**Ionoregulatory responses of *Menidia menidia* to seawater acidification and temperature**

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**Abstract**

Establishing cellular and physiological mechanisms of fish tolerance or sensitivity to environmental change is a critical component of predicting individual- and population-level consequences of global change. Early life stages do not have the fully developed organs and systems that adult fishes have, and yet some species exhibit tolerance of a range of conditions despite widely held expectations that embryos and larvae would be more sensitive to stressors such as ocean acidification and warming. We tested the hypothesis that increased density of skin ionocytes is a mechanism for responding to high CO2 across a range of temperatures in embryos and larvae of the Atlantic silverside (*Menidia menidia*). Embryos, newly hatched larvae, and mature (10-mm) larvae were sampled from factorial treatment combinations of pCO2 (~400, 2200, and 4200 µatm) and temperature (17, 20, 24, and 28°C) and ionocytes rich in Na+/K+-ATPase (NKA) were stained via whole-mount immunohistochemistry on the skin and gills (when present). Temperature and CO2 had significant interactive effects on the skin of embryos and newly hatched larvae, with high CO2 increasing temperature-dependence of ionocyte density. Embryonic ionocyte density decreased with increasing temperature, with embryos from the 28°C treatments having particularly sparse ionocytes. Conversely, newly hatched larvae in the 28°C treatments had more ionocytes per mm skin surface than the lower temperatures. By the time larvae reached 10-mm standard length, three gill arches per side with filaments and lamellae had formed and the density of skin surface ionocytes had declined substantially, with no temperature or CO2 effects. The overall high abundance of ionocytes on embryo and larvae skin (up to ~700 cells mm-2) provides evidence of a mechanism for this species’ ability to persist in high CO2 conditions, but warming may pose a challenge by speeding up hatch timing while delaying the proliferation of ionocytes until after hatching.

**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/or slower 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 and gills. 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 and greater overall ionocyte abundances in the gills of high-CO2 reared fish due to faster gill development. 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 believe more energy was redirected from growth to maintenance at the embryo stage, and therefore hypothesized that effects on ionocytes would be more pronounced then.

**Methods**

*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 Long Island Sound salt marsh. Adults were transported to laboratory facilities at the Rankin Seawater Facility at University of Connecticut’s Avery Point campus in Groton, CT, 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 molecular biology analysis and 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, to correct for the effects of temperature treatments on growth rate. 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. Individual larvae for gene expression analysis were quickly strained, transferred to a 1.7 mL microfuge tube, snap frozen in liquid nitrogen, and stored at -82°C.

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, 2021). 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.

Graphical user interface

Description automatically generated

**Figure 1.** Spawning dates, target temperature and pCO2 levels, and stages sampled for each response variable in each experiment. At center, microscope photographs of *M. menidia* with ionocytes stained purple at the embryo, newly hatched larva, and 10-mm larva stages (from top to bottom).

*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. The image sequence for a sample was loaded as a stack and projected into a single image with all ionocytes in focus. We selected and measured a section of upward-facing skin surface in the 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.

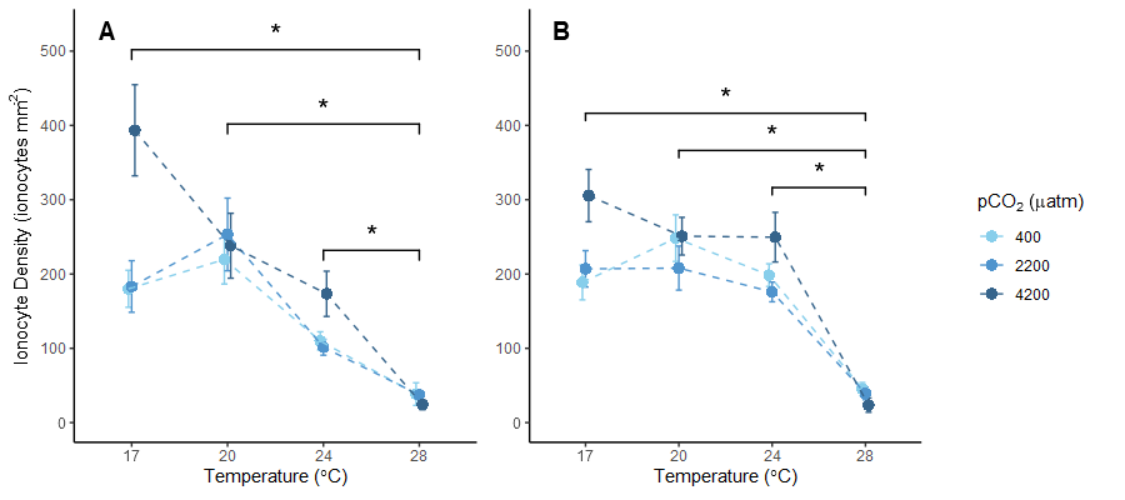
*Data analysis*

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 fitted 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. When the regression models detected a significant effect, the estimated marginal means (EMM) function from the emmeans package in R was used to characterize pairwise differences between treatment groups using Tukey comparisons. 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’, ‘emmeans’ (Lenth, 2023), and ‘olsrr’ (Hebbali, 2020) packages.

**Results**

Embryos and newly hatched larvae had higher maximum 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. Yolk data were more skewed than body data. Therefore, to meet the assumptions of normality and homoskedasticity the embryo yolk data were log-transformed and embryo body 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.

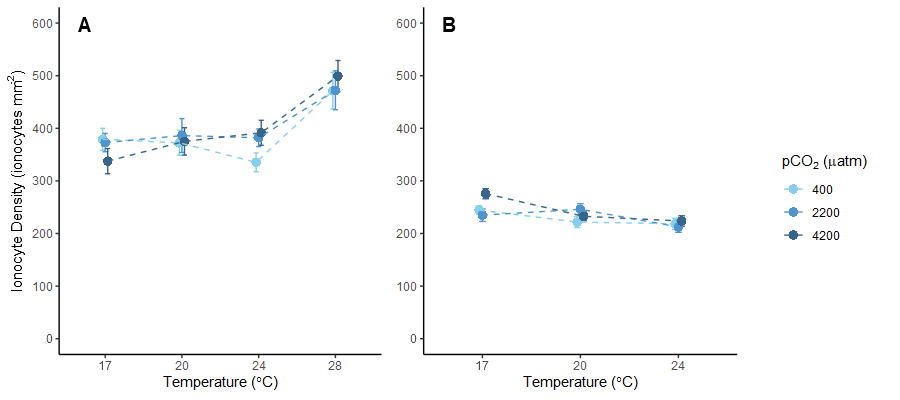
Temperature and pCO2 had a significant interactive effect on embryo yolk sac (linear regression, *F*(3,280)=23.94, *p* < 0.01) and body (linear regression, *F*(3,280)=16.3, *p* < 0.001) skin surface ionocyte density. Ionocyte density decreased with increasing temperature, with 28°C embryos having significantly lower ionocyte densities than the lower temperature treatments across all pCO2 levels (EMM, *p*<0.05). The greatest mean yolk sac ionocyte density occurred in the highest pCO2 and 17°C treatment combination, while body 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 body ionocyte density at 4200 µatm is only 61.9% greater than the density at 400 µatm. In both cases it appears that high CO2 increases the temperature-dependence of ionocyte density, making it increase more sharply as temperature decreases. Within the 4200 µatm pCO2 level, yolk sac ionocyte density is significantly lower at 24°C than at 17°C (EMM, *p*<0.05). This trend is absent in body ionocytes, however, illustrating greater response of yolk sac ionocytes to elevated pCO2.



**Figure 2.** Embryo yolk sac (A) and body (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).

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), but not those of 10-mm larvae (linear regression, *F*(3,337)=7.946, *p* > 0.05). Newly hatched larvae show the opposite temperature effect of embryos, with ionocyte density increasing with temperature, particularly in the 28°C treatment (Figure 3). However, the 24°C and 400 µatm pCO2 treatment group has slightly but significantly lower ionocyte density than larvae reared at 17 and 20°C (EMM, p<0.05). 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. Three gill arches on each side, with filaments and lamellae, have developed by this stage, and skin surface ionocyte densities decreased across treatments to just over half the density of newly hatched larvae.

Embryos and newly hatched larvae in this study were also used for respirometry measurements (full results reported in Schwemmer et al., 2020). We used Pearson’s correlation test to test whether ionocyte density is correlated with mass-specific routine metabolic rates (RMR) in individuals for which both quantities were measured. In embryos, RMR significantly decreased with 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).



**Figure 3.** Newly hatched larvae (A) and 10-mm larvae (B) ionocyte density means plotted with respect to temperature and pCO2. Error bars show standard error.

**Table S2.** Mean, standard error, sample size, and age at sampling (range across all experiments) for ionocyte density of embryo yolk sac and body skin surface in each treatment.

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

**Table S3.** Mean, standard error, sample size, and age at sampling (range across all experiments) for ionocyte density of newly hatched and 10-mm larvae in each treatment.

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

**Table 3.** 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 (body)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** |
| Newly hatched larvae | 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

**Discussion**

Across four experiments exposing *M. menidia* embryos and larvae to pCO2 and temperature treatments, ionocyte density was primarily influenced by temperature with some low magnitude CO2 effects. In embryos sampled about one day before hatching, high CO2 increased temperature-sensitivity of ionocyte density, 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 CO2 to promote H+ removal and HCO3- accumulation, particularly in the yolk sac area. Previous studies support both an increase in ionocyte density (Kwan et al., 2021) and accumulation of HCO3- (Kwan and Tresguerres, 2022) in fish early life stages under acidified conditions, counter to the idea that incomplete development of organs would prevent substantial ion transport in response to high CO2. 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 CO2 (Dahlke et al., 2020). The CO2 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.

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. We also 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 rather an indirect effect of temperature through its influence on developmental rate. Although we set our sampling timepoints to account for effects of temperature on development rates – sampling 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 of larvae at hatching 28°C relative to larvae reared at 24°C (Murray and Baumann, 2018). 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. The large impact of 28°C compared to differences among the other temperature treatments aligns with a pattern of quick hatching time, small larval size, and high metabolism in *M. menidia* from the same set of experiments (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, the survival of these 28°C fish regardless of CO2 treatment (Murray and Baumann, 2018) is evidence that the existing 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 newly hatched larvae. 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 if it is the plasticity may become centralized in the gills as this will have more long-term utility and more direct proximity to blood vessels. 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. [I will have more to add about gills when finish analyzing].

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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