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

Teresa G. Schwemmer, Maya Peña-Lobel, Megan A. Hahn, Nolwenn M. Dheilly, Janet A. Nye

To be submitted to: *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*

**Abstract**

**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 et al., 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 intracellular 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 paragraph should hone in on why we got interested in looking at ionocytes. Give examples of mechanistic/physiological papers, such as GABA/neurological stuff behind behavior effects, metabolism and OCLTT/other theories (tie in the fact that we are interested in warming x CO2?), Heuer and Grosell 2014, Esbaugh 2018. It can reveal how/if energy is being spent, implications for energy budget. Also can help us understand how current fluctuations they experience may confer tolerance/make them more adaptable.

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 low DO and a synergistic negative effect of static high CO2 and low DO (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).

Rodriguez-Dominguez et al 2018

Fish maintain ionic homeostasis, both for osmoregulation and acid-base balance, by transporting ions in and out of the body with active and passive transport.

Explain gills, ionocytes, and the enzymes involved for menidia.

Describe existing research on OA and these functions – e.g. Dahlke et al., 2020

Explain differences between yolk sac, skin surface, and gill ionocytes – e.g. Thermes et al., 2010.

The Cattano et al (2018) paper mentions effects on yolk – see if there’s anything relevant to ionocytes/ionic regulation at all.

Gene expression work

Existing gene expression knowledge for OA and fish, especially need to find out if there is any for the genes we used.

Types of genes we used and the enzymes they control.

Background on the methods – check Maya’s regeneron paper for any crucial background details.

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 and gene expression of seven ionoregulatory enzymes. 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. 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 this effect would be more pronounced in earliest life stages, as we have reason to believe more energy was expended then. We expected that the effects on gene expression would vary for each gene based on the type of enzyme it regulates. Specifically, we hypothesized that NHE and VHA would be downregulated due to a lower pH gradient in acidified water, and that NBC would be upregulated and NKCC1/2 downregulated under high pCO2 based on previous research (Liu et al., 2016).

**Methods**

**Treatments, animals, and sampling**

Collection, rearing, and harvesting of live Atlantic silverside (*Menidia 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 ∼120 h 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, given 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.

[describe how treatments were achieved – respirometry paper]. Target treatment levels and spawning dates for each experiment and life stages sampled for each response variable are summarized in Figure 1. Measured seawater conditions are shown 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).

**Gene expression**

*Determination of* M. menidia *Gene Sequences*

At the time of this experiment, the genome of *M. menidia* had not been sequenced, although it later was (Tigano et al., 2021) and only an extremely limited number of gene sequences from this organism had been determined and published (CITATION/EXAMPLES?). However, the raw transcriptome had previously been generated, and the Transcriptome Shotgun Assembly was deposited in Genbank (accession # GEVY00000000; Therkildsen et al., 2016).

A list of effector proteins that play a role in regulation of acidity and salt balance in fish was generated to study possible molecular adaptations to environmental variables. Chosen genes are NKAα1, VHA1, NBC1, NHE1, NHE3, NKCC1l, NKCC1s, NKCC2, and NCC-like. In addition, elongation factor 1-alpha 1 (ef-1a), glyceraldehyde 3-phosphate dehydrogenase (gapdh), 40S ribosomal protein S4 (rps4), 60S ribosomal protein L13 (rpl13), ADP-Ribosylation Factor Guanine Nucleotide-Exchange Factor 1 (arfgef1), 40S ribosomal protein S8 (rps8), and ubiquitin - 40S ribosomal protein S27a (rps27a) sequences were selected as candidate reference genes. Gene sequences were available for either the zebrafish model species *Danio rerio* or the Japanese rice fish medaka *Oryzias latipes* in GenBank [1]. We used the Basic Local Alignment Search Tool (BLAST) [21] of nucleotide sequences (blastn) to extract *M. menidia* cDNA sequences. When needed, contiguous sequences were manually assembled to obtain full cDNA sequence. Transcripts were then translated into proteins before conducting a reciprocal BLAST search against the non-redundant database (tblastn).

*Primer Design and Analysis*

Primer Express 3.0.1 software (Applied Biosystems) was used to design PCR primers to amplify *M. menidia* cDNA. An internal algorithm ranked candidate sequences according to the most likely predicted success and the highest ranking pairs were chosen for further work. SeaView 4.0 [7] was used to align primer sequences to check for overlaps or other obvious issues. Efficiency and linear dynamic range of primer pairs were calculated using a dilution series ranging from to 1 to 1/14580 that encompassed the entire range of template concentration in our samples. The specificity of the primer pairs was confirmed by melting curve analysis. After two rounds of testing, it was determined that reference genes ef-1a, gapdh, rps4, rpl13, and interest genes NKCC1s, and NCC-like would be omitted from the study. The primers used for real-time quantitative PCR are shown in Table 1.

*RNA extraction*

RNA was extracted from all 234 control and experimental samples of larvae (90 from Experiment 3, performed in 2018; and 144 from Experiment 2, performed in 2019) using the QIAGEN RNeasy MiniKit and following manufacturer instructions. Frozen vials containing larvae were placed in an ice bath, 350 μL of Qiagen RLT lysis buffer was added, and the sample was manually homogenized with a mini-pestle. RNA quality was confirmed by 1% TBE gel electrophoresis. Amount of extracted RNA for each sample was determined by Thermo Scientific NanoDrop 2000/2000c. Many samples were found to contain guanidine isothiocyante; thus, samples were additionally processed with the Ambion TURBO DNA-free Kit Treatment and Removal Reagents. Some samples were diluted or concentrated depending on the initial amount or RNA as determined by the NanoDrop.

*Real-time quantitative PCR*

One microgram of total DNase-treated RNA was used as a template for reverse transcriptase (RT) reactions using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR. Real-time qPCR was performed with an Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System in a 10 μl reaction volume using 1/20 diluted cDNA template, 200 nM forward and reverse primers and 5 μl of Invitrogen™ Express SYBR GreenER qPCR Supermix with premixed ROX. Each tissue sampled from 5 individual fish for each treatment condition was run in duplicate for each gene (n =10). Thermal cycling conditions were initiated at 95°C for 10 min to activate Taq polymerase; followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. Amplification was followed by a melting curve analysis to confirm the specificity of the PCR reactions. Quantitative PCR data were analyzed using the comparative quantitation method, and the relative expression level of each gene was calculated from 2−ΔCt where ΔCt equals Cycle Threshold of Gene of Interest minus Cycle Threshold of Mean Reference Genes.

*Choice of reference gene*

Quantitative RT-PCR was performed to identify the best candidate control genes. Ct values were normalized across samples by calculating the ratio of the Ct value to the mean of the Ct value obtained for all genes for a given sample and logarithmically transformed (base 10). The Coefficient of Variation was calculated for each gene using normalized Ct values.

**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**

*Gene Expression*

Ct values for technical replicates were condensed, with outliers sorted out. The geometric mean of Ct values for the three reference genes was calculated. In order to obtain the ΔΔCt value, the Ct values of the geometric means were compared to the ΔCt values of the gene of interest, using 20°C and 8.2 pH as the control group. Relative gene expression was then calculated using 2−ΔΔCt data that had been transformed by Tukey’s Ladder of Powers. Normality of the data distribution was tested with Bartlett and Shapiro-Wilk normality tests. Then, statistical analyses by ANOVA and linear mixed effect models were performed using R programming.

*Ionocyte density*

Statistical analysis of the ionocyte density data was conducted using linear 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. A significance threshold of α=0.05 was used for all statistical tests. All statistical tests were performed in R statistical software (v4.0.2) using the ‘stats’, ‘emmeans’, and ‘olsrr’ packages.

**Results**

**Gene expression**

**Ionocyte density**

Embryos and newly hatched larvae had similar maximum ionocyte densities, 662.0 and 703.2 ionocytes mm-2, respectively, while 10-mm larvae had lower ionocyte densities, with a maximum of 424 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. Therefore, to meet the assumptions of normality and homoskedasticity the embryo data were square-root transformed in the linear model.

Temperature and pCO2 had a significant interactive effect on embryo yolk sac (linear regression, p < 0.01) and body (linear regression, p < 0.001) skin surface ionocyte density. Ionocyte density decreased with increasing temperature, and pCO2 increased ionocyte density at 17°C but not at the higher temperatures. The yolk sac ionocyte density was more strongly affected by high pCO2 at 17°C than the body ionocytes were. 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.

Temperature and pCO2 had a significant interactive effect on ionocyte densities of newly hatched larvae (linear regression, p < 0.01), but not those of 10-mm larvae (linear regression, p = 0.08). For 10-mm larvae, however, there was a significant effect of pCO2 on ionocyte density (p = 0.049). Newly hatched larvae show the opposite temperature effect of embryos, with ionocyte density increasing with temperature (Figure 3). Differences between pCO2 levels are small and temperature-dependent. At 17°C, ionocyte density decreases with high pCO2 but at 24°C it increases with pCO2. Like the embryos, temperature-dependence overall increases with pCO2, but in this case greater temperature-dependence means increasing ionocyte density with temperature, rather than decreasing. At the 10-mm sampling the larval ionocyte densities are no longer affected by temperature, and differences between pCO2 treatments are small. The most substantial difference is that at 17°C ionocyte density at 4200 µatm pCO2 is 12.7% and 17.3% greater than at 400 µatm and 2200 µatm, respectively.

**Chart, line chart, scatter chart

Description automatically generated**

**Figure 2.** Embryo yolk sac and body ionocyte density data plotted with respect to temperature and grouped by pCO2 treatment. Linear regressions plotted to each pCO2 group to visualize interaction effect. **Chart, line chart

Description automatically generated**

**Figure 2.** Embryo yolk sac and body ionocyte density means plotted with respect to temperature and pCO2. Error bars are standard error.

**Chart

Description automatically generatedFigure 3.** Newly hatched and 10-mm larvae ionocyte density data plotted with respect to temperature and grouped by pCO2 treatment. Linear regressions plotted to each pCO2 group to visualize interaction in 1dph larvae and pCO2 effect in 10-mm larvae. *Note different y-axis scales.*

**Chart, scatter chart

Description automatically generatedFigure 3.** Newly hatched and 10-mm larvae ionocyte density means plotted with respect to temperature and pCO2. Error bars are standard error. *Note different y-axis scales.*

**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 | 65 | 108.8 | 12.9 |
| 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 | 67 | 340.8 | 14.8 |
| 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 | 39 | 223.8 | 11.3 |
| 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)\* | pCO2 | 3.9e-3 | 1.3-3 | 5.9 | **0.0026** |
| Temp | -0.38 | 0.14 | -2.7 | **0.0072** |
| pCO2 x Temp | -1.5e-4 | 5.7e-5 | -2.7 | **0.0078** |
| Embryo (body)\* | pCO2 | 4.6e-3 | 1.2e-3 | 3.7 | **0.00023** |
| Temp | -9.7e-2 | 0.13 | -0.73 | 0.47 |
| pCO2 x Temp | -2.0e-4 | 5.4e-5 | -3.6 | **0.00034** |
| Newly hatched larvae | pCO2 | -5.7e-2 | 2.0e-2 | -2.8 | **0.0048** |
| Temp | -0.42 | 2.4 | -0.18 | 0.86 |
| pCO2 x Temp | 2.8e-3 | 9.0e-4 | 3.1 | **0.0019** |
| 10-mm larvae | pCO2 | 2.9e-2 | 1.5e-2 | 2.0 | **0.049** |
| Temp | -1.8 | 1.9 | -0.95 | 0.34 |
| pCO2 x Temp | -1.2e-3 | 7.0e-4 | -1.7 | 0.084 |

\*Square-root transformed

**Discussion**

Outline of discussion points:

Ionocyte density data patterns: embryo data were distributed differently than larvae because they are developing, so most of them have lower densities while a smaller number of presumably more advanced/developed embryos have densities approaching those of the newly hatched larvae. By the time they reached 10-mm SL skin-surface densities are decreasing (means and maxima), likely because gills are better developed – they have ionocytes and more surface area so skin ionocytes are no longer needed.

Yolk differences from rest of skin surface: existing data for this, and possible explanations or meaning for the yolk epithelial ionocytes to be more responsive to environmental conditions and ionoregulatory demands than the rest of the body.

Recommendations for future research and shortcomings of this study

Study how combinations of CO2 and salinity levels affect fish.

Enzyme activity

Unbalanced data/lack of true replication

Results notes to consider for discussion/further analysis:

Embryos: significant interaction between CO2 and temperature. Ionocyte density decreases slightly with increasing temperature, but high CO2 intensifies the temperature-dependence. Ionocyte density is highest when low temperature and high CO2 are combined, and this response is stronger in the yolk sac skin but also present in the rest of the body. Ionocyte density is lowest at the highest temperature (28°C), and CO2 has little to no effect at this temperature.

Caveats: transformation required to meet assumptions of linear model; there may be outliers; Experiment 1 did not have 4200 µatm pCO2 treatment so there are two experiments influencing the 400 and 2200 µatm results, but only one influencing 4200 µatm (when looking at 17-24°C). This means the 17°C x 4200 µatm pCO2 effect may be artificially magnified by not having a second replicate.

Larvae, 1dph: There is a significant interaction between temperature and pCO2. Ionocyte density increases with temperature, mainly in a sharp increase in ionocytes at 28°C compared to the other temperatures. There is no pCO2 effect except at 17°C (front only), in which ionocyte density decreases slightly as pCO2 increases. This is the opposite effect of embryos for both temperature and pCO2, and the data are much more tightly distributed in larvae than in embryos.

Caveats: when using treatments as quantitative variables there is a significant interaction, and the significant effect is there for front and total but not when looking at back (trunk/tail) only.

The data do not seem to require transformation. Why are the embryo data more heavily skewed than the larvae? That dataset has a long positive tail – there are mostly lower ionocyte densities but several in each treatment group that are higher. Are they more developed and closer to hatching? These seem not to be outliers because there are so many of them and it is gradual, not a spike of a few that are extremely high compared to the majority – it is just the distribution of the data. Is this zero-inflation? Or near-zero inflation? There are so many that have <100 ionocytes/mm2, but also a good number that have >500 ionocytes/mm2.

I think justify the transformation of only the embryos by saying that the distribution is different for embryos and that it biologically makes sense (they are still developing their embryos and small differences in developmental stage could mean great differences in ionocyte development).

Larvae, 10mm: LMER analysis shows significant temperature effect, and significant interaction for back section and whole body. Continuous analysis shows no effect on front, significant CO2 and temp interaction for back, and significant CO2 effect (with p=0.08 for interaction) for total body. Overall ionocyte density decreases slightly with increasing temperature, and CO2 effects flip flop for different temperatures but are small. Overall ionocyte densities are lower than for embryos and 1dph larvae (makes sense as gills develop). CO2 effect seems to be that at 17°C the ionocyte density is highest at 4200 µatm. CO2 effects are smaller/nonexistent at other temperatures.

Caveats: LMER analysis has singular fit for back half; no significant experiment effect (does that mean I can remove it as the random effect and use lm+anova?)

Need to look at post hoc comparisons for this – CO2 effect seems very small.

**Acknowledgements**

The authors would like to thank Samantha Murphy, Amanda Ackermann, Yuchen Zhang, Jason Chan, Jeffrey Casey, Nicole Margolis, and Delphine Mossman for their assistance with staining and image analysis. We would also like to thank Nita Wong for her technical assistance with the gene expression work. We also acknowledge the Developmental Studies Hybridoma Bank (DSHB) at University of Iowa and the hybridoma contributor D. M. Fambrough for the a5 antibody used in ionocyte staining, as well as Stephen McCormick of United States Geological Survey and Karla Daniels of DSHB for their helpful advice during the development of the ionocyte staining and analysis protocols.

**References**

Baumann, H., Cross, E. L., and Murray, C. S. 2018. Robust quantification of fish early life CO2 sensitivities via serial experimentation. *Biol. Lett.* 14: 20180408. doi:10.1098/rsbl.2018.0408

Bengtson, D. A., Barkman, R. C., and Berry, W. J., 1987. Relationships between maternal size, egg diameter, time of spawning season, temperature, and length at hatch of Atlantic silverside, *Menidia menidia*. *J. Fish. Biol.*, 31: 697-704.

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., and Sayers, E. W. 2017. GenBank. *Nucleic Acids Res.*, 45(D1): D37-D42. doi:10.1093/nar/gkw1070

Cattano, C., Claudet, J., Domenici, P., and Milazzo, M. 2018. Living in a high CO2 world: a global meta-analysis shows multiple trait-mediated fish responses to ocean acidification. *Ecol. Monogr.*, 88(3): 320-335.

Clark, T. D., Raby, G. D., Roche, D. G., Binning, S. A., Speers-Roesch, B., Jutfelt, F., and Sundin, J. 2020. Ocean acidification does not impair the behavior of coral reef fishes. *Nature*, 577: 370-375. doi:10.1038/s41586-019-1903-y

Cottingham, K. L., Lennon, J. T., and Brown, B. L. 2005. Knowing when to draw the line: designing more informative ecological experiments. *Front. Ecol. Environ.*, 3: 145-152. doi:10.1890/1540-9295(2005)003[0145:KWTDTL]2.0.CO;2

Couturier, C. S., Stecyk, J. A. W., Rummer, J. L., Munday, P. L., and Nilsson, G. E. 2013. Species-specific effects of near-future CO2 on the respiratory performance of two tropical prey fish and their predator. *Comp. Biochem. PHysiol. A*, 166: 482-489. doi:10/1016/j.cbpa.2013.07.025

Cross, E. L., Murray, C. S., and Baumann, H. 2019. Diel and tidal *p*CO2 x O2 fluctuations provide physiological refuge to early life stages of a coastal forage fish. *Sci. Rep.*, 9: 18146. doi:10.1038/s41598-019-53930-8

Dixon, R. L., Grecay, P. A., and Targett, T. E. 2017. Responses of juvenile Atlantic silverside, striped killifish, mummichog, and striped bass to acute hypoxia and acidification: Aquatic surface respiration and survival. *J. Exp. Mar. Biol. Ecol.*, 493: 20-30.

Espinel-Velasco, N., Hoffmann, L., Agüera, A., Byrne, M., Dupont, S., Uthicke, S., Webster, N. S., and Lamare, M. 2018. Effects of ocean acidification on the settlement and metamorphosis of marine invertebrate and fish larvae: a review. *Mar. Ecol. Prog. Ser.*, 606: 237-257. https://doi.org/10.3354/meps12754

Gouy, M., Guindon, S., and Gascuel, O. 2010. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol. Biol. Evol.*, 27(2): 221-224. doi:10.1093/molbev/msp259

Gunderson, A. R., Armstrong, E. J., and Stillman, J. H. 2016. Multiple stressors in a changing world: the need for an improved perspective on physiological responses to the dynamic marine environment. *Annu. Rev. Mar. Sci.*, 8: 357-378. doi:10/1146/annurev-marine-122414-033953

Havenhand, J., Dupont, S., and Quinn, G. P. 2010. Designing ocean acidification experiments to maximize inference. In *Guide to Best Practices for Ocean Acidification Research and Data Reporting* (ed. U. Riebesell, V. J. Fabry, L. Hansson and J.-P. Gattuso), pp. 67-136. Luxembourg: Publications Office of the European Union Luxembourg.

Hsu, S.-M., Raine, L., and Fanger, H. 1981. Use of Avidin-Biotin-Peroxidase Complex (ABC) in Immunoperoxidase Techniques: A Comparison between ABC and Unlabeled Antibody (PAP) Procedures. *J. Histochem. Cytochem.*, 29(4): 577-580.

Hurlbert, S. H. 2004. On misinterpretations of pseudoreplication and related matters: a reply to Oksanen. *Oikos*, 104: 591-597. doi:10.1111/j.0030-1299.2004.12752.x

Jarrold, M. D. and Munday, P. L. 2018. Diel CO2 cycles do not modify juvenile growth, survival and otolith development in two coral reef fish under ocean acidification. *Mar. Biol.*, 165: 49. https://doi.org/10.1007/s00227-018-3311-5

Liu, S.-T., Horng, J.-L., Chen, P.-Y., Hwang, P.-P., and Lin, L.-Y. 2016. Salt secretion is linked to acid-base regulation of ionocytes in seawater acclimated medaka: new insights into the salt-secreting mechanism. *Sci. Rep.*, 6: 31433. doi:10/1038/srep31433

Middaugh. D. P., Hemmer, M. J., and Goodman, L. 1987. Methods for Spawning, Culturing and Conducting Toxicity-Tests with Early Life Stages of four Atherinid Fishes: The Inland Silverside, *Menidia beryllina*, Atlantic silverside, *M. Menidia*, Tidewater silverside, *M. peninsula*, and California grunion, *Leuresthes tenuis*. Gulf Breeze, FL: United States Environmental Protection Agency.

Murray, C. S. and Baumann, H. 2018. You Better Repeat It: Complex CO2 x Temperature Effects in Atlantic Silverside Offspring Revealed by Serial Experimentation. *Diversity*, 10: 69. doi:10.3390/d10030069.

Murray, C. S., Malvezzi, A., Gobbler, C. J., and Baumann, H. 2014. Offspring sensitivity to ocean acidification changes seasonally in a coastal marine fish. *Mar. Ecol. Prog. Ser.*, 504: 1-11. doi:10.3354/meps10791

NCBI Resource Coordinators. 2013. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res.*, 41(D1): D8-D20. doi:10.1093/nar/gks1189

Rummer, J. L., Stecyk, J. A. W., Couturier, C. S., Watson, S.-A., Nilsson, G. E., and Munday, P. L. 2013. Elevated CO2 enhances aerobic scope of a coral reef fish. *Conserv. Physiol.*, 1: cot023. doi:10.1093/conphys/cot023

Schultz, E. T., Conover, D. O., and Ehtisham, A. 1998. The dead of winter: size-dependent variation and genetic differences in seasonal mortality among Atlantic silverside (Atherinidae: *Menidia menidia*) from different latitudes. *Can. J. Fish. Squat. Sci.*, 55: 1149-1157.

Schwemmer, T. G., Baumann, H., Murray, C. S., Molina, A. I., and 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

Sundin, J., Amcoff, M., Mateos-González, F., Raby, G. D., and Clark, T. D. 2019. Long-term acclimation to near-future ocean acidification has negligible effects on energetic attributes in a juvenile coral reef fish. *Oecologia*, 190: 689-702. https://doi.org/10.1007/s00442-019-04430-z

Therkildsen NO, Palumbi SR, Baumann H. Menidia menidia transcriptome. Genbank Direct Submission. 31-JUL-2016. Accession number GEVY00000000.1 https://www.ncbi.nlm.nih.gov/nuccore/GEVY00000000