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Elevated pCO_2 and hypoxia alter the acid–base regulation of developing sheepshead minnows *Cyprinodon variegatus*

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ABSTRACT: Lowered dissolved oxygen and pH levels are 2 environmental variables that concomitantly change in an estuarine environment and both are exacerbated by nutrient pollution and subsequent eutrophication. To better understand how estuarine residents compensate for daily fluctuations in these environmental variables, the interactive effects of elevated partial pressure of CO_2 (pCO_2) and hypoxia were assessed in developing sheepshead minnows *Cyprinodon variegatus* using a 2 by 2 factorial design over a 42 d exposure. Embryos were exposed to either acidic (pCO_2: ~2000 µatm), hypoxic (reduced dissolved oxygen, ~2 mg l^{-1}), or combined acidic and hypoxic conditions and monitored for development, hatch rate, and survival. Measurements of anaerobic pathway use, oxidative stress, and acid–base regulatory enzymes were evaluated at 3 life stages (embryo, larva, and juvenile) to discern if and how fish compensate for these stressors during development. The combination of elevated pCO_2 and hypoxia delayed hatching in embryos but did not impact survival. Neither elevated pCO_2, hypoxia, nor the combination of the stressors elicited an increase in anaerobic metabolic pathways or impacted oxidative stress of juvenile fish. Measurements of enzymes related to acid–base regulation were elevated in all 3 treatments in larval fish. Elevated carbonic anhydrase activity was observed in the multi-stress treatment in embryos and larval fish, but not in juvenile fish. These results show that developing sheepshead minnows can compensate for acidified and hypoxic waters.

KEY WORDS: *Cyprinodon variegatus* · Elevated pCO_2 · Hypoxia · Oxidative stress · Acid–base balance

1. INTRODUCTION

Coastal ecosystems such as estuaries, saltmarshes, and mangroves naturally experience wide ranges of acidity and have the capacity to temper the physical and chemical conditions of the environment (Gutiérrez et al. 2011). The carbonate chemistry parameters (pH, partial pressure of CO_2 [pCO_2], total alkalinity, and dissolved inorganic carbon) of estuarine environments are driven largely by processes such as primary production, respiration, and calcification. Greater changes in pH levels occur frequently in these environments through these processes compared to acidification caused by atmospheric deposition alone. Additionally, tidal influence, river input, and residence time influence the variability of pH in these ecosystems (Borges & Gypens 2010, Cai et al. 2011, Duarte et al. 2013, Baumann et al. 2015, Hendriks et al. 2015). Furthermore, the influence of nutrient-enhanced acidification in coastal systems, i.e. a decrease in pH caused by the enhanced release of CO_2 from microbes feeding upon phytoplankton and organic matter, has been widely documented (Borges & Gypens 2010, Doney 2010, Cai et al. 2011, Hof-

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mann et al. 2011, Duarte et al. 2013). Studies investigating the impacts of coastal acidification on estuarine organisms have mainly focused on organismal survival, growth, and embryonic hatch rates, whereas the physiological responses and potential compensation mechanisms of these organisms to environmental perturbations remain largely unexplored.

Dissolved oxygen (DO) and pH levels naturally change concomitantly in coastal systems, and anthropogenic influences can exacerbate this interaction (Rabalais et al. 2009, Howarth et al. 2011, Baumann et al. 2015). Organismal and microbial respiration lower DO levels, creating hypoxic zones, that are coupled with the decline in pH caused by the release of CO_2 during respiration (Cochran & Burnett 1996, Pörtner et al. 2005, Melzner et al. 2013, Baumann et al. 2015). Hypoxic events that are linked to increased levels of CO_2 and dissolved inorganic carbon can reduce the pH of coastal waters by 0.5 pH units or more (Howarth et al. 2011). Nutrient pollution can cause eutrophication and may exacerbate hypoxia and pH declines (Rabalais et al. 2009, Borges & Gypens 2010, Howarth et al. 2011). These changes in DO and pH can occur over a matter of hours to days (Baumann et al. 2015, Miller et al. 2016) and often occur more frequently over summer months when temperatures are highest (Rabalais et al. 2009, Cai et al. 2011, Baumann et al. 2015). Coastal estuaries have been shown to experience diel changes in pH of over 1 unit a day, and fluctuations in DO up to 6 mg l^{-1} over the course of a day (Baumann et al. 2015, Miller et al. 2016). Human activities that amplify acidic conditions and the subsequent hypoxic zones in coastal waters are projected to increase over the coming decades (IPCC 2013), and extreme decreases in DO and pH levels currently seen in summer months are projected to become more frequent (Rabalais et al. 2009, Duarte et al. 2013).

Organisms that reside either permanently or seasonally in an estuarine environment are adapted to dynamic fluctuations in environmental variables including temperature, salinity, and, in some cases, DO (Jensen et al. 1993). These adaptations allow estuaries to serve as a refuge from predators that may be less able to tolerate environmental extremes. Therefore, organisms that reside in an estuary may possess the capacity to physiologically adjust to changes in pCO_2 and hypoxia levels under projected eutrophication scenarios. However, predicting the interactive effects of hypoxia and elevated pCO_2 in estuarine organisms can be challenging because of the opposing physiological responses these stressors can elicit. Hypoxia typically causes an increase in ventilation and/or metabolic depression, and in some cases, causes organisms to use anaerobic energy sources (Hochachka 1986, Burnett & Stickle 2001, Mandic et al. 2009, Ekau et al. 2010). In teleost fishes, elevated pCO_2 exposure typically causes an alteration of blood CO_2 chemistry, followed by respiratory acidosis, which in turn is alleviated by retaining additional HCO_3^- in the blood (Evans et al. 2005, Perry & Gilmour 2006, Esbaugh et al. 2016, Esbaugh 2018). The additional bicarbonate is sequestered by direct transfer of this base for an acid (H^+) across the gill, kidney, and intestinal epithelia (Heisler 1984, Karnaky 1986), by way of Na^+/K^+ ATP-ase (NKA) pumps, and catalyzation of HCO_3^- and H^+ from water and CO_2 via carbonic anhydrase (Claiborne et al. 2002, Deigweiher et al. 2008). Additionally, both stressors have been known to increase the oxidative stress within an organism, and subsequently can enhance damage from reactive oxygen species (Abele & Puntarulo 2004). The combination of elevated pCO_2 and hypoxia can affect organisms either additively or synergistically, increasing physiological stress and decreasing organism survival (DePasquale et al. 2015, Gobler & Baumann 2016).

Sheepshead minnows *Cyprinodon variegatus* Lacépède, 1803 are teleost fish that permanently inhabit estuaries and can withstand a wide array of environmental parameters. These fish can tolerate temperatures ranging from −1.9 to 45°C, salinities from 0 to >125‰, and DO levels below 1 mg l^{-1} (see Nordlie 2006 for review). Sheepshead minnows occupy an intermediate trophic step between zooplankton and larger predators (Pikitch et al. 2014), functioning as both predator and prey, making them an important link in estuarine food webs. Given the range of environmental conditions these fish can tolerate, sheepshead minnows were selected as a model species to determine how the interactive effects of hypoxia and acidification affect estuarine organism development and physiological homeostasis.

We tested hypoxia (low DO, ~2 mg l^{-1}) and acidification (elevated pCO_2, ~2000 µatm) occasionally seen during summer months in estuaries in the northern Gulf of Mexico (Cai et al. 2011, IPCC 2013, Baumann et al. 2015) (www.nerrsdata.org; accessed August 1, 2018). To investigate the interactive effects of acidification and hypoxia on development, we exposed fish over a 42 d period that included embryo (<24 h old) through juvenile life stages. For each life stage, we investigated how treatment exposure affected acid–base regulatory enzymes (carbonic anhydrase and NKA activities), anaerobic pathway use (lactate

dehydrogenase activity), and the oxidative stress response (protein carbonyl formation, superoxide dismutase, and catalase activities) of fish.

2. MATERIALS AND METHODS

2.1. Sheepshead minnow culture

Adult sheepshead minnows were collected from local saltmarsh swales in Santa Rosa Sound, northwest Florida, USA, transported to the laboratory, and quarantined for 1 mo. Culture waters were continuously renewed from a water intake in Santa Rosa Sound and maintained at approximately 25 ± 0.5°C by titanium heaters. Salinity was maintained at 20 ± 1‰ by the addition of fresh water or high-salinity seawater. DO (mg l^{-1}) and pH levels were checked once a month in culture tanks (average DO of ~7 mg l^{-1}/95% air saturation and pH ~7.9), and these tanks were monitored for DO and pH daily for 1 wk before experiments were started. Fish were fed Tetramin® flake food and frozen brine shrimp. At the end of the quarantine period, fish were spawned by placing 2 males and 3 females into a 6 mm mesh-bottom spawning chamber (20.5 cm × 26.5 cm × 22.5 cm) positioned atop a separate 450 µm mesh screen to which released eggs adhered. Fertilized eggs were collected each morning and hatched in aquaria. This F_1 generation was reared in ambient laboratory conditions, then spawned as described above to initiate the stressor exposures (F_2 generation). Due to a low number of available embryos, the first experiment (herein 'larval-juvenile') measured effects only on the larval and juvenile stages, although embryos were reared in treatment waters. A second experiment ('embryo-only') monitored only the embryonic stage. Each experiment was performed separately, using embryos from the same parental stock of fish.

2.2. Experimental design

The effects of elevated pCO_2 and hypoxia were investigated over a 42 d exposure using a 2 × 2 factorial experimental design. The exposure system was comprised of 4 polymer fiberglass headboxes (250 l, Aquatic Ecosystems) that served as gas mixing reservoirs for treatment waters. The system was maintained at 25°C and 20‰ salinity as described above, supplied with the same Santa Rosa Sound water as culture tanks. Each headbox gravity-fed 5 flow-through experimental chambers that served as treatment replicates. Each experimental chamber (7.8 l) was constructed of glass and housed 20 organisms in glass mason jars with 300 µm mesh bottoms to allow for water and gas exchange across embryos. Experimental chambers were randomly distributed within a water bath. Flow to each chamber (300 ml min^{-1}) was independently controlled and monitored 3 times a week. Target DO levels of 6–7 mg l^{-1} (ambient, ~95% air saturation) and 2 mg l^{-1} (low DO; hypoxic, ~27–28% air saturation), and target pCO_2 levels of 300–400 µatm (ambient) and 2000 µatm (elevated) were used to create the following 4 treatments: (1) ambient conditions, (2) ambient DO/elevated pCO_2, (3) low DO/ambient pCO_2, and (4) low DO/elevated pCO_2.

We used a pCO_2 generation system first described by Fangue et al. (2010) to create elevated pCO_2 and low DO treatments. To create elevated pCO_2 levels, air was stripped of moisture (using Drierite®) and carbon dioxide (using Sodasorb®) and blended with pure CO_2 using digital mass flow controllers (Aalborg Instruments). The air/CO_2 blend was infused into headboxes via Venturi injectors. For the low DO treatments, nitrogen gas was metered using mass flow controllers and bubbled using Venturi injectors to displace oxygen. Air without nitrogen or CO_2 addition was bubbled with a Venturi injector into the control treatment headbox. Headboxes and experimental chambers were set up at least 24 h prior to each test to ensure DO and pCO_2 were at target levels before organisms were loaded into chambers.

Water samples were collected twice a week from experimental chambers at the same time each morning from 2 randomly selected chambers which served as replicates for carbonate chemistry measurements for each treatment. The pH from each chamber was measured using the m-cresol spectrophotometric method (Dickson et al. 2007: SOP 6b; UV-Vis 1700, Shimadzu) adjusted for lower salinities (Hammer et al. 2014). Total alkalinity (potentiometric titration; Dickson et al. 2007: SOP 3b) was measured and used to evaluate carbonate chemistry. Alkalinity titrations were verified using certified reference materials (provided by Andrew Dickson, Scripps Institution of Oceanography), and all samples were run on a Metrohm Titrando 905 titrator. pCO_2 values were calculated using the program CO2Calc and using the CO_2 constants from Mehrbach et al. (1973) refit by Dickson & Millero (1987). Measured pH and total alkalinity values were combined with measurements of DO and temperature (PreSens FIBOX 4 DO meter) as well as salinity (YSI Pro2030) to assess experimental DO and pCO_2 levels. Mean water quality parame-

ters measured over the course of the experiments for each treatment are shown in Table 1.

2.3. Fish condition

To determine if treatments had any effect on fish condition and growth, standard length (mm) and weight (g) were measured on all juvenile fish at the end of the experiment. Fulton's condition index, *K*, was calculated using the following equation (Hopkins 1992):

$$K = \frac{100\,000 \times W}{L^3} \quad (1)$$

where W = fish wet weight (g) and L = standard length (mm).

2.4. Biochemical analyses

To evaluate how fish were responding on a cellular level to treatments, measurements of anaerobic pathways (lactate dehydrogenase activity), acid–base regulatory enzymes (carbonic anhydrase and NKA activity), and oxidative stress and antioxidant response (protein carbonyl formation, total superoxide dismutase activity, catalase activity) were quantified on protein extracts from all 3 life stages. A protein extract was prepared from flash-frozen samples (6 d post fertilization embryos, 14 d post hatch larval fish, and 42 d old juvenile fish) with approximately 100 mg of whole embryos or whole fish. Samples were homogenized with 100 mM phosphate buffer containing a protease inhibitor (cOmplete™ EDTA-free tablets, Roche Diagnostics), then centrifuged for 5 min at 13 000 × *g*. To evaluate NKA activity, samples were homogenized with 50 mM Imidazole buffer (pH = 7.3), then centrifuged for 2 min at 2000 × *g*. Aliquots of supernatants were stored at −20°C until ready for use in individual assays. Protein content of samples was analyzed using the Bradford method for protein quantification (Bradford 1976) on a SpectraMax 190 Microplate reader (Molecular Devices).

2.5. Lactate dehydrogenase activity

Specific lactate dehydrogenase (LDH) activity was quantified using a spectrophotometric method adapted from Yancey & Somero (1978). Total enzyme activity was measured by combining 5 µl of protein extract with 2.00 ml of LDH cocktail (0.2 M imidazole/HCl buffer, pH = 7.00, 5.5 mM nicotinamide adenine dinucleotide [NADH], 2.00 mM sodium pyruvate), and the change of absorbance at 340 nm was measured over 3 min. Absorbances were used to calculate enzyme activity using the following equation:

$$\text{Activity} = S \times \left(\frac{V}{\varepsilon}\right) \quad (2)$$

where S = the slope describing the rate of conversion, V = volume of LDH cocktail buffer added to each cuvette (2 ml), and ε = micromolar extinction coefficient for NADH (6.22 µmol cm^{-1} at 340 nm). All samples were processed in duplicate, and the averaged activities are reported as International Units (IU) per gram fresh tissue weight (gfw).

2.6. Measurement of oxidative stress and antioxidant response

Protein carbonyl (PC) formation was quantified as an indicator of oxidative stress in extracted pro-

Table 1. Mean (±SD) temperature, salinity, dissolved oxygen (DO), pH, total alkalinity (T_A; SW: seawater), and pCO_2 in control (400 µatm; DO: 7 mg l^{-1}), elevated pCO_2 (2000 µatm; DO: 7 mg l^{-1}), low DO (2 mg l^{-1}; pCO_2: 400 µatm), and multi-stress treatments (pCO_2: 2000 µatm; DO: 2 mg l^{-1}) during larval-juvenile and embryo-only experiments

Treatment	Temperature (°C)	Salinity (ppt)	DO (mg l^{-1})	pH (total scale)	T_A (µmol kg^{-1} SW)	pCO_2 (µatm)
Larval-juvenile						
Control	25.00 ± 0.14	20.64 ± 0.38	7.03 ± 0.48	7.97 ± 0.01	1700.97 ± 28.96	422.97 ± 10.23
Elevated pCO_2	25.16 ± 0.24	20.64 ± 0.35	7.01 ± 0.04	7.37 ± 0.05	1704.53 ± 25.81	1923.31 ± 211.24
Low DO	25.14 ± 0.15	20.65 ± 0.33	2.03 ± 0.13	7.94 ± 0.01	1698.58 ± 30.37	457.12 ± 7.61
Multi-stress	25.19 ± 0.21	20.71 ± 0.33	2.04 ± 0.15	7.36 ± 0.02	1705.79 ± 26.76	1940.82 ± 94.11
Embryo-only						
Control	24.88 ± 0.17	20.85 ± 0.43	7.02 ± 0.02	8.00 ± 0.00	1689.29 ± 34.82	390.85 ± 6.20
Elevated pCO_2	24.89 ± 0.14	21.07 ± 0.34	7.02 ± 0.01	7.38 ± 0.07	1704.44 ± 37.42	1888.64 ± 264.90
Low DO	24.88 ± 0.10	21.07 ± 0.34	2.09 ± 0.32	7.97 ± 0.00	1696.82 ± 30.82	421.47 ± 4.59
Multi-stress	24.97 ± 0.11	21.09 ± 0.32	2.00 ± 0.07	7.39 ± 0.05	1710.34 ± 27.48	1843.28 ± 197.304

tein samples using the alkaline method from Mesquita et al. (2014) at 450 nm. The PC concentrations were based on the average absorbances and the micromolar extinction coefficient of 2,4-dinitrophenylhydrazine reagent under alkaline conditions (0.022308 µmol cm^{-1} at 450 nm) and reported as nmol PC (mg protein)$^{-1}$. All samples were processed in duplicate and averaged activities were normalized by gfw.

Total superoxide dismutase (SOD) activity was measured as the first step in the antioxidant pathway in response to oxidative stress. SOD activity was quantified in extracted protein samples at 560 nm using the 96-well spectrophotometric assay described by Ewing & Janero (1995). Each sample was processed in duplicate with averaged activities normalized to units of SOD gfw^{-1}, where 1 unit of SOD is defined as the amount required to give 50% of maximal inhibition of nitro-blue tetrazolium reduction.

Catalase activity was measured as a second measure of changes to antioxidant capacity using the spectrophotometric method of Beers & Sizer (1952). Protein extracts were run in quartz cuvettes due to the low absorption wavelength (240 nm) used to measure enzyme activities. Total catalase activity was calculated using Eq. 2, where S = the slope describing the rate of disappearance of H_2O_2, V = volume of H_2O_2-phosphate buffer added to each cuvette (3 ml), and ε = micromolar extinction coefficient of H_2O_2 (0.0436 µmol cm^{-1} at 240 nm). All samples were processed in duplicate, and averaged activities were normalized by gfw.

2.7. Measurements of acid–base regulatory enzymes

Carbonic anhydrase (CA) activity was measured to discern the degree to which fish were sequestering bicarbonate after exposure to treatments using an assay modified from Capasso et al. (2012). Briefly, ice-cold CO_2-saturated MilliQ® water was quickly added to ice-cold culture water containing either 10 µl of culture water (control) or protein extract (sample). Phenol red (2 mmol) was used as the color indicator, and the time for the solution to change from pink (approximately pH = 8) to a straw-yellow (approximately pH = 6) is inversely related to the amount of CA present in the sample. The catalyzed conversion of CO_2 to bicarbonate was used as a surrogate indicator for CA activity.

All samples were processed in duplicate, and the mean time to achieve the color change indicator was used to calculate the CA activity (in Wilbur-Anderson units [WAU]) of the sample using the following equation:

$$\mathrm{WAU} = \frac{(T_0 - T)}{T} \quad (3)$$

where T_0 = time (in seconds) for an uncatalyzed reaction and T = time (in seconds) for the catalyzed reaction. Each sample was then normalized by gfw.

Total NKA activity was quantified as a second measurement of acid–base regulation after exposure to treatments, using the 96-well assay method of McCormick (1993). All samples were processed in duplicate, and the change of absorbance at 340 nm was measured over 10 min. Absorbances were used to calculate enzyme activity using Eq. 2, where S = the slope describing the rate of conversion, V = volume of NKA cocktail buffer added to each well (0.2 ml), and ε = micromolar extinction coefficient for NADH (6.22 µmol cm^{-1} at 340 nm). All samples were processed in duplicate, and the averaged activities are reported as IU gfw^{-1}.

2.8. Statistical analyses

All data were analyzed using a 2-way ANOVA with treatment (control, elevated pCO_2, hypoxia, or multi-stress) and life stage (embryo, larva, or juvenile) as main level effects, using SigmaPlot statistical software (version 13.0). A post hoc Holm-Sidak multiple comparison procedure was used to explore differences between treatments and life stages if a significant main effect was found. A Shapiro-Wilk test was used to evaluate assumptions of normality, and a Brown-Forsythe test was used to explore equal variance ($p = 0.05$ in both cases). If any data had not met assumptions, they would have been transformed only if the level of significance was close to $p = 0.05$, but there were no cases where this applied.

3. RESULTS

3.1. Hatch rate, survival, and fish condition

Hatching began 4 d after the start of the experiment in all treatments, and >90% of embryos hatched by Day 6 of exposure in the control and elevated pCO_2 treatments in both experiments (Table 2). Limited hatching occurred in the multi-stress treatment on Day 4 in both the larval-juvenile (8%) and embryo-only (5%) exposures. Compared to the controls,

Table 2. Mean survival and calculated percent hatch rates of sheepshead minnow *Cyprinodon variegatus* embryos in response to control, elevated pCO_2, low dissolved oxygen (DO), and multi-stress treatments (see Table 1 for treatment details) at 4, 6, and 8 d post fertilization. *Significant differences from control treatment

Day post fertilization	Control		Elevated pCO_2		Low DO		Multi-stress	
	Survival (%)	Hatch rate (%)	Survival (%)	Hatch rate (%)	Survival (%)	Hatch rate (%)	Survival (%)	Hatch rate (%)
Larval-juvenile								
4	99.5	30.8	100	14.5	100	25.5	99.5	8*
6	99.5	99.5	100	99.5	97.5	89.5	97	79.5*
8	99.5	100	99.5	100	96	99.5	96.5	98.4
Embryo-only								
4	94.6	20	93.1	7.4	97.1	20	94.6	5*
6	92.3	92.6	92	91.4	86.3	65.1	86.3	59.4*
8	92	100	92	100	84	84.6	83.4	85.1

Table 3. Mean (±SD) length, weight, and calculated Fulton's condition index (K) of juvenile (42 d old) sheepshead minnows *Cyprinodon variegatus* in control, elevated pCO_2, low dissolved oxygen (DO), and multi-stress treatments (see Table 1 for treatment details) during the larval-juvenile experiment

Treatment	Length (mm)	Weight (g)	K (g mm^{-3})
Control	14.0 ± 0.07	0.07 ± 0.02	2.37 ± 0.17
Elevated pCO_2	13.6 ± 1.25	0.06 ± 0.02	2.36 ± 0.20
Low DO	14.0 ± 1.13	0.07 ± 0.02	2.37 ± 0.34
Multi-stress	13.88 ± 1.46	0.06 ± 0.02	2.33 ± 0.32

hatching was significantly delayed in both experiments until Day 8 of exposure in the majority of embryos in the multi-stress treatments (larval-juvenile $p = 0.009$, embryo-only $p < 0.001$; Table 2). Embryonic survival was assessed by examining embryos under a microscope for a heartbeat and spontaneous movement. In the larval-juvenile exposure, embryo survival was greater than 95% across treatments. A total of 4 fish died after embryos hatched (2 in control, 2 in the multi-stress), and survival was >90% across all treatments. In the embryo-only exposure, embryonic survival exceeded 90% in the control and elevated pCO_2 treatments at the start of embryonic hatching and was slightly lower in the DO and multi-stress treatments (Table 2). After 42 d of exposure, juvenile fish showed no differences in fish weight, length, or condition, regardless of treatment (Table 3).

3.2. Biochemical analyses

Analyses of LDH activity showed a significant main level effect of life stage ($p < 0.001$), with no significant effect of treatment ($p = 0.525$). LDH levels steadily increased as fish developed from embryo to juvenile (Fig. 1).

3.3. Oxidative stress and antioxidant response

A significant main level effect of life stage was noted in PC formation ($p < 0.001$), with no overall treatment effects ($p = 0.099$, Fig. 2). However, within the embryo life stage, PC levels in the multi-stress treatment were significantly different than control fish ($p = 0.009$). Additionally, PC formation was notably decreased in larval fish compared to embryos and juvenile fish (Fig. 2).

Only a main level effect of life stage was noted for SOD activity and catalase activity ($p < 0.001$ in both

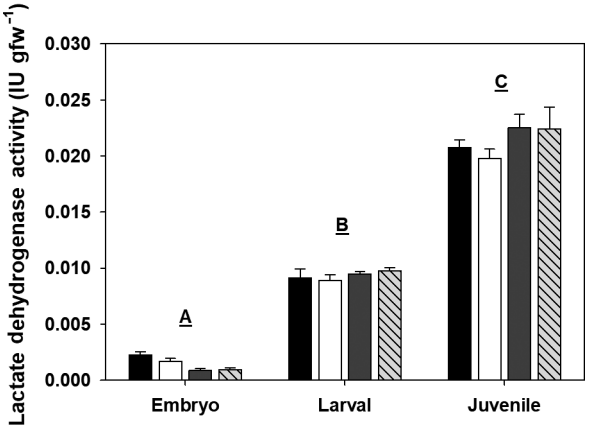

Fig. 1. Total lactate dehydrogenase activity (±SE) of embryo, larval, and juvenile sheepshead minnows *Cyprinodon variegatus,* (n = 5 for each life stage) exposed to control conditions (ambient dissolved oxygen [DO] and pCO_2; black bars), elevated pCO_2 (white bars), low DO (grey bars), and low DO and elevated pCO_2 (cross-hatched bars). Data were analyzed by 2-way ANOVA, and letters indicate significant differences between life stages; gfw: g fresh tissue weight

cases). Embryos had an almost 3-fold higher SOD activity level compared to larval and juvenile fish regardless of treatment (Fig. 3). In contrast, catalase activity levels were higher in larval and juvenile fish when compared to embryos (Fig. 4).

3.4. Carbonic anhydrase and Na^+/K^+ ATPase

Two-way ANOVA showed a significant effect of life stage ($p < 0.001$) in both CA and NKA enzyme activities. A significant effect of treatment and interaction between life stage and treatment was also seen in the NKA data. Embryonic fish showed no significant changes, regardless of treatment, although CA levels were much higher compared to larval and juvenile fish (Fig. 5). Larval fish showed significant increases in NKA activity compared to embryo and juvenile fish, across all treatments ($p < 0.001$ in all cases), with the multi-stress treatment showing a synergistic interaction between acidification and hypoxia (Fig. 6).

4. DISCUSSION

Understanding and predicting the interactive effects of hypoxia and elevated pCO_2 in estuarine organisms are critical for forecasting responses to such anthropogenically driven environmental changes. We investigated the capacity of embryo through juvenile developmental stages in sheepshead minnows to compensate for exposure of elevated levels of pCO_2 (2000 µatm) and low DO (2 mg l^{-1}) that are expected to occur under projected acidification and eutrophication scenarios (Rabalais et al. 2009, Duarte et al.

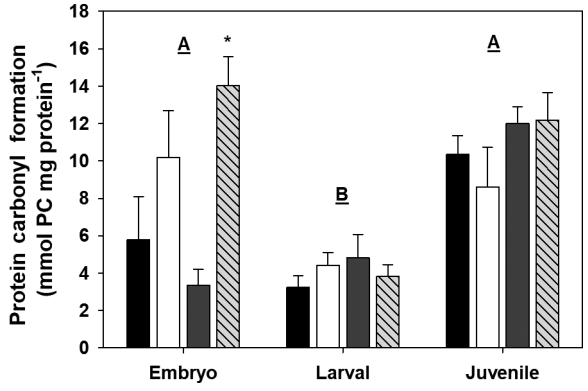

Fig. 2. Protein carbonyl (PC) formation (±SE) of embryo, larval, and juvenile sheepshead minnows *Cyprinodon variegatus* (n = 5 for each life stage). Details as in Fig. 1. Asterisk indicates a significant difference from the control treatment

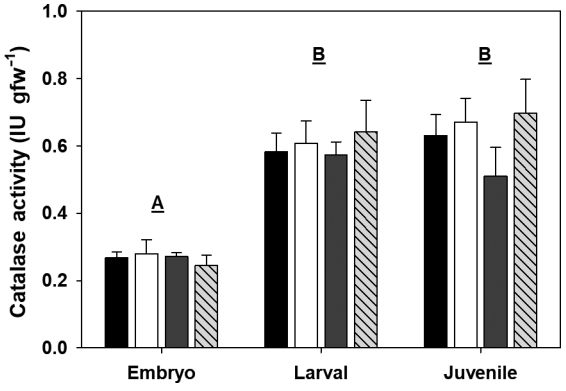

Fig. 4. Catalase activity (±SE) of embryo, larval, and juvenile sheepshead minnows *Cyprinodon variegatus* (n = 5 for each life stage). Details as in Fig. 1

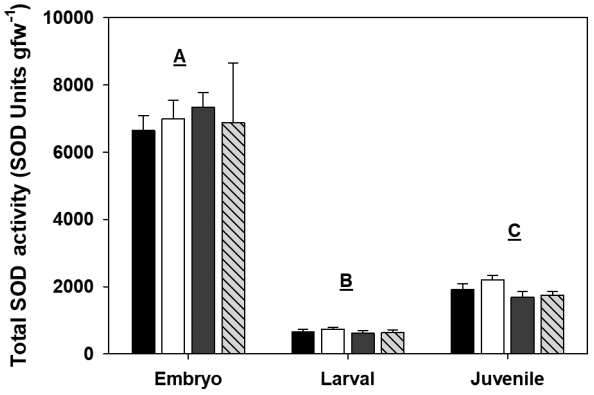

Fig. 3. Total superoxide dismutase (SOD) activity (±SE) of embryo, larval, and juvenile sheepshead minnows *Cyprinodon variegatus* (n = 5 for each life stage). Details as in Fig. 1

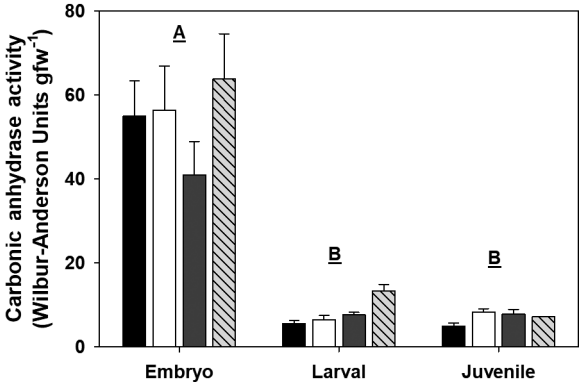

Fig. 5. Carbonic anhydrase activity (±SE) of embryo, larval, and juvenile sheepshead minnows *Cyprinodon variegatus* (n = 5 for each life stage). Details as in Fig. 1

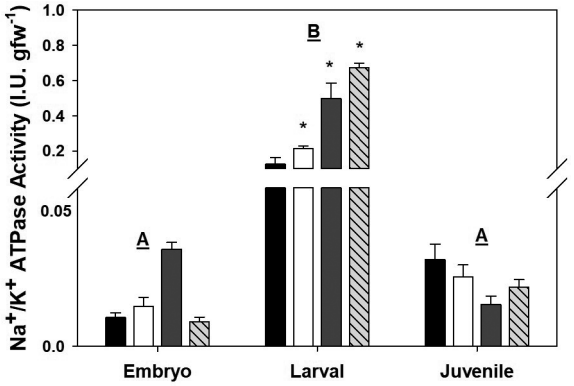

Fig. 6. Na^+/K^+ATP-ase activity (±SE) of embryo, larval, and juvenile sheepshead minnows *Cyprinodon variegatus* (n = 5 for each life stage). Details as in Fig. 1. Asterisks indicate significant differences from the control treatment

2013, IPCC 2013). The interactive effects of elevated pCO_2 and hypoxia on sheepshead minnow development and cellular homeostasis were determined using a suite of biochemical assessments. Hypoxia and the combination of hypoxia and elevated pCO_2 had minimal effects on embryonic hatch rate and survival, and no effect on fish survival or condition after hatching. No significant effects were seen in LDH activity, PC formation, or antioxidant activity of larval or juvenile fish. Acid–base regulation was the only metabolic process influenced by hypoxia and/or elevated pCO_2 in this species, specifically in larval fish.

The majority of studies examining the effects of elevated pCO_2 on early life stages of teleost fish have focused on metrics such as hatch rate, development, and overall survival, producing variable results. Atlantic silverside *Menidia menidia* and inland silverside *M. beryllina* showed decreased survival and altered development at exposures of ~1000 and 2300 µatm (Baumann et al. 2012, Malvezzi et al. 2015), yet no negative effects were noted in separate studies at exposures of 2000 or 2200 µatm in these same species (DePasquale et al. 2015, Murray & Baumann 2018). Elevated pCO_2 levels of ~2000 µatm did not influence hatch rate or survival in sheepshead minnows (DePasquale et al. 2015, Gobler et al. 2018); our results also indicated no detrimental effects of elevated pCO_2 on embryonic development or hatch rate.

In contrast to elevated pCO_2, exposure to hypoxia delays hatching in teleost fish, regardless of species. DO levels of 2.5 mg l^{-1} significantly delayed hatching in *M. menidia*, *M. beryllina*, and sheepshead minnows by 1–3 d, and reduced hatching success by approximately 50% in all 3 species in both hypoxic and multi-stress treatments (DePasquale et al. 2015). We also determined that exposure to hypoxia singly or combined with elevated pCO_2 delayed hatching by approximately 2 d. However, our hatch success rates in hypoxia and multi-stress treatments (95 and 85%) were much higher than the approximately 50% reported by DePasquale et al. (2015). Additionally, post-hatch survival of larvae was substantially higher than reported by DePasquale et al. (2015), despite similar targeted treatment levels. This may be due to the source of fish used. Our embryos were spawned from wild-caught stock, versus the fish in DePasquale's study, which were purchased from a laboratory facility.

LDH activity, a widely used anaerobic pathway in teleost fishes (Hochachka & Somero 2002, Evans et al. 2005), was expected to increase under low DO concentrations. However, other than a decrease in LDH activity noted in embryonic fish, we saw no treatment effects. We did observe an overall increase in LDH activity as fish developed. In embryos and early larval fish, metabolism is almost wholly aerobic via cutaneous diffusion of oxygen versus diffusion across gill lamellae (Wieser 1995). As fish and gills develop, anaerobic pathways are more heavily relied upon due to increased transportation costs and time of delivery of oxygen to tissues (Vetter & Lynn 1997). The results of our study demonstrate this concept, shown by the increase in LDH activity during sheepshead minnow development from embryo to juvenile.

PC formation can be used as an indicator of reactive oxygen species formation and subsequent oxidative stress (Dalle-Donne et al. 2003). PCs are expected to increase when organisms are exposed to acidification and/or hypoxia, as both of these stressors impair mitochondrial function (Guzy & Schumacker 2006, Murphy 2009, Tomanek et al. 2011). Fish in our study had varying levels of PC formation, and only embryo multi-stress treatment values differed from control fish. Low oxygen availability has been shown to increase PC formation in hypoxia-tolerant adult goby *Perccottus glenii* (Lushchak & Bagnyukova 2007), and exposure to elevated pCO_2 markedly increased PC formation in 3 species of Antarctic notothenioid adults through 28 d of exposure (Enzor & Place 2014). The lack of PC formation noted in the present study indicates that larval and juvenile fish were capable of physiologically compensating for any mitochondrial impairment caused by treatment exposure. The overall decrease in PC formation noted in larval fish compared to the embryonic and juvenile life stages requires further exploration.

While no treatment effects were noted in the 2 antioxidant pathways examined, SOD and catalase, enzyme activity varied with life stage. Embryonic fish appear to use SOD more, while larval and juvenile fish rely more on catalase. Studies on the estuarine species spot croaker *Leiostomus xanthurus* and pinfish *Lagodon rhomboides* showed an increase in SOD activity in response to hypoxia, with no clear trend of catalase activity (Ross et al. 2001, Cooper et al. 2002). Both of these studies examined individual tissues for these enzymes, and the whole-fish responses noted in our study may have diluted a tissue-specific response. Another possible explanation for the lack of response is the chronic hypoxic exposure of our study. Borowiec et al. (2015) noted that oxidative stress and antioxidant activities became apparent after mummichog *Fundulus heteroclitus* exposed to hypoxia were returned to normoxic conditions; i.e. that oxidative stress became apparent only when oxygen debt could be repaid. The constant hypoxic exposure in our study could have masked this effect.

In teleost fish, ionic and osmotic balance are often disrupted during exposure to acidification, resulting in increased metabolic costs of re-establishing acid–base balance via osmoregulation (see Perry & Gilmour 2006, Heuer & Grosell 2014 for reviews). CA, a metalloenzyme which catalyzes the hydrolysis of CO_2, combined with NKA pumps found in the chloride cells, are largely responsible for maintaining and regulating ion balance. While no differences in CA activity were noted in treatment fish versus control fish in this study, levels of CA were notably higher in embryos than in larval and juvenile fish, likely due to the egg capsule preventing CO_2 diffusion out of the embryo (Rombough 1988). NKA activity was the only enzyme to show significant changes in response to all treatments in sheepshead minnow larvae. This increase during the larval phase is likely due to fish moving from cutaneous to branchial respiration, relying more on the NKA pumps for osmoregulation (Alderdice 1988). Juvenile sheepshead showed no changes in NKA activity, a finding similar to other teleost fishes. Gulf toadfish *Opsanus beta* showed complete compensation within 2 h of exposure to 1900 µatm (Esbaugh et al. 2012), and woolly sculpin *Clinocottus analis* showed no difference in hypoxia tolerance or NKA activity after 7 d of acclimation at elevated pCO_2 levels (~1000 µatm, Hancock & Place 2016). Interestingly, a 14 d exposure to 1000 µatm in red drum *Sciaenops ocellatus* showed an increase of NKA activity even after 2 wk at elevated pCO_2 levels (Esbaugh et al. 2016).

Understanding the interactive effects of elevated pCO_2 and hypoxia on physiological and biochemical compensation processes of estuarine organisms is critical to predicting impacts of environmental alteration of these stressors by eutrophication. An important facet is identifying sensitive species and life stages that may be significantly impacted and understanding their ability to adapt to future stressor conditions. Within the estuarine environment, DO and pCO_2 levels are invariably linked; large variation of DO and pCO_2 levels can occur on a daily basis due to primary production and respiration. Hypoxic zones are projected to become more frequent due to nutrient pollution, which causes declines in pH levels (Rabalais et al. 2009, Borges & Gypens 2010, Howarth et al. 2011). Both hypoxic zones and pH level declines are further exacerbated by nutrient influxes and anthropogenic CO_2 emissions (Cai et al. 2011, Hofmann et al. 2011). Several studies have examined the interactive effects of low DO and elevated pCO_2 on estuarine teleost survival, embryonic development, and physiology. These studies have produced variable results, with some studies providing evidence of susceptibility in *M. menidia* and *M. beryllina* (DePasquale et al. 2015, Miller et al. 2016) and other research demonstrating adaptability and compensation in weakfish *Cynoscion regalis* (Lifavi et al. 2017), *F. heteroclitus* (Cochran & Burnett 1996), and sheepshead minnows (DePasquale et al. 2015, this study).

The results of our study indicate that embryonic, larval, and juvenile sheepshead minnows are capable of compensating for the detrimental effects of acidification (2000 µatm) and hypoxia (2 mg l^{-1}) exposure. The lack of measured anaerobic activity and oxidative stress observed here point toward physiological compensation in these fish. However, minor changes in early life stages, such as the increase in NKA activity noted in larval fish, may significantly alter or impact later life stages (Pechenik 2006), which were not tested here. Additionally, any delay in hatching, as noted in this study and that of DePasquale et al. (2015), may influence population numbers, as an increase in the length of embryonic or larval stages may increase mortality (Raimondo 2012). Despite these possibilities, sheepshead minnows appear to be inherently more tolerant to the combination of elevated pCO_2 and hypoxia than other teleost species.

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LITERATURE CITED

Abele D, Puntarulo S (2004) Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. Comp Biochem Physiol Part A Mol Integr Physiol 138: 405–415

Alderdice DF (1988) Osmotic and ionic regulation in teleost eggs and larvae. In: Hoar WS, Randall DJ (eds) Fish physiology. Academic Press, San Diego, CA, p 163–252

Baumann H, Talmage SC, Gobler CJ (2012) Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. Nat Clim Change 2:38–41

Baumann H, Wallace RB, Tagliaferri T, Gobler CJ (2015) Large natural pH, CO_2 and O_2 fluctuations in temperate tidal salt marsh on diel, seasonal, and interannual time scales. Estuar Coast 38:220–231

Beers RF, Sizer IW (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 195:133–140

Borges AV, Gypens N (2010) Carbonate chemistry in the coastal zone responds more strongly to eutrophication than to ocean acidification. Limnol Ocean 55:346–353

Borowiec BG, Darcy KL, Gillette DM, Scott GR (2015) Distinct physiological strategies are used to cope with constant hypoxia and intermittent hypoxia in killifish (*Fundulus heteroclitus*). J Exp Biol 218: 1198–1211

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Burnett LE, Stickle WB (2001) Physiological responses to hypoxia. In: Rabalais NN, Turner RE (eds) Coastal and estuarine studies, coastal hypoxia: consequences for living resources and ecosystems. American Geophysical Union, Washington, DC, p 101–114

Cai WJ, Hu X, Huang WJ, Murrell MC and others (2011) Acidification of subsurface coastal waters enhanced by eutrophication. Nat Geosci 4:766–770

Capasso C, De Luca V, Carginale V, Cannio R, Rossi M (2012) Biochemical properties of a novel and highly thermostable bacterial α-carbonic anhydrase from *Sulfurihydrogenibium yellowstonense YO2AOP1*. J Enzyme Inhib Med Chem 27:892–897

Claiborne JB, Edwards SL, Morrison-Shetlar AI (2002) Acid-base regulation in fishes: cellular and molecular mechanisms. J Exp Zool 293:302–319

Cochran RE, Burnett LE (1996) Respiratory responses of the salt marsh animals, *Fundulus heteroclitus*, *Leiostomus xanthurus*, and *Palaemonetes pugio* to environmental hypoxia and hypercapnia and to the organophosphate pesticide, azinphosmethyl. J Exp Mar Biol Ecol 195: 125–144

Cooper RU, Clough LM, Farwell MA, West TL (2002) Hypoxia-induced metabolic and antioxidant enzymatic activities in the estuarine fish *Leiostomus xanthurus*. J Exp Mar Biol Ecol 279:1–20

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R (2003) Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta 329:23–38

Deigweiher K, Koschnick N, Pörtner HO, Lucassen M (2008) Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia. Am J Physiol Regul Integr Comp Physiol 295:R1660–R1670

DePasquale E, Baumann H, Gobler CJ (2015) Vulnerability of early life stage Northwest Atlantic forage fish to ocean acidification and low oxygen. Mar Ecol Prog Ser 523: 145–156

Dickson AG, Millero FJ (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res A 34:1733–1743

Dickson AG, Sabine CL, Christian JR (eds) (2007) Guide to best practices for ocean CO_2 measurements. PICES Special Publication 3, IOCCP Rep 8. North Pacific Marine Science Organization, Sidney

Doney SC (2010) The growing human footprint on coastal and open-ocean biogeochemistry. Science 328: 1512–1516

Duarte CM, Hendriks IE, Moore TS, Olsen YS and others (2013) Is ocean acidification an open-ocean syndrome? Understanding anthropogenic impacts on seawater pH. Estuaries Coasts 36:221–236

Ekau E, Anuel H, Pörtner HO, Gilbert D (2010) Impacts of hypoxia on the structure and processes in pelagic communities (zooplankton, macro-invertebrates and fish). Biogeosciences 7:1669–1699

Enzor LA, Place SP (2014) Is warmer better? Decreased oxidative damage in notothenioid fish after long-term acclimation to multiple stressors. J Exp Biol 217: 3301–3310

Esbaugh AJ (2018) Physiological implications of ocean acidification for marine fish: emerging patterns and new insights. J Comp Physiol B Biochem Syst Environ Physiol 188:1–13

Esbaugh AJ, Heuer R, Grosell M (2012) Impacts of ocean acidification on respiratory gas exchange and acid-base balance in a marine teleost, *Opsanus beta*. J Comp Physiol B Biochem Syst Environ Physiol 182:921–934

Esbaugh AJ, Ern R, Nordi WM, Johnson AS (2016) Respiratory plasticity is insufficient to alleviate blood acid-base disturbances after acclimation to ocean acidification in the estuarine red drum, *Sciaenops ocellatus*. J Comp Physiol B Biochem Syst Environ Physiol 186: 97–109

Evans DH, Piermarini PM, Choe KP (2005) The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation and excretion of nitrogenous waste. Physiol Rev 85:97–177

Ewing JF, Janero DR (1995) Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. Anal Biochem 232:243–248

Fangue NA, O'Donnell MJ, Sewell MA, Matson PG, MacPherson AC, Hofmann GE (2010) A laboratory-based, experimental system for the study of ocean acidification effects on marine invertebrate larvae. Limnol Oceanogr Methods 8:441–452

Gobler CJ, Baumann H (2016) Hypoxia and acidification in ocean ecosystems: coupled dynamics and effects on marine life. Biol Lett 12:20150976

Gobler CJ, Merlo LR, Morrell BK, Griffith AW (2018) Temperature, acidification, and food supply interact to negatively affect the growth and survival of the forage fish, *Menidia beryllina* (inland silverside), and *Cyprinodon variegatus* (sheepshead minnow). Front Mar Sci 5:86

Gutiérrez JL, Jones CG, Byers JE, Arkema KK and others (2011) Physical ecosystem engineers and the functioning of estuaries and coasts. In: Wolanski E, McLusky DS (eds) Treatise on estuarine and coastal science Vol 7. Academic Press, Waltham, MA, p 53–81

Guzy RD, Schumacker PT (2006) Oxygen sensing by mitochondria at complex. III. The paradox of increased reactive oxygen species during hypoxia. Exp Physiol 91: 807–819

Hammer K, Schneider B, Kuliński K, Schulz-Bull DE (2014) Precision and accuracy of spectrophotometric pH measurements at environmental conditions in the Baltic Sea. Estuar Coast Shelf Sci 146:24–32

Hancock JR, Place SP (2016) Impact of ocean acidification on the hypoxia tolerance of the woolly sculpin, *Clinocottus analis.* Conserv Physiol 4:cow040

Heisler N (1984) Acid-base regulation in fishes. In: Hoar W, Randall D (eds) Fish physiology, Vol 10A. Academic Press, Orlando, FL, p 315–360

Hendriks IE, Duarte CM, Olsen YS, Steckbauer A and others (2015) Biological mechanisms supporting adaptation to ocean acidification in coastal ecosystems. Estuar Coast Shelf Sci 152:A1–A8

Heuer RM, Grosell M (2014) Physiological impacts of elevated carbon dioxide and ocean acidification on fish. Am J Physiol Regul Integr Comp Physiol 307:R1061–R1084

Hochachka PW (1986) Defense strategies against hypoxia and hypothermia. Science 231:234–241

Hochachka PW, Somero GN (2002) Biochemical adaptation: mechanism and process in physiological evolution. Oxford University Press, Oxford

Hofmann GE, Smith JE, Johnson KS, Send U and others (2011) High-frequency dynamics of ocean pH: a multi-ecosystem comparison. PLOS ONE 6:e28983

Hopkins KD (1992) Reporting fish growth: a review of the basics. J World Aquacult Soc 23:173–179

Howarth R, Chan F, Conley DJ, Garnier J, Doney SC, Marino R, Billen G (2011) Coupled biogeochemical cycles: eutrophication and hypoxia in temperate estuaries and coastal marine systems. Front Ecol Environ 9: 18–26

IPCC (2013) Climate change 2013: the physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge

Jensen FB, Nikinmaa M, Weber RE (1993) Environmental perturbations of oxygen transport in teleost fish: causes, consequences and compensations. In: Rankin JC, Jenson FB (eds) Fish ecophysiology. Springer, Dordrecht, p 161–179

Karnaky KJ Jr (1986) Structure and function of the chloride cell of *Fundulus heteroclitus* and other teleosts. Am Zool 26:209–224

Lifavi DM, Targett TE, Grecay PA (2017) Effects of diel-cycling hypoxia and acidification on juvenile weakfish *Cynoscion regalis* growth, survival, and activity. Mar Ecol Prog Ser 564:163–174

Lushchak VI, Bagnyukova TV (2007) Hypoxia induces oxidative stress in tissues of a goby, the rotan *Perccottus glenii.* Comp Biochem Physiol B Biochem Mol Biol 148: 390–397

Malvezzi AJ, Murray CS, Feldheim KA, DiBattista JD and others (2015) A quantitative genetic approach to assess the evolutionary potential of a coastal marine fish to ocean acidification. Evol Appl 8:352–362

Mandic M, Todgham AE, Richards JG (2009) Mechanisms and evolution of hypoxia tolerance in fish. Proc R Soc B 276:735–744

McCormick SD (1993) Methods for nonlethal gill biopsy and measurement of Na^+, K^+-ATPase activity. Can J Fish Aquat Sci 50:656–658

Mehrbach C, Culberson CH, Hawley JE, Pytkowicz RM (1973) Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol Oceanogr 18:897–907

Melzner F, Thomsen J, Koeve W, Oschlies A and others (2013) Future ocean acidification will be amplified by hypoxia in coastal habitats. Mar Biol 160:1875-1888

Mesquita CS, Oliveira R, Bento F, Geraldo D, Rodrigues JV, Marcos JC (2014) Simplified 2,4-dinitrophenylhydrazine spectrophotometric assay for quantification of carbonyls in oxidized proteins. Anal Biochem 458:69–71

Miller SH, Breitburg DL, Burrell RB, Keppel AG (2016) Acidification increases sensitivity to hypoxia in important forage fishes. Mar Ecol Prog Ser 549:1–8

Murphy MP (2009) How mitochondria produce reactive oxygen species. Biochem J 417:1–13

Murray CS, Baumann H (2018) You better repeat it: Complex CO_2 × temperature effects in Atlantic silverside offspring revealed by serial experimentation. Diversity (Basel) 10:69–88

Nordlie FG (2006) Physicochemical environments and tolerances of cyprinodontoid fishes found in estuaries and salt marshes of eastern North America. Rev Fish Biol Fish 16:51–106

Pechenik JA (2006) Larval experience and latent effects—metamorphosis is not a new beginning. Integr Comp Biol 46:323–333

Perry SF, Gilmour KM (2006) Acid–base balance and CO_2 excretion in fish: unanswered questions and emerging models. Respir Physiol Neurobiol 154:199–215

Pikitch EK, Rountos KJ, Essington TE, Santora C and others (2014) The global contribution of forage fish to marine fisheries and ecosystems. Fish Fish 15:43–64

Pörtner HO, Langenbuch M, Michaelidis B (2005) Synergistic effects of temperature extremes, hypoxia and increases in CO_2 on marine animals: from Earth history to global change. J Geophys Res 110:C09S10

Rabalais NN, Turner RE, Díaz RJ, Justi D (2009) Global change and eutrophication of coastal waters. ICES J Mar Sci 66:1528–1537

Raimondo S (2012) Incorporating temperature-driven seasonal variation in survival, growth, and reproduction into population models for small fish. Mar Ecol Prog Ser 469: 101–112

Rombough PJ (1988) Respiratory gas exchange, aerobic metabolism, and effects of hypoxia during early life. In: Hoar WS, Randall DJ (eds) The physiology of developing fish: eggs and larvae. Academic Press, San Diego, CA, p 59–161

Ross SW, Dalton DA, Kramer S, Christensen BL (2001) Physiological (antioxidant) responses of estuarine fishes to variability in dissolved oxygen. Comp Biochem Physiol C Toxicol Pharmacol 130:289–303

Tomanek L, Zuzow MJ, Ivanina AV, Beniash E, Sokolova IM (2011) Proteomic response to elevated P_{CO2} level in eastern oysters, *Crassostrea virginica*: evidence for oxidative stress. J Exp Biol 214:1836–1844

Vetter RD, Lynn EA (1997) Bathymetric demography, enzyme activity patterns, and bioenergetics of deep-living scorpaenid fishes (genera *Sebastes* and *Sebastolobus*): paradigms revisited. Mar Ecol Prog Ser 155:173–188

Wieser W (1995) Energetics of fish larvae, the smallest vertebrates. Acta Physiol Scand 154:279–290

Yancey PH, Somero GM (1978) Temperature dependence of intracellular pH: its role in the conservation of pyruvate apparent K_m values of vertebrate lactate dehydrogenases. J Comp Physiol 125:129–134

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