17 | >20 | 11-12 | 14-15 | 400 | 26 | 0.016(±0.002) | 41 | 0.078(±0.004) |
| 2200 | 27 | 0.020(±0.001) | 35 | 0.071(±0.005) |
| 4200 | 12 | 0.026(±0.002) | 24 | 0.066(±0.005) |
| 20 | >20 | 8 | 10-11 | 400 | 12 | 0.027(±0.002) | 23 | 0.071(±0.007) |
| 2200 | 12 | 0.020(±0.002) | 26 | 0.088(±0.006) |
| 4200 | 13 | 0.024(±0.002) | 24 | 0.087(±0.009) |
| 24 | >20 | 5 | 6-7 | 400 | 55 | 0.024(±0.001) | 65 | 0.126(±0.006) |
| 2200 | 46 | 0.021(±0.002) | 68 | 0.118(±0.006) |
| 4200 | 16 | 0.017(±0.002) | 33 | 0.118(±0.009) |
| 28 | >20 | 4 | 5 | 400 | 11 | 0.039(±0.004) | 9 | 0.196(±0.018) |
| 2200 | 11 | 0.035(±0.004) | 10 | 0.191(±0.018) |
| 4200 | 11 | 0.034(±0.002) | 10 | 0.207(±0.022) |
| 24 | 23.0 | 5 | 6-7 | 400 | 26 | 0.022(±0.002) | 21 | 0.165(±0.013) |
| 2200 | 28 | 0.028(±0.003) | 19 | 0.148(±0.018) |
| 4200 | 29 | 0.034(±0.003) | 19 | 0.174(±0.017) |
| 24 | 12.0 | 5-6 | 7-8 | 400 | 26 | 0.018(±0.002) | 15 | 0.182(±0.019) |
| 2200 | 31 | 0.026(±0.002) | 22 | 0.179(±0.013) |
| 4200 | 30 | 0.025(±0.002) | 18 | 0.173(±0.019) |
| 24 | 9.0 | 7 | 9 | 400 | 13 | 0.025(±0.004) | 11 | 0.126(±0.030) |
| 2200 | 14 | 0.022(±0.003) | 11 | 0.142(±0.020) |
| 4200 | 8 | 0.027(±0.004) | 10 | 0.130(±0.024) |
| 24 | 7.5 | 7 | - | 400 | 18 | 0.025(±0.003) | - | - |
| 2200 | 17 | 0.018(±0.003) | - | - |
| 4200 | 19 | 0.017(±0.002) | - | - |

Dashes (-) indicates treatments for which too few embryos survived to hatching for larval respirometry to be done.

1. **Metabolic response of the coastal fish *Menidia menidia* reared in elevated CO2 to progressive hypoxia**
   1. **Introduction**

Acidification of coastal waters and estuaries is both gradually increasing as the oceans absorb atmospheric carbon dioxide (CO2) emissions (IPCC, 2022; 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 et al., 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).

* 1. **Methods**
     1. *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.

* + 1. *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.

* + 1. *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.

* 1. **Results**
     1. *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).

* + 1. *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). 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).

* 1. **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. This 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.

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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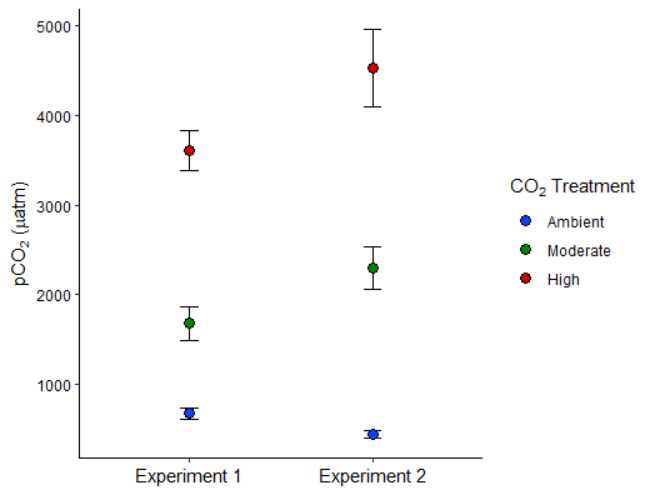
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* 1. **Figures**



**Figure 2.1.** 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.

Diagram

Description automatically generated

**Figure 2.2.** 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.

Diagram, engineering drawing

Description automatically generated

**Figure 2.3.** 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.

Diagram

Description automatically generated

**Figure 2.4.** 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.

* 1. **Tables**

**Table 2.1.** 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 2.2.** 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 |

1. **Ionoregulatory responses of *Menidia menidia* to seawater acidification and temperature**
   1. **Introduction**

Ocean acidification, the gradual decrease in global ocean pH due to dissolution of atmospheric carbon dioxide (CO2), has highly variable effects on fishes, both within (Frommel et al., 2012; Dahlke et al., 2017; Baumann et al., 2018) and among species (Kroeker et al., 2010; Couturier et al., 2013; Heuer and Grosell, 2014), and this only becomes more complex when multiple co-occurring stressors are involved, such as high temperature and hypoxia (Gunderson et al., 2016). Ocean acidification affects responses such as growth, mortality, metabolism, and behavior in many studies (Cattano et al., 2018; Esbaugh, 2018; Espinel-Velasco et al., 2018) while in other studies fish are unaffected (Jarrold and Munday, 2018; Sundin et al., 2019; Clark et al., 2020). The earliest life stages tend to have greater sensitivity to high CO2 (Ishimatsu et al., 2005; Baumann et al., 2012; Rodriguez-Dominguez et al., 2018), owing to their lower mobility as well as incomplete development of regulatory organ systems and internal buffering capacity (Melzner et al., 2009; Marshall et al., 2016). In some cases, effects of elevated CO2 are only detected in combination with another stressor (Cross et al., 2019; Schwemmer et al., 2020). Although the growing number of studies on fish responses to acidification seem to paint an increasingly complicated picture, this information will be critical to anticipating how ecologically and economically valuable species will be impacted as pH changes alongside warming, deoxygenation, pollution, and overfishing (Baumann, 2019).

One way to increase understanding of such variable, subtle, and complex responses to stressors and, importantly, their implications for fitness, is to find evidence of the mechanisms behind observed sensitivity or tolerance. Knowledge of physiological mechanisms can enhance predictive models that link organismal processes to scales relevant for management, such as population models and energy budgets (Le Quesne and Pinnegar, 2012; Lavaud et al., 2021). This knowledge will help answer the question of whether the underlying processes are subject to phenotypic plasticity that could mitigate negative responses under persistent acidification (Esbaugh, 2018). Like many of the invertebrates most sensitive to ocean acidification, formation of calcium carbonate in some teleosts is impacted by increased acidity (Grosell, 2019). Unlike in invertebrates, however, the primary threat posed to fish by ocean acidification is thought to come directly from hypercapnia rather than the changes to ocean pH and calcium carbonate formation (Esbaugh et al., 2012). Fish have effective mechanisms of acid-base homeostasis and CO2 removal, enabling them to survive higher CO2 levels than predicted with global ocean acidification in the coming centuries (e.g. Randall et al., 1976; Ishimatsu et al., 2004). When CO2 enters the blood it reacts with water to form bicarbonate (HCO3-) and hydrogen (H+) ions, which reduces the blood pH. This is rapidly compensated by exchanging bicarbonate ions (HCO3-) for chloride ions and by hemoglobin (Hb) and other compounds that buffer H+ (Brauner et al., 2019). A variety of enzymes in the gills and kidneys transport ions to support proton removal for pH regulation, often requiring ATP for active transport (Deigweiher et al., 2008; Brauner et al., 2019). While these processes may confer some tolerance, studies documenting changes in growth, percent survival, behavior, and more suggest that acid-base regulation has costs that could impact populations and ecology. For example, additional energy required for H+ transport may require additional food or yolk consumption and may result in slower somatic growth (Deigweiher et al., 2008; Lefevre, 2019). Even effective compensation may entail at least brief disturbances to extra- and intracellular pH that could disrupt physiological processes, such as Hb-oxygen binding (Root and Bohr effect; Brauner and Randall, 1996), and cause malformations or tissue damage (Frommel et al., 2012; Pimentel et al., 2016). There is great interspecies variability in the efficacies of these regulatory mechanisms so it cannot be assumed that what is observed in one species applies to all (Brauner et al., 2019), although there may be some commonalities in tolerances and mechanisms in species with similar habitat conditions (Cattano et al., 2018; Baumann, 2019).

The Atlantic silverside, *Menidia menidia*, is a useful model species with numerous studies on its responses to acidification and other environmental stressors (Bengtson et al., 1987; Schultz et al., 1998; Dixon et al., 2017; Baumann et al., 2018). Abundant throughout the estuaries of the North American east coast, *M. menidia* is easy to collect in the wild, spawn, and maintain for laboratory experiments (Middaugh et al., 1987). It is adapted to great environmental fluctuations, so using wild *M. menidia* can provide unique information that is representative of how fish in the wild would respond to stressors compared to using captive fish from genetically homogeneous populations/broodstocks. A pressing question in ocean acidification research is whether CO2 fluctuations experienced in the wild mediate species’ sensitivities to long term exposure to the higher levels that will occur as the global oceans acidify (Baumann, 2019). One study showed that diel cycling CO2 and dissolved oxygen (DO) had a less severe effect on *M. menidia* early life growth and survival metrics when compared to the detrimental effects of static treatments (Cross et al., 2019). Earlier work suggested a parental effect in which offspring of silversides that were collected at more acidified times of the spawning season were more tolerant of static high CO2 (Murray et al., 2014). A study quantifying metabolic rates found that high CO2 only impacted embryonic metabolism through an interaction effect with hypoxia (Schwemmer et al., 2020). Although this species was unaffected by high CO2 in some experiments (Murray and Baumann, 2018), a meta-analysis of 20 acidification experiments on *M. menidia* showed reductions in early life survival and growth that tended to be strongest near the beginning and end of the spawning season (Baumann et al., 2018).

Observed declines in early life growth and increases in metabolism under elevated CO2 suggest tradeoffs between maintenance costs and growth may be occurring in *M. menidia*. Fish eggs and larvae are highly subject to diffusion relative to adults, lack gills, and have little control over ventilation or mobility to remove CO2. Prior to cell differentiation, pH disturbances may be passively buffered by respiratory proteins and ions in the perivitelline fluid (Alderdice, 1988; Melzner et al., 2009). Embryos later form epithelial ionocytes, specialized mitochondria-rich cells with enzymes that transport ions, thus allowing more active control over both acid-base balance and osmoregulation (Alderdice, 1988). The use of ATP to actively transport H+ and other ions involved in acid-base balance may be one mechanism behind energetic tradeoffs leading to observed changes in metabolism and growth. Buffering by Hb can preserve intracellular pH under moderate CO2 elevations, and this is enhanced in teleosts through the Bohr effect, in which low pH reduces Hb-oxygen affinity (Brauner et al., 2019). Low pH can also lead to low internal oxygen levels due to reduced oxygen carrying capacity (Root effect; Berenbrink et al., 2005), so another tradeoff associated with acid-base regulation may be reduced oxygen uptake. In *M. menidia* embryos, oxygen consumption increased with CO2 unless hypoxia was simultaneously imposed (Schwemmer et al., 2020), suggesting that maintenance demands associated with ion exchange outweigh any reduction in oxygen uptake ability due to Hb buffering.

Ionocytes typically form on the yolk sac epithelium of the embryo but may also be found in other areas of the body, especially in late embryonic and larval stages (Alderdice, 1988). In some species embryonic ionocytes are particularly abundant near the blood vessels of the yolk sac (Guggino, 1980; Alderdice, 1988). A number of ion transporters can be found in ionocytes, and their composition varies between species and habitat salinity, as many of these transporters are also involved in osmoregulation (Christensen et al., 2012). Marine teleosts are thought to use Na+/K+-ATPase (NKA), carbonic anhydrase (CAc), apical Na+/H+ exchangers (NHE2/3), vacuolar H+-ATPase (VHA) and Na+/HCO3- cotransporter (NBC) to hydrate CO2 and remove the resulting ions, H+ and HCO3- (Esbaugh, 2018). Basolateral NKA is involved in acid-base balance through secondary active transport, moving Na+ from the ionocyte cytoplasm into the blood to create a gradient that allows apical NHE to remove H+ to the external seawater by bringing in Na+ from seawater (Silva et al., 1977; Claiborne et al., 2002; Catches et al., 2006). Because of their role in acid-base regulation and use of ATP to do so, ionocytes and their ion transporting proteins have become a mechanism of interest for high-CO2 tolerance (Esbaugh, 2018). NKA-positive ionocyte abundance was found to decrease with increasing temperature but not respond to CO2 in the yolk sac of newly hatched Atlantic cod (*Gadus morhua*) larvae reared in combinations of temperature and CO2 treatments (Dahlke et al., 2017). Another study on *G. morhua* found similar levels of ion pump enzyme activity in larvae to those of adult gill tissue, demonstrating great capacity for ion regulation despite their lack of fully developed organs (Dahlke et al., 2020). This study also highlighted the importance of measuring multiple metrics of ion regulation, such as ionocyte abundance, enzyme activity, and gene expression of ion transporting enzymes; enzyme activity of NKA, VHA, and ATP-synthase increased significantly with CO2 in cod embryos and larvae (Dahlke et al., 2020), even though ionocyte abundance was not affected in the previous study (Dahlke et al., 2017). A study on white seabass (*Atractoscion nobilis*) eggs and larvae from a broodstock that had previously been exposed to high CO2 found that rearing offspring in elevated CO2 increased ionocyte abundance and density, but that accounting for ionocyte size to calculate total ionocyte area negated this effect (Kwan et al., 2021). This suggests that populations historically experiencing periods of high CO2, including estuarine fishes like *M. menidia*, may already have sufficient mechanisms in place to cope with predicted global CO2 increases without making substantial changes to their physiology.

With this study, we aimed to mechanistically explain previously recorded growth and survival responses of *M. menidia* to elevated CO2 by quantifying the temperature-dependent effects of seawater acidification on ionic regulation in the early life stages. We sampled embryos and larvae that were reared in four experiments combining factorial combinations of temperature and partial pressure of CO2 (pCO2) and measured ionocyte density on the skin. A separate study also measured gene expression of seven ionoregulatory enzymes in fish from these experiments (Schwemmer et al., in prep). We hypothesized that early life *M. menidia* reared in elevated pCO2 would have increased density of ionocytes as a mechanism for maintaining internal acid-base balance. We expected to see greater ionocyte densities on the skin surface to increase capacity for ion exchange in the absence of gills. Based on previous results showing reduced growth and survival and increased embryonic routine metabolic rates under high pCO2 (Murray et al., 2014; Murray and Baumann 2018; Schwemmer et al., 2020), we hypothesized that more energy was redirected from growth to maintenance at the embryo stage, and therefore there would be fewer ionocytes in embryos exposed to high CO2.

* 1. **Methods**
     1. *Animals, Sampling, and Treatments*

Collection, rearing, and harvesting of live *M. menidia* were conducted as previously described in Murray and Baumann (2018) and Schwemmer et al. (2020). For the experiments in this study, wild, spawning ripe *M. menidia* adults were collected in 2016 from Mumford Cove, a salt marsh adjacent to Long Island Sound, New York, USA. Adults were transported to laboratory facilities at the Rankin Seawater Facility at University of Connecticut’s Avery Point campus in Groton, Connecticut, USA. Females were strip-spawned at room temperature by gently squeezing their eggs into shallow spawning dishes containing pieces of plastic window screen (1 mm mesh). Milt from several males was collected, pooled, mixed in seawater, and poured into the spawning dishes. After 15 minutes, fertilized eggs attached to the screen via uncoiled chorionic filaments while unfertilized eggs could be gently rinsed off later. Screens were cut into pieces which were randomly divided and hung in 20-L rearing containers with mesh-covered holes for water to overflow without fish escaping. One rearing container containing 100 viable embryos was placed in each tank of treatment seawater within 2 hours of fertilization (filtered to 1 μm and UV sterilized).

Larvae hatched 4-11 days postfertilization and were immediately fed *ad libitum* with newly hatched brine shrimp nauplii (*Artemia* spp.). Embryos were collected one day before hatching for ionocyte staining. Larvae were collected for ionocyte staining at 1-day post hatch (dph) and once the larvae within a temperature treatment reached ~10 mm standard length (SL). The final sampling point was determined by SL rather than time to allow assessment of treatment effects at a similar developmental stage (Tables S2 and S3). Temperature strongly affects rates of growth and development, and this allowed us to correct for that so that differences in development would not be confounding treatment effects. Consequently, age at final sampling ranged from 10 to 23 dph. Embryos and larvae used for ionocyte analysis first underwent respirometry (Schwemmer et al., 2020), then were fixed and preserved for staining.

Experimental treatment conditions are described in detail in Murray and Baumann (2018) and Schwemmer et al. (2020). Briefly, four factorial experiments were conducted in 2016 and 2017 combining environmentally relevant CO2 and temperature levels as currently experienced in Long Island Sound estuaries and predicted worldwide by 2100 (IPCC, 2022). Target treatment levels and spawning dates for each experiment and life stages sampled for each response variable are summarized in Figure 1. CO2 levels were manipulated by continuously bubbling 100% CO2 (bone dry grade, AirGas) mixed with air via gas proportioners (Cole Parmer, Vernon Hills, IL, USA) into rearing containers (Experiments 1 and 4) or by controlling bubbling 100% CO2 into sumps of treatment tanks with an automated program designed in LabView software (National Instruments, Austin, TX, USA; Experiments 2 and 3). In Experiments 1 and 4, pH was monitored daily with a handheld probe and in Experiments 2 and 3 pH was measured once per hour automatically by pumping water from each tank to a central pH electrode, which was then used to control CO2 input to maintain static treatments. Laboratory air was also bubbled continuously to maintain oxygen at 100% air saturation. Thermostats controlled aquarium heaters or chillers (DeltaStar, Lynchburg, VA, USA) to maintain temperature treatments. Carbonate chemistry conditions were characterized by measuring pH, temperature, salinity, and total alkalinity (TA). TA samples were collected three times per experiment and measured using an endpoint titration (G20 Potentiometric Titrator, Mettler Toledo, Columbus, OH, USA). CO2SYS (V2.1) was used to calculate pCO2 (µatm), fugacity of CO2 (*f*CO2, µatm), dissolved inorganic carbon (DIC, µmol kg-1), and carbonate ion concentration (CO32-, µmol kg-1). Measured seawater conditions are reported in Table S1.

* + 1. *Ionocyte Density*

At the time of sampling, embryos and larvae were fixed in 4% paraformaldehyde (buffered, pH 7.4) and stored at 4°C for 4 hours, then transferred to 70% ethanol and stored at 4°C. Embryos were manually dechorionated before staining. Samples were equilibrated in phosphate buffered saline (PBS) for 5 minutes, blocked in normal horse serum for 20 minutes to minimize background staining, blotted dry, then incubated for 30 minutes in α5 primary antibody, a mouse monoclonal antibody raised against the alpha subunit of chicken NKA (D. M. Fambrough, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). The primary antibody was diluted to 1 μg/mL for embryos and 2 μg/mL for larvae, in PBS with 1% bovine serum albumin. Primary antibody binding was detected using the avidin-biotin complex (ABC) method (Hsu et al., 1981). Samples were incubated for 30 minutes each in a secondary antibody and ABC reagent (Vectastain® ABC HRP Kit, Peroxidase, Mouse IgG, Vector Laboratories, Burlingame, CA) and rinsed for 5 minutes in PBS between each step. The specific binding was then visualized with a peroxidase substrate kit (Vector® VIP HRP Substrate Kit, Vector Laboratories, Burlingame, CA). Conducting this protocol in the absence of primary antibody produced no staining, confirming that the staining indicated presence of the antigen NKA.

         All samples were photographed through a compound microscope (Nikon Eclipse E200) at several depths of focus to capture the entirety of the three-dimensional structure. Images were analyzed using the software ImageJ. For each individual fish, the images from all focus depths were loaded as a stack and projected into a single image with all ionocytes in focus. We selected and measured the area (mm2) of a section of upward-facing skin surface in the resulting photo and counted the ionocytes within that area. For larvae, the ionocytes on the head, abdomen, and trunk skin surface, excluding the eyes and mouth, were enumerated. For embryos, yolk sac epithelium was analyzed separately from the rest of the skin. Ionocyte density was calculated as ionocytes mm-2 of skin surface area. Ionocyte density was quantified by two different people or twice by the same person with at least one month in between to account for subjectivity and uncertainty in ionocyte identification. Standard deviation between the two densities was calculated and if it was greater than three standard deviations above the mean, the sample was analyzed again by the same methods to rectify differences in analysis.

* + 1. *Data Analysis*

Statistical analysis of the ionocyte density data was conducted using linear regression models with the formula: Ionocyte Density ~ pCO2 x Temperature. The independent variables were input as quantitative variables using the mean measured pCO2 and temperatures for each treatment. In each experiment we sampled one experimental unit (n=1) per treatment, and not all treatment levels were repeated in multiple experiments (e.g. 28°C) so we had pseudoreplication. When tests that use categorical independent variables, such as analysis of variance, cannot be used due to pseudoreplication, a model can instead be fit with quantitative (continuous) independent variables (Hurlbert, 2004), which in fact provides greater statistical power and more informative results than an analysis with categorical independent variables would (Cottingham et al., 2005; Havenhand et al., 2010). Assumptions of normally distributed residuals and homoskedasticity were confirmed using the Kolmogorov-Smirnov test and Breusch-Pagan test, respectively. Linear model residuals and Cook’s distance were used to identify extreme and highly influential datapoints, which resulted in one outlier being removed from the data for each stage. A significance threshold of α=0.05 was used for all statistical tests. All statistical tests were performed in R statistical software (v4.2.2, R Core Team, 2022) using the ‘stats’ and ‘olsrr’ (Hebbali, 2020) packages.

* 1. **Results**

Embryos and hatchlings had higher maximum skin ionocyte densities, 722.6 and 611.4 ionocytes mm-2, respectively, while 10-mm larvae had lower ionocyte densities, with a maximum of 418.8 ionocyte mm-2. However, embryo data were distributed around a lower mean with a strong positive skew while both stages of larvae had normally distributed ionocyte densities (Figure 1). Yolk data were more skewed than skin data. Therefore, to meet the assumptions of normality and homoskedasticity the embryo yolk data were log-transformed and embryo skin data were square-root transformed in the linear model. Additionally, across all treatments combined the non-yolk portion of the embryos’ bodies had significantly greater ionocyte density than the yolks (t-test, p<0.001, t(558)=-3.7), and progression of ionocyte development commonly differs between the yolk sac and rest of the body (Alderdice, 1988) so we analyzed these regions separately.

Temperature and pCO2 had a significant interactive effect on embryo yolk sac (linear regression, *F*(3,280)=23.94, *p* < 0.01) and skin (linear regression, *F*(3,280)=16.3, *p* < 0.001) surface ionocyte density. Ionocyte density decreased with increasing temperature, with 28°C embryos having the lowest ionocyte densities across all temperature and pCO2 levels. The greatest mean yolk sac ionocyte density occurred in the highest pCO2 and 17°C treatment combination, while skin ionocytes exhibited a weaker pCO2 effect at this temperature. Within the 17°C treatments, the mean yolk ionocyte density at 4200 µatm pCO2 is 118.4% greater than that at 400 µatm, while the skin ionocyte density at 4200 µatm is only 61.9% greater than the density at 400 µatm. In the yolk and body skin it appears that high CO2 increases the temperature-dependence of ionocyte density, making it decrease more sharply as temperature increases. Within the 4200 µatm pCO2 level, yolk sac ionocyte density decreases more dramatically with temperature than skin ionocyte density does, illustrating the greater response of yolk sac ionocytes to elevated pCO2.

Temperature and pCO2 had a significant interactive effect on ionocyte densities of newly hatched larvae (linear regression, *F*(3,393)=9.461, *p* = 0.001). Hatchlings show the opposite temperature effect of embryos, with ionocyte density increasing with temperature, particularly in the 28°C treatment (Figure 3A). The 400 µatm pCO2 treatment groups have a slight decrease in ionocytes as temperature decreases up to 24°C before increasing sharply at 28°C. In contrast, the 4200 µatm larvae have a more steady increase in ionocytes with temperature across all temperature levels. Within the 24°C treatments, ionocyte density at 4200 µatm pCO2 is 16.7% higher than at 400 µatm. At the 10-mm larval sampling point the ionocyte densities are no longer affected by temperature or pCO2 (linear regression, *F*(3,337)=7.946, *p*>0.05). The most substantial difference is that at 17°C ionocyte density at 4200 µatm pCO2 is 12.7% and 17.4% greater than at 400 µatm and 2200 µatm, respectively. At this stage, three gill arches with filaments and lamellae had developed on each side and skin surface ionocyte densities decreased to just over half the density of newly hatched larvae, regardless of treatments.

Embryos and hatchlings in this study were also used for respirometry measurements (full results reported in Chapter 1). We used Pearson’s correlation test to test whether ionocyte density is significantly correlated with mass-specific routine metabolic rates (RMR) in individuals for which both quantities were measured. In embryos, RMR significantly decreased with increasing ionocyte density (Pearson’s correlation coefficient = -0.21, *t*(241)=-3.83, *p*<0.001) while in larvae RMR significantly increased with ionocyte density (Pearson’s correlation coefficient = 0.13, *t*(353)=2.55, *p*=0.01). Both response variables are strongly affected by temperature, as RMR increases with temperature while ionocyte density’s relationship with temperature is stage-dependent. Before hatching ionocyte density is lower at the highest temperatures, leading to a negative correlation of RMR with ionocyte density (Figure 4A). After hatching ionocyte density is highest at high temperature and the RMR-ionocyte correlation becomes positive (Figure 4B).

* 1. **Discussion**

Across four experiments exposing *M. menidia* embryos and larvae to pCO2 and temperature treatments, ionocyte density was primarily influenced by temperature with some interacting, low magnitude pCO2 effects. In embryos sampled about one day before hatching, high pCO2 increased overall temperature-sensitivity of ionocyte density. Embryos reared at the lowest temperature had greater ionocyte density at the highest pCO2 level, particularly on the yolk sac epithelium. Embryonic ionocytes have been noted to occur on the yolk sac in proximity to vitelline blood vessels (Guggino, 1980; Alderdice, 1988), likely to facilitate ion transport for blood pH regulation. Low temperature slows growth, with offspring in our 17°C taking the longest to hatch, so the additional incubation time and delay in gill development may have led to increased ionocyte formation in response to high pCO2 to promote H+ removal and HCO3- accumulation, particularly in the yolk sac area. Previous studies support the idea that substantial ion transport is possible in early life stages exposed to high pCO2, despite incomplete development of organ systems. Acidified conditions have been shown to elicit both an increase in ionocyte density (Kwan et al., 2021) and accumulation of HCO3- (Kwan and Tresguerres, 2022). While fish in the earliest stages of embryogenesis and gastrulation may rely on passive defenses such as non-HCO3- buffers (Alderdice, 1988; Melzner et al., 2009), differentiation of cells like ionocytes and plasticity in homeostatic mechanisms seem to confer sufficient tolerance for high pCO2 (Dahlke et al., 2020). The pCO2 effect observed in *M. menidia* embryos from 17°C treatments, as well as the overall abundance of NKA-rich ionocytes on the skin of near-hatching embryos and newly hatched larvae, indicate a substantial capacity for removing H+ ions by creating a Na+ gradient to drive NHE2/3.

Although ionocyte density was low among most embryos, at 17°C embryos were able to produce more ionocytes in response to high pCO2, which could aid acid-base balance and improve chances of hatch survival. Ionocyte density is subject to phenotypic plasticity, as demonstrated by past studies that have shown changes in ionocyte density, distribution, and functioning in response to environmental conditions such as salinity in euryhaline species (Schwerdtfeger and Bereiter-Hahn, 1978; Zydlewski et al., 2003; Hiroi and McCormick, 2012) and air exposure in amphibious species (Tunnah et al., 2022). Our results support our hypothesis that chronic, environmentally relevant high CO2 can similarly induce a plastic response in skin ionocytes of *M. menidia*, although this response was only detected at the lowest temperature. Without measurements of enzyme activity levels, energetic costs of additional ionocytes cannot be ascertained. Nonetheless, this finding implies is that additional energy may be directed to pumping ions under these conditions, which may boost chances of survival but could also be expected to divert energy away from growth, which could have implications for post hatch survival, predation vulnerability, and ultimately recruitment (Houde, 1997).

Temperature evidently affects ionocyte density either directly, such as by increasing enzyme activity rates and reducing the need for greater quantities of ionocytes, or indirectly through impacts on development rate. We observed a decrease in embryonic ionocytes with increasing temperature and an increase in larval ionocyte density at the highest temperature treatment (28°C). However, it is uncertain if these differences in ionocyte density are a direct result of temperature, and thus further evidence of phenotypic plasticity, or instead an indirect effect of temperature through its influence on developmental rate. Although we set our sampling timepoints to compensate for effects of temperature on development rates – sampling based on hatching and total length rather than equal times from fertilization – we have still observed temperature effects on body size at each time point, for example reduced length at hatching for 28°C relative to those reared at 24°C (Murray and Baumann, 2018).

The differences in distribution of ionocyte abundances directly before and after hatching are potentially an important mechanism by which sensitivity improves once larvae have hatched. Before hatching, the frequency distribution has a strong positive skew, particularly on the yolk sac, showing that while the majority of embryos have a relatively low ionocyte density, a smaller subset have great ionocyte abundances that are similar to those of hatchlings. By the day of hatching, which in our experiments was only one or two days after sampling of embryos, the skew is gone and ionocyte densities are symmetrically distributed around a much higher mean (Figure 2). There are two possible explanations for this rapid shift, one being that ionocytes proliferate around the time of hatching and only low numbers of the embryos we sampled had such high abundances. The second explanation is that there is broad natural variability in ionocyte production and embryos with lower ionocytes are less likely to survive to hatching because of their reduced capacity for homeostasis, leaving the remaining hatchlings with greater ionocyte abundances. Such selective mortality would not be restricted to fish in acidified conditions, as ionocytes are also central to salt secretion for osmoregulation (Hwang and Lin, 2013; Liu et al., 2016), but it still could favorably produce survivors that are better prepared to regulate acid-base balance. The reality may be a combination of these two explanations, and both help explain why post-hatch larvae were less sensitive to both pCO2 and temperature. This pattern also may be responsible for the reversal in temperature effect on ionocyte density. Mean hatch survival in these experiments was lower at 28°C than the other temperatures (Table 4; Murray and Baumann, 2018) and these embryos also had the lowest ionocyte density. The rapid shift to 28°C fish having the greatest ionocyte densities makes sense if the individuals with less ionocytes were less likely to survive hatching.

The large impact of 28°C compared to differences among the other temperature treatments aligns with results for other response variables from the same set of experiments: quick hatching time, small larval size, and high metabolism (Table 4; Figure 4; Murray and Baumann, 2018; Schwemmer et al., 2020). Combined, these results indicate that the temperature of 28°C – rarely encountered in the spawning season and regional habitat of this species – requires high maintenance rates that quickly deplete the yolk while reserving less energy for growth and development. The rapid increase we observed in ionocyte density after hatching at 28°C may serve as evidence of delayed ionocyte formation, either due to temperature-related energetic limitations or growth and hatching exceeding the limits of ionocyte formation speed. At the same time, evidence for *G. morhua* show increased enzyme activity and reduced ionocyte density with high temperature (Dahlke et al., 2017; Dahlke et al., 2020), suggesting that fish reared at high temperature have fewer ionocytes that are more active to keep up with ionoregulatory needs. While we did not measure enzyme activity in this study, metabolic rates of hatchlings slightly increased with ionocyte density, suggesting a low magnitude increase in energy used for ionic regulation in fish with more ionocytes. More likely, however, is that this correlation is related to the high ionocyte densities we found at high temperature. As shown in Figure 5, RMR increases with temperature (Schwemmer et al., 2020), and the correlations of RMR with ionocyte density are likely an indirect result of how temperature affects both; ionocyte density is negatively correlated with temperature before hatching and positively correlated with it after hatching. Even if we cannot be certain that the additional ionocytes at 28°C are accompanied by additional energy spent on pH regulation at high CO2, the lack of CO2 effects on survival in these experiments (Murray and Baumann, 2018) provides further evidence that the ionocytes were sufficient for acid-base regulation, or at least a bare minimum for survival.

When larvae reached 10-mm standard length, skin surface ionocyte densities had decreased to just over half of the densities quantified in hatchlings. These mature larvae consistently had three gill arches on each side of the body, with several gill filaments and secondary lamellae usually visible and ionocytes present. These trends are consistent with the transition from reliance on cutaneous to branchial ionocytes as fish progress through the larval stage (Alderdice, 1988; Hiroi et al., 1998). There were no CO2 or temperature effects on skin ionocyte density of 10-mm larvae. This is not surprising, as surface area is increasing in the gills and the number of ionocytes may no longer be a limiting factor in ion exchange or plasticity may become centralized in the gills, which are more efficient at ion regulation due to their proximity to both the blood and outside environment. The oldest individuals sampled at this stage were 23 dph, so the latest most silversides would be reaching this stage is around the end of July. In Long Island Sound, this is approaching the hottest time of year with greatest incidences of eutrophication, stratification, hypoxia, and coastal acidification. It bodes well for larvae that the gills are already developing, mobility is increasing, and reliance on skin surface ionocytes appears to be declining by this stage. We did not sample mature larvae in Experiments 3 or 4, the ones that included 28°C treatments, so it is possible this prevented us from detecting a high temperature effect as was observed in the earlier stages. Future work should continue to include such high temperatures to aid understanding of the interactions between temperature stress and acid-base regulation and further establish the environmental limits of this species with physiological mechanisms.

This study presents a mechanism for the generally high tolerance *M. menidia* have for high CO2 while showing some evidence for temperature-dependent plasticity in ionocyte density, potentially exchanging energy that would have gone towards growth for maintenance costs under high CO2. Further support for this mechanism could be found by measuring activity of ion transporting enzymes, which could confirm whether the ionocyte differences we did observe can be considered a proxy for energy expended on ion exchange. This could also improve understanding of how responsive *M. menidia* ionocytes are to changes in CO2 when density is unaffected, as it is possible the ionocytes simply increase activity without the fish needing to produce more cells. There are two types of ionocytes that marine fish use to transport ions for pH and osmoregulation. We stained for Type-I ionocytes, in which NKA play a role in pH compensation by creating a Na+ gradient that allows NHE2/3 to passively exchange H+ in the cell for Na+ from the seawater (Silva et al., 1977; Claiborne et al., 2002; Kwan et al., 2020). Type-II ionocytes are high in carbonic anhydrase and VHA, which allows them to contribute to both transport of HCO3- into the endolymph and H+ out of the body (Kwan et al., 2020). Although logistical constraints prevented us from staining for Type-II ionocytes, it would be useful to know the extent to which the cellular mechanisms of *M. menidia* early life CO2 responses include HCO3- transport and whether Type-II ionocytes exhibit similar patterns of sensitivity and resistance to acidification and high temperature to those of Type-I ionocytes. Quantifying VHA-rich cells would also contribute to our knowledge of the energy expended on ion exchange early in life. Consistent with what is expected for an estuarine species that has historically experienced periods of high CO2 (Baumann, 2019; Kwan et al., 2021), *M. menidia* develop ionocytes before hatching that likely confer the ability to tolerate a wide range of acidity levels. While CO2 effects on ionocyte density were minimal in the early life stages of *M. menidia*, extreme high temperatures and the accompanying high rates of development and yolk depletion may compromise the ability of embryos and larvae to develop cells for active ionic regulation. Knowledge of the mechanisms behind interacting stressor impacts such as these has important implications for accurately predicting energetically determined responses, such as reduced body size or survival, that may have population-level consequences, such as predation mortality or reproductive output.

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* 1. **Figures**

Graphical user interface

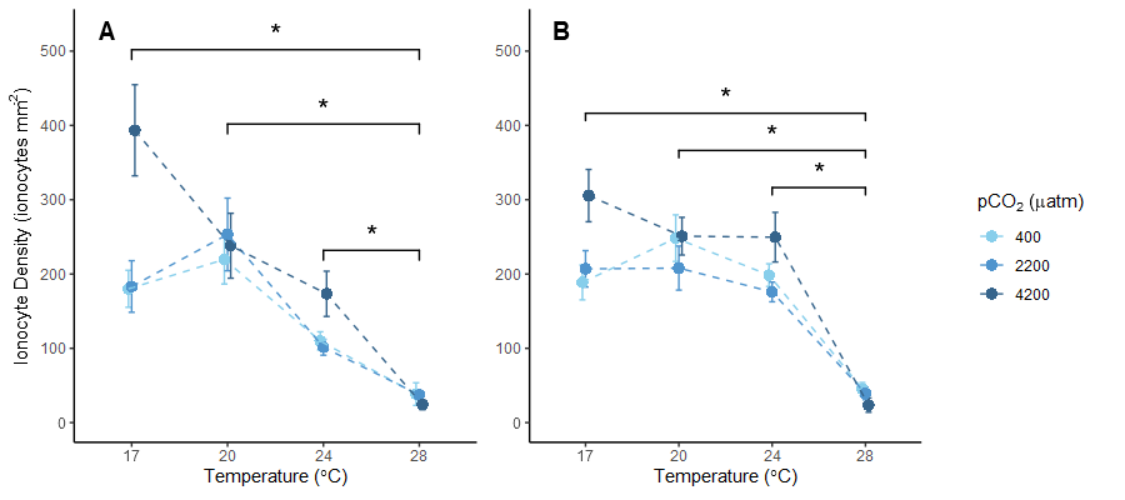
Description automatically generated

**Figure 3.1.** Microscope images of an *M. menidia* embryo, hatchling, and 10-mm larva (from top to bottom) with ionocytes stained dark purple.

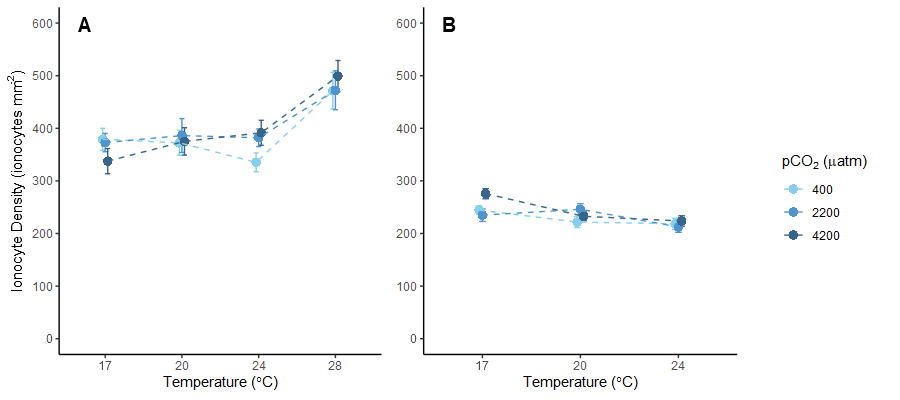
Chart, histogram

Description automatically generated

**Figure 3.2.** Frequency distribution of ionocyte densities at the embryo stage (yolk and skin) and hatchlings. In embryos the data are positively skewed, particularly for the yolk, but immediately after hatching the ionocyte densities are more normally distributed. The 10-mm larvae also have normally distributed ionocyte densities, although around a lower mean, but we do not display them here so the difference in distribution before and after hatching can be emphasized.



**Figure 3.3.** Embryo yolk sac (A) and skin (B) ionocyte density means plotted with respect to temperature and pCO2. Error bars show standard error and brackets with asterisks represent significant differences between temperature treatments (EMM, p<0.05).



**Figure 3.4.** Hatchling (A) and 10-mm larvae (B) ionocyte density means plotted with respect to temperature and pCO2. Error bars show standard error.

Chart, scatter chart

Description automatically generated

**Figure 3.5.** Metabolic rates of embryos (A) and mass-specific metabolic rates of hatchlings (B) plotted with respect to ionocyte density, with shades of blue indicating the treatment temperature ranging from 17°C (lightest shade) to 28°C (darkest shade). Each point represents an individual fish for which both RMR and ionocyte density were quantified. The black line and gray shading are a linear regression of RMR ~ Ionocyte Density with 95% confidence intervals.

* 1. **Tables**

**Table 3.1.** Spawning dates, target temperature, target pCO2 levels, and stages sampled in each experiment. E stands for embryos, H stands for hatchlings, and L stands for mature (10-mm) larvae.

|  |  |  |  |
| --- | --- | --- | --- |
|  | 400 µatm | 2200 µatm | 4200 µatm |
| Experiment 1 – April 22, 2016 | | | |
| 17 | E, H | E, H | - |
| 20 | E, H | E, H | - |
| 24 | E, H | E, H | - |
| Experiment 2 – May 3, 2016 | | | |
| 17 | E, H, L | E, H, L | E, H, L |
| 20 | E, H, L | E, H, L | E, H, L |
| 24 | E, H, L | E, H, L | E, H, L |
| Experiment 3 – May 19, 2016 | | | |
| 17 | E, H, L | E, H, L | E, H, L |
| 20 | E, H, L | E, H, L | E, H, L |
| 24 | E, H, L | E, H, L | E, H, L |
| Experiment 4 – May 26, 2017 | | | |
| 24 | E, H | E, H | E, H |
| 28 | E, H | E, H | E, H |

**Table 3.2.** Mean, standard error, sample size, and age at sampling (range in days post fertilization) for ionocyte density of embryo yolk sac and body skin surface in each treatment.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Temp (°C)** | **pCO2 (µatm)** | **N** | **Age (dpf)** | **Mean** | **SE** |
| Embryo (yolk sac) | 17 | 400 | 29 | 14-15 | 180.1 | 24.9 |
| 2200 | 30 | 14-15 | 183.2 | 34.7 |
| 4200 | 10 | 14-15 | 393.4 | 61.3 |
| 20 | 400 | 13 | 10-11 | 219.6 | 33.0 |
| 2200 | 13 | 10-11 | 253.3 | 48.9 |
| 4200 | 13 | 10-11 | 238.1 | 43.7 |
| 24 | 400 | 60 | 6-7 | 110.0 | 12.2 |
| 2200 | 64 | 6-7 | 101.4 | 10.7 |
| 4200 | 24 | 6-7 | 173.3 | 30.5 |
| 28 | 400 | 9 | 5 | 38.4 | 15.2 |
| 2200 | 9 | 5 | 37.4 | 5.9 |
| 4200 | 10 | 5 | 24.2 | 6.8 |
| Embryo (skin) | 17 | 400 | 29 | 14-15 | 188.7 | 23.4 |
| 2200 | 30 | 14-15 | 207.0 | 24.4 |
| 4200 | 10 | 14-15 | 305.5 | 35.2 |
| 20 | 400 | 13 | 10-11 | 248.3 | 31.3 |
| 2200 | 13 | 10-11 | 208.0 | 29.6 |
| 4200 | 13 | 10-11 | 250.9 | 25.3 |
| 24 | 400 | 60 | 6-7 | 198.5 | 15.3 |
| 2200 | 65 | 6-7 | 184.3 | 15.6 |
| 4200 | 24 | 6-7 | 249.6 | 33.4 |
| 28 | 400 | 9 | 5 | 45.2 | 8.7 |
| 2200 | 9 | 5 | 39.2 | 8.4 |
| 4200 | 10 | 5 | 23.6 | 9.6 |

**Table 3.3.** Mean, standard error, sample size, and age at sampling (range in days post hatching) for ionocyte density of newly hatched and 10-mm larvae in each treatment.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Temp (°C)** | **pCO2 (µatm)** | **N** | **Age (dph)** | **Mean** | **SE** |
| Hatchlings | 17 | 400 | 42 | 1 | 379.2 | 14.5 |
| 2200 | 42 | 1 | 372.8 | 11.8 |
| 4200 | 27 | 1 | 337.5 | 16.8 |
| 20 | 400 | 27 | 1 | 372.3 | 11.8 |
| 2200 | 27 | 1 | 386.1 | 17.4 |
| 4200 | 25 | 1 | 375.1 | 16.9 |
| 24 | 400 | 66 | 1 | 335.4 | 15.1 |
| 2200 | 69 | 1 | 382.0 | 12.2 |
| 4200 | 37 | 1 | 391.4 | 18.1 |
| 28 | 400 | 12 | 1 | 471.5 | 22.4 |
| 2200 | 12 | 1 | 472.3 | 23.6 |
| 4200 | 11 | 1 | 499.3 | 14.7 |
| 10-mm larvae | 17 | 400 | 40 | 15-23 | 244.6 | 8.5 |
| 2200 | 37 | 15-23 | 234.8 | 9.0 |
| 4200 | 33 | 15-23 | 275.7 | 8.9 |
| 20 | 400 | 40 | 12-14 | 221.7 | 9.2 |
| 2200 | 39 | 12-14 | 246.0 | 10.0 |
| 4200 | 38 | 12-14 | 232.7 | 7.7 |
| 24 | 400 | 38 | 10 | 218.5 | 10.2 |
| 2200 | 37 | 10 | 212.4 | 8.4 |
| 4200 | 39 | 10 | 223.7 | 8.4 |

**Table 3.4.** Linear model coefficients and p-values for ionocyte density

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | **Coefficient** | **St. Err.** | **t-value** | **p-value** |
| Embryo (yolk sac)1 | pCO2 | 7.8e-4 | 2.5e-4 | 3.1 | **0.0020** |
| Temp | -7.2e-2 | 2.7e-2 | -2.7 | **0.0082** |
| pCO2 x Temp | -3.2e-5 | 1.1e-5 | -2.9 | **0.0039** |
| Embryo (skin)2 | pCO2 | 4.6e-3 | 1.2e-3 | 3.8 | **0.0002** |
| Temp | -0.11 | 0.13 | -0.82 | 0.415 |
| pCO2 x Temp | -1.9e-4 | 5.3e-5 | -3.7 | **0.0003** |
| Hatchlings | pCO2 | -5.9e-2 | 2.0e-2 | -3.0 | **0.0031** |
| Temp | -0.88 | 2.3 | -0.38 | 0.706 |
| pCO2 x Temp | 3.0e-3 | 9.0e-4 | 3.3 | **0.0010** |
| 10-mm larvae | pCO2 | 2.5e-2 | 1.4e-2 | 1.7 | 0.0818 |
| Temp | -2.6 | 1.9 | -1.4 | 0.169 |
| pCO2 x Temp | -1.0e-3 | 7.0e-4 | -1.5 | 0.145 |

1Square-root transformed

2Natural log-transformed

**Table 3.5.** Comparison of ionocyte density results with previously reported effects of pCO2 and temperature on growth and survival of fish from the same experiments. Experiment 2 is omitted because growth and survival were not quantified. Green symbol is sole pCO2 effect and orange symbol is sole temperature effect, ‘↑’=positive effect, ‘↓’=negative effect, ‘↑↓’=both positive and negative, ‘×’=interaction, and ‘–’=no effect. The reference numbering is the experiment number used in the publication where the growth and survival data are reported, Murray and Baumann (2018), because the numbering for the same experiments differs in this study.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Embryo ionocytes | Hatchling ionocytes | Hatch survival | Hatch length | Larval survival | Larval growth | Reference  numbering |
| Exp. 1 | **–** | **×** | **–** | **–** | **↑** | **↑** | ‘Exp. 2’ |
| Exp. 3 | **↓** | **↓** | **–** | **×** | **↑↓** | **↑** | ‘Exp. 3’ |
| Exp. 4 | **↓** | **–** | **–** | **↑, ↓, ×** | **↓** | **↑** | ‘Exp. 5’ |

1. **Attributing hypoxia responses of early life *Menidia menidia* to energetic mechanisms with Dynamic Energy Budget theory**
   1. **Introduction**