Journal of Experimental Marine Biology and Ecology 493 (2017) 20–30

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Responses of juvenile Atlantic silverside, striped killifish, mummichog, and striped bass to acute hypoxia and acidification: Aquatic surface respiration and survival

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ARTICLE INFO

Keywords:
Aquatic surface respiration
Diel-cycling
Hypoxia
Dissolved oxygen
Acidification

ABSTRACT

Diel fluctuations in dissolved oxygen (DO) and pH create hypoxic conditions that alter the quality of shallow estuarine nursery habitats for juvenile fishes. Understanding how different species in these environments mitigate stress associated with intermittent hypoxia through compensatory behaviors, such as aquatic surface respiration (ASR), is important in determining the effect of these stressors on estuarine ecosystems. Behavioral responses of Atlantic silversides (*Menidia menidia*), striped killifish (*Fundulus majalis*), mummichog (*Fundulus heteroclitus*), and juvenile striped bass (*Morone saxatilis*) were independently observed during exposure to two levels of diel-cycling DO (3–9 mg O_2 l^{-1} and 1–11 mg O_2 l^{-1}) each tested with both the corresponding pH cycle (7.2–7.8 and 6.8–8.1, respectively) and static pH (7.5) under controlled laboratory conditions. In treatments in which DO declined to ~3 mg O_2 l^{-1}, none of the species examined exhibited ASR behavior either with or without the associated pH decline. However, ASR was observed during both 4-hour and extended 16-hour exposure where DO declined to ~1.0–1.6 mg O_2 l^{-1} in *M. menidia* and both *Fundulus* species. *M. saxatilis* did not exhibit ASR and no mortalities occurred during 4-hour low DO/pH treatments or during 16 hour exposure to 1.5 mg O_2 l^{-1}. During extended 16-hour treatments, DO thresholds for ASR were not found to be different between *F. majalis* and *F. heteroclitus*, but both differed significantly from *M. menidia*. Across both 4-hour and 16-hour treatments, the onset of ASR was observed in *M. menidia* at or near lethal levels (1.31–1.62 mg O_2 l^{-1}). No evidence of a pH (pCO_2) effect on ASR or survival was found in any species in response to naturally co-varying DO and pH swings, despite pH as low as 6.8 and high pCO_2 levels of > ~12,000 µatm. These results suggest that utilization of ASR is a species-specific response influenced by the magnitude and duration of hypoxic exposure. ASR may serve as a last-ditch strategy by *M. menidia* to prolong survival for minutes to hours, but function as a means for *F. heteroclitus* to mitigate or reduce negative effects of hypoxia on a scale of days to weeks, with *F. majalis* exhibiting an intermediate response.

1. Introduction

Estuarine environments fluctuate widely in dissolved oxygen (DO), pH, pCO_2, salinity, and temperature across a range of spatial and temporal scales as a function of both biotic and abiotic processes (Boynton et al., 1996; Stierhoff et al., 2009a; Tyler et al., 2009; Howarth et al., 2011; Baumann et al., 2015). Along the U.S. east coast, estuaries commonly experience some extent of severe hypoxia (≤ 2.0 mg O_2 l^{-1}) and anoxia (0 mg O_2 l^{-1}) during warm and highly productive summer months (Eby and Crowder, 2002; Breitburg, 2002; Bell and Eggleston, 2005; Diaz and Rosenberg, 2008). Exacerbated by anthropogenic nutrient loading, hypoxic conditions are increasingly prevalent in estuaries worldwide (Diaz, 2001; Bell and Eggleston, 2005; Diaz and Rosenberg, 2008). Diel fluctuations in DO, driven by day-night cycles of photosynthesis and respiration, occur primarily in the photic zone in deep-water environments but can extend to the benthos in shallow water estuarine margins (Tyler et al., 2009; Baumann et al., 2015). During these diel cycles, DO can range from hyperoxia (≥ 15 mg O_2 l^{-1}) during the day and drop to hypoxia or anoxia overnight, with the lowest concentrations just after dawn (Kemp and Boynton, 1980; Beck and Bruland, 2000; Tyler et al., 2009; Baumann et al., 2015; Miller et al., 2016).

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http://dx.doi.org/10.1016/j.jembe.2017.04.001
Received 5 October 2015; Received in revised form 2 April 2017; Accepted 10 April 2017

pH co-varies with diel hypoxia cycles due to diel fluctuation in pCO_2 as a result of nighttime respiration and daytime photosynthesis in highly productive shallow-water estuaries and coastal areas (Howarth et al., 2011; Baumann et al., 2015). pCO_2 concentrations can rise to > 20,000 µatm and associated pH values drop from ~8 to < 7 (Howarth et al., 2011; Ullman et al., 2013; Baumann et al., 2015; Miller et al., 2016) over the course of just a few hours. These combined cycles expose estuarine organisms to both potential stressors simultaneously, and the importance of understanding their individual and interactive effects has received recent attention (Wallace et al., 2014; DePasquale et al., 2015; Gobler and Baumann, 2016; Davidson et al., 2016).

One adaptation to low DO exhibited by some fishes is aquatic surface respiration (ASR), which involves rising to the surface and irrigating the gills with the thin, oxygen-rich surface layer (Kramer and McClure, 1982). Several species which engage in ASR possess morphological adaptations such as upturned mouths and dorso-ventrally flattened heads (Lewis, 1970; Kramer and McClure, 1982; Stierhoff et al., 2003; Chapman and McKenzie, 2009). DO thresholds for initiation of ASR are highly variable among species; fishes which initiate ASR at higher pO_2 levels are likely to have a lower hypoxia tolerance, suggesting that ASR begins when oxygen becomes physiologically-limiting (Chapman and McKenzie, 2009). The widespread occurrence of ASR among teleosts indicates that it provides an advantage to fishes by reducing mortality and mitigating negative effects on growth of hypoxia exposure (Kramer and McClure, 1982; Kramer, 1987; Wannamaker and Rice, 2000; Secor and Gunderson, 1998; Stierhoff et al., 2003).

Many ecologically and economically important fishes utilize shallow estuarine areas as nursery habitat during their first late spring, summer, and early fall growth season, coinciding with their larval and juvenile stages (Weinstein, 1979; Minello et al., 2003; Ross, 2003). To mitigate negative effects on growth and survival, fish may either avoid intermittently hypoxic environments or use compensatory behaviors such as ASR (Bell and Eggleston, 2005; Chapman and McKenzie, 2009; Stierhoff et al., 2009b). ASR poses clear benefits and costs (Kramer, 1987); however its occurrence and details of the DO and associated pH (pCO_2) thresholds in many fishes which experience diel-cycling hypoxia and acidification in estuarine nursery areas are incomplete or unknown.

Among estuarine fishes along the U.S. east coast, very little comparative research has been done on the independent and interactive impacts of DO and associated pH (pCO_2) levels on ASR thresholds and survival in species routinely exposed to summertime diel cycles of hypoxia and pH. Smith and Able (2003) found that ASR was the dominant behavior of Atlantic silversides (*Menidia menidia*) during 5 h exposure to DO that declined from ~7 to ~1 mg $O_2\,l^{-1}$, but concluded that *M. menidia* is less tolerant of hypoxia than other species of Atherinopsidae, Cyprinodontidae and Fundulidae studied. Killifishes (Fundulidae) are well known to employ ASR under low DO conditions (Lewis, 1970; Wannamaker and Rice, 2000; Stierhoff et al., 2003; Smith and Able, 2003; Chapman and McKenzie, 2009). Fundulids possess flattened heads and upturned mouths, as do *M. menidia*, which facilitate ASR behavior (Lewis, 1970; Stierhoff et al., 2003). While striped killifish (*Fundulus majalis*) have been reported to engage in ASR, they typically exhibit an overall lower tolerance to prolonged hypoxia compared to their congeners (Woodley and Peterson, 2003; Nordlie, 2006). Comparatively, previous research has shown that mummichog (*Fundulus heteroclitus*) spend proportionally more time performing ASR in low DO, allowing them to survive near anoxic conditions (Wannamaker and Rice, 2000). ASR has not been investigated in juvenile striped bass (*Morone saxatilis*), which possess no obvious anatomical adaptations for ASR, although the behavior has been observed in species of the closely related temperate perch family (Percichthyidae) (McNeil and Closs, 2007).

This study investigated the ASR responses of *M. menidia, F. majalis, F. heteroclitus,* and *M. saxatilis* to declining DO and pH (increased pCO_2). These species are common in shallow estuarine environments along the U.S. east coast and display an apparent range of morphological capacity to engage in ASR as a compensatory behavior in low DO. We hypothesize, based on the presence of specific morphological adaptations (i.e., upturned mouths and dorso-ventrally flattened heads) and previous literature, that the tendency to perform ASR will be observed in *F. heteroclitus* > *F. majalis* > *M. menidia* > *M. saxatilis*. Specifically, the objectives were to determine (1) the extent to which each of the four species performs ASR under different diel-cycling DO and pH/pCO_2 regimes (4 hour exposure), (2) the effects of prolonged hypoxia (16 hour exposure) on the frequency and extent of ASR behavior, (3) the DO threshold for ASR, when present, and whether a co-varying diel pH cycle exacerbates the impact of hypoxia on ASR behavior, and (4) the relationship between ASR behavior and survival.

2. Methods

2.1. Fish collection and acclimation

Young of the year (YOY) *F. majalis* and *M. menidia* were collected by seine from lower Delaware Bay, and *F. heteroclitus* were collected in minnow traps from Canary Creek (Lewes, Delaware, USA) in September 2013. Juvenile *M. saxatilis* were obtained from aquaculture facilities at the University of Maryland's Horn Point Laboratory in Cambridge, Maryland, USA. Fish were held at normoxia; 14L:10D photoperiod; 25 °C; and salinity = 12 ppt for *M. saxatilis* and 25 ppt for *M. menidia, F. majalis, F. heteroclitus* for ≥ 14 days prior to experiments. Fish were fed frozen mysid shrimp (*Mysis relicta*), or Ziegler Finfish Silver® 3 mm sinking pellets (*M. saxatilis* only), *ad libitum* twice daily at 09:00 and 17:00.

2.2. Laboratory aquarium systems

Experiments were conducted in computer-controlled recirculating aquarium systems in a temperature- and photoperiod-controlled laboratory. The apparatus was a redesign of the computer-interfaced DO monitoring and control device (Grecay and Stierhoff, 2002) previously developed for investigations of hypoxia effects on estuarine fishes (Stierhoff et al., 2006; Stierhoff et al., 2009a, 2009b). At 5 minute intervals, DO and pH (NBS scale) were simultaneously monitored and adjusted through the solenoid-controlled flow of water past DO and pH probes (Hach® LDO dissolved oxygen probe and Hach® Differential pH/ORP sensor, respectively) (Davidson et al., 2016). Following each reading, corresponding gas solenoids injected the appropriate compressed gases to regulate DO and pH in treatment systems (~415 l each): CO_2 and air are bubbled to lower and raise pH, while N_2 and O_2 were added to lower and raise DO, respectively. To observe behavioral responses, two observation tanks (56 × 22.86 × 44.45 cm; 56.8 l) were positioned one atop each of two treatment systems (Dixon, 2014). Water was delivered to observation tanks via tubing from their treatment systems, returning via a standpipe in the tank to create a single recirculating system. Vexar mesh separated the standpipe from the field of view for video observation. Observation tanks were sealed with a glass lid to create a single continuous atmosphere with the treatment system. A handheld dissolved oxygen meter (YSI 556 MultiProbe System) was used daily to verify DO and pH in both systems.

As raising the pH required bubbling compressed air, this prevented maintenance of supersaturated DO concentrations. Thus, in treatments where both DO and pH fluctuated on diel cycles, pH reached the target level before DO supersaturated (see Fig. 1b). There was usually slight variability in the minimum DO values achieved among some trials within a given species/treatment combination (see Figs. 1a,b; 2a; 3a,b). The potential relevance of this variability is noted in the Results.

Fish were selected at random from the holding tanks and placed into single-species groups of three for each trial, because a) early tests indicated that solitary fish did not perform consistent or typical

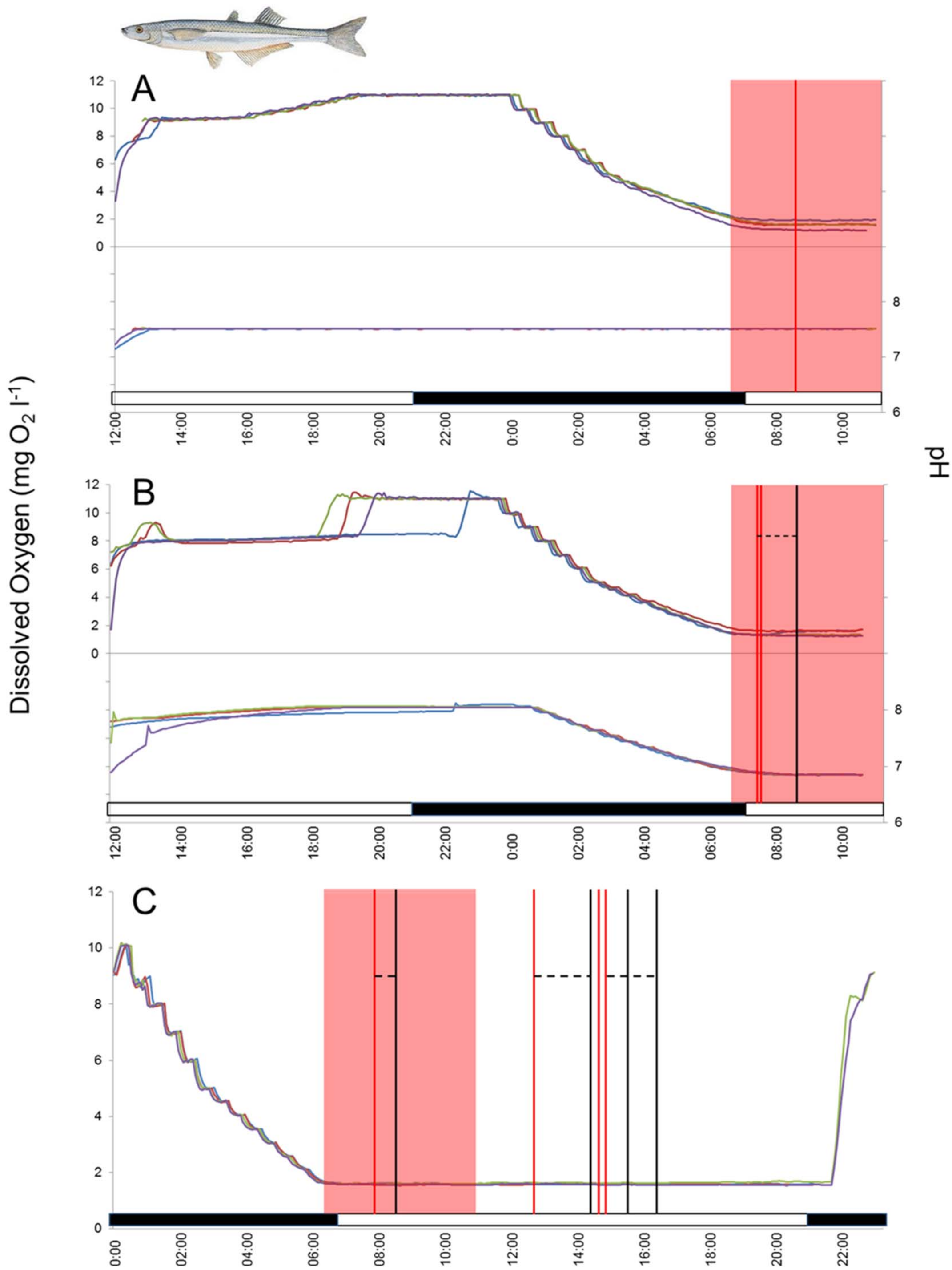

Fig. 1. *Menidia menidia* behavioral responses to treatments: (a) 4-hour low DO with static pH (7.5), (b) 4-hour low DO with cycling pH (6.8–8.1), and (c) extended 16-hour low DO with static pH (7.5). DO/pH levels are shown for each replicate. Shaded/unshaded horizontal bar indicates light (07:00–21:00) and dark (21:00–7:00) photoperiods, respectively. Red shaded region indicates minimum DO in the diel cycle. Vertical red line indicates first ASR by an individual in a particular trial in (a) [1] (08:23, 1.49 mg $O_2\,l^{-1}$, pH 7.1); (b) [1] (07:12, 1.40 mg $O_2\,l^{-1}$, pH 6.95) and [2] (07:14, 1.42 mg $O_2\,l^{-1}$, pH 6.94); and (c) [1] (7:58, 1.59 mg $O_2\,l^{-1}$, pH 7.52); [2] (12:37, 1.59 mg $O_2\,l^{-1}$, pH 7.52); [3] (14:49, 1.6 mg $O_2\,l^{-1}$, pH 7.51); and [4] (15:02, 1.59 mg $O_2\,l^{-1}$, pH 7.52). Corresponding vertical black line indicates subsequent mortality if applicable in (b) [1] (08:20, 1.31 mg $O_2\,l^{-1}$, pH 6.88); and (c) [1] (8:29, 1.57 mg $O_2\,l^{-1}$, pH 7.51); [2] (14:46, 1.57 mg $O_2\,l^{-1}$, pH 7.51); [3] (15:35, 1.59 mg $O_2\,l^{-1}$, pH 7.52); and [4] (16:33, 1.62 mg $O_2\,l^{-1}$, pH 7.52). Horizontal dashed line indicates when the first individual to initiate ASR in a trial was also the first to reach mortality. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

behavior regardless of DO level (RLD personal obs.) and b) fish in small groups was more representative of natural conditions. Fish were not re-used. Prior to observation, food was withheld for 12 h and fish standard length (SL; Tables 1, 2) was measured using a digital caliper (± 0.1 mm). Fish were allowed to adjust to the tanks for 10–15 min before video recording (per Wannamaker and Rice, 2000). A 14L:10D photoperiod was maintained during all trials.

2.3. 4-hour low DO and pH trials

Treatments were designed to be representative of the magnitudes and rate of decline in DO and pH (~4 hour low DO exposure) occurring

Table 1
4-hour low DO/pH treatments. (n = 4 replicate trials per treatment, each with 3 fish).

	DO (mg O_2 l^{-1})	pH
Extreme-Range DO, static pH	1–11	7.5
Extreme-Range DO, cycling pH	1–11	6.8–8.1
Moderate-Range DO, static pH	3–9	7.5
Moderate-Range DO, cycling pH	3–9	7.2–7.8
Control	7.5	7.5

during diel cycles in shallow estuarine tributaries in the U.S. Mid-Atlantic Bight (Tyler et al., 2009; Maryland Department of Natural Resources, 2017; Miller et al., 2016). Two ranges of DO were used: (1) "Extreme-Range" = 1–11 mg O_2 l^{-1} and (2) "Moderate-Range" = 3–9 mg O_2 l^{-1}. Each DO treatment was paired with either static pH (7.5) or a corresponding magnitude of cycling pH: (1) "Extreme pH Range" = 6.8–8.1, or (2) "Moderate pH Range" = 7.2–7.8. A normoxic control treatment (7.5 mg O_2 l^{-1}; pH = 7.5) was run for comparison. Each species was therefore exposed to five treatments (Table 1). Four replicate trials were run for each treatment, producing a total of 20 trials (5 treatment levels × 4 replicate trials) for each species.

Observations began at 12:00; fish were introduced when DO in the aquarium systems had returned to normoxia (7.5 mg O_2 l^{-1}) following early-morning lows. Therefore, in each trial, behavior was observed in response to a single ensuing diel cycle (Fig. 1a,b).

2.4. Extended 16-hour low DO trials

A second set of trials was conducted to observe behavior under sustained, extended hypoxia, designed to represent circumstances where temperature, tide, wind, and precipitation can contribute to deviation from the typical diel-cycling DO pattern (Tyler et al., 2009) and low DO can extend well into the daytime. For each species, the target DO minimum during extended hypoxia was determined from results of the 4-hour low DO and pH trials. These minima (for *M. menidia*: 1.5 mg O_2 l^{-1}; *F. majalis* and *F. heteroclitus*: 1.0 mg O_2 l^{-1}) were lower than those at which ASR was initially observed in the 4-hour trials, but above mortality thresholds; pH remained constant at 7.5. In *M. saxatilis,* the DO minimum was initially set at 1.5 mg O_2 l^{-1}, but was reduced to 1.0 mg O_2 l^{-1} for the last two trials, when no change in behavior from control was initially observed.

In these trials, observations began at midnight as DO was decreasing from super-saturation (~10 mg O_2 l^{-1}) to the oxygen minimum over the following 6 h. Low DO levels were then held for 16 h before returning to saturation (see Fig. 1c).

2.5. Video analyses

Continuous video recordings (Sony Handycam DCR-SR88) were captured for each trial. Minimum DO and pH concentrations were reached 30 min prior to the start of each light period (07:00). It was imperative to adequately illuminate fish as DO decreased overnight, particularly during this critical period just before lights-on. Red LED light (630 nm peak; full-width half-max 620–640 nm) was used to illuminate tanks during dark hours.

Time intervals and watch durations were chosen by pre-screening recordings for each species. During 4-hour low DO trials, video recordings were watched for 1 min each hour during DO incline from normoxia and high daytime DO (12:00–00:00), and during overnight DO decline (00:00–06:30). Observation frequency was increased to every 15 min during the period from 06:30 to ~10:30 as DO approaches minimum levels for each range (1 or 3 mg O_2 l^{-1}) (Table 1), as this was determined to be the primary ASR observation period. In total, 42 discrete 1-minute observations were made for each 24 h recording. For extended 16-hour treatments, videos were similarly watched for 1 min each hour during overnight DO decline from super-saturation to the DO minimum (00:00–06:00), then increased to every 15 min from 06:00 to ~22:00 as DO minimum was maintained. 87 discrete 1 min observations were made for extended 16-hour trials.

ASR thresholds are reported as the DO and pH levels where at least one of the three individuals first engaged in ASR behavior, defined as when fish were in direct contact with the water surface coupled with ventilation at the air-water interface. When ASR was observed in a particular fish, video watch continued until ASR ceased, to determine duration of each event for each individual. In the event that a watch interval included fish already engaged in ASR, the video was rewound to determine the point at which ASR began. On occasions where loss of equilibrium (LOE) was observed, the time and DO/pH level were recorded as mortality. Notes were made on general fish behavior.

2.6. Measurement of pCO_2 in the laboratory and Pepper Creek

In order to avoid disturbance of fish during the ASR trials, water samples were collected from the systems during precisely created blank runs subsequent to completion of the ASR trials. Water samples were collected over the range of DO and pH conditions tested. Water samples were also collected from Pepper Creek in the Delaware Coastal Bays, a nursery area for many estuarine fish species, to compare carbonate chemistry parameters in the field and laboratory. Triplicate samples were taken on September 5, 2014 at 08:09, 12:03, and 16:41 to capture the diel range of DO, pH, and pCO_2 on that day. Temperature and salinity were ~25 °C and ~22.5 ppt, respectively. Water was collected in 20 ml scintillation vials, sterilized with 0.2 ml of 5% $HgCl_2$, and refrigerated. For DIC (dissolved inorganic carbon) analysis, 0.5–1 ml samples were acidified and the obtained CO_2 gas was measured using an infrared CO_2 gas detector (Li-Cor 7000). DIC values were checked against certified reference material from A. Dickson. CO2SYS software (Pierrot et al., 2006) was used to determine pCO_2 values using known values of salinity, temperature, pH, and DIC.

Table 2
Mean fish sizes across "Extreme-Range" treatments tested in 4-hour low DO/pH trials (Table 1) and 16-hour low DO trials (Methods section). The range of dissolved oxygen (DO) at onset of aquatic surface respiration (ASR), mean DO at ASR onset, duration of ASR events, range of DO at loss of equilibrium (LOE), and mean DO at LOE across all 4-hour and 16-hour treatments are provided. Range of DO at ASR onset and at LOE are reported as the range of DO values at which at least one individual in a trial first exhibited the response, reported as the lowest-highest DO thresholds observed across all trials. Duration range refers to duration of discrete ASR bouts exhibited by individuals across trials. When loss of equilibrium (LOE) was observed, the time and DO/pH level were recorded as mortality.

	Fish size (mm SL ± SE)	DO range (mg O_2 l^{-1}) at ASR onset	Mean DO (mg O_2 l^{-1}) at ASR Onset	Duration range of ASR events (min)	DO range (mg O_2 l^{-1}) at LOE	Mean DO (mg O_2 l^{-1}) at LOE
Menidia menidia	79 ± 0.9	1.31–1.62	1.52	17–226	1.31–1.62	1.55
Fundulus majalis	67 ± 1.3	1.05–1.46	1.33	1–18	–	–
Fundulus heteroclitus	60 ± 0.9	1.05–1.19	1.13	5–30	–	–
Morone saxatilis	115 ± 1.0	–	–	–	–	1.42

2.7. Statistical analyses

(1) A linear mixed model was used to compare the effect of treatment (Extreme-Range DO; static vs. cycling pH) on DO threshold for ASR in *F. majalis*.

(2) For the extended 16-hour treatments, a one-way mixed effects model was used to test the fixed effect (species) for differences in mean DO threshold (mg $O_2\,l^{-1}$) at ASR initiation for *M. menidia* and both *Fundulus* species.

(3) To compare DO thresholds for ASR and mortality (LOE) in *M. menidia* during extended 16-hour treatments, a two-way replicated mixed model with DO as the dependent variable and response (ASR or mortality) and trial as independent variables (fixed and random, respectively) was used.

3. Results

In both Moderate-Range DO treatments with either static or cycling pH (Table 1), none of the species performed ASR, and no mortality occurred. Behavior in these treatments was indistinguishable from fish in the control, so they were excluded from further analyses.

3.1. 4-hour low DO and pH trials

3.1.1. Menidia menidia

M. menidia aggregated in mid-water when DO levels were high during the day, and rested on the bottom with occasional rapid surfacing as DO dropped below normoxia (~7.5 mg $O_2\,l^{-1}$). *Menidia* exposed to Extreme DO/static pH treatment only engaged in ASR in the trial with the lowest minimum DO, beginning at 08:23, DO = 1.49 mg $O_2\,l^{-1}$, pH = 7.51 (Fig. 1a). The threshold for ASR varied among individuals, but once all three fish began to use ASR, the behavior continued in all fish until the end of the trial (Table 3). No mortalities occurred. When exposed to the Extreme DO/cycling pH treatment, fish in one trial engaged in ASR at 07:14, DO = 1.42 mg $O_2\,l^{-1}$, pH = 6.94 (Fig. 1b; Table 3). In a second trial, fish initiated ASR at 07:12, DO = 1.40 mg $O_2\,l^{-1}$, pH = 6.95, and a single mortality was observed ~70 min later at DO = 1.31 mg $O_2\,l^{-1}$, pH = 6.88. At near lethal limits, all individuals were performing ASR, lasting until either the end of the trial or until mortality occurred. For the latter trial, mortality occurred in the same individual that had first initiated ASR (Fig. 1b; Table 3), however, this observation is not uniform across all trials or species.

3.1.2. Fundulus majalis

F. majalis exposed to Extreme DO/static pH initiated ASR in one trial at 08:45, DO = 1.46 mg $O_2\,l^{-1}$, pH = 7.52 and in a second trial at 10:20, DO = 1.40 mg $O_2\,l^{-1}$, pH = 7.5 (Fig. 2a; Table 3). Individuals performed ASR intermittently; once an individual began ASR, the fish performed the behavior in discrete bouts lasting from 1 min to a maximum-observed 18 min (Table 2), with duration increasing as the trial progressed. No mortality occurred. *F. majalis* in Extreme DO/cycling pH treatment exhibited ASR in all four replicates, first initiated as follows: (1) 07:10, DO = 1.46 mg $O_2\,l^{-1}$, pH = 6.93; (2) 07:40, DO = 1.40 mg $O_2\,l^{-1}$, pH = 6.94; (3) 07:50, DO = 1.31 mg $O_2\,l^{-1}$, pH = 6.87; and (4) 08:01, DO = 1.39 mg $O_2\,l^{-1}$, pH = 6.88 (Fig. 2b; Table 3). Again, once initiated all individuals performed ASR intermittently for the remainder of the trial. There was no detectable effect of treatment (static vs. cycling pH) on ASR threshold ($p = 0.47$).

3.1.3. Fundulus heteroclitus

During Extreme DO/static pH treatment, *F. heteroclitus* generally

Table 3

DO level (mg $O_2\,l^{-1}$) at first onset of aquatic surface respiration (ASR) and mortality in each of the four replicate trials per treatment: "Extreme-Range" (1–11 mg $O_2\,l^{-1}$) 4-hour low DO treatments with either static (7.5) or cycling (6.8–8.1) pH and extended 16-hour low DO treatments with static pH (7.5). For each trial, levels are reported in the order in which each of the three individuals first engaged in ASR. DO min refers to minimum DO value attained in each trial; n = number of fish in each treatment. Target DO minimum in extended 16-hour trials was determined from results of 4-hour trials (*M. menidia* 1.5 mg $O_2\,l^{-1}$, *Fundulus* spp. 1.0 mg $O_2\,l^{-1}$). In *M. saxatilis*, DO minimum was reduced from 1.5 to 1.0 mg $O_2\,l^{-1}$ for the last two trials.

		Trial 1			Trial 2			Trial 3			Trial 4		
Treatment	n	DO Min	DO ASR	DO Mortality	DO Min	DO ASR	DO Mortality	DO Min	DO ASR	DO Mortality	DO Min	DO ASR	DO Mortality
M. menidia													
4h low DO/static pH	12	1.84	–	–	1.55	–	–	1.55	–	–	1.17	1.49, 1.46, 1.45	–
4h low DO/cycling pH	12	1.34	–	–	1.59	–	–	1.29	1.42, 1.41, 1.31	–	1.23	1.40, 1.41, 1.36	1.31
Extended 16h low DO/static pH	12	1.57	1.59, 1.58, 1.59	1.57, 1.58, 1.57	1.53	1.60, 1.61, 1.60	1.57, 1.59, 1.59	1.59	1.62, 1.62, 1.61	1.62, 1.62, 1.61	1.53	1.59, 1.59, 1.57	1.57, 1.60, 1.55
F. majalis													
4h low DO/static pH	12	1.86	–	–	1.42	–	–	1.41	1.40, 1.40, 1.41	–	1.37	1.46, 1.41, 1.40	–
4h low DO/cycling pH	12	1.34	1.46, 1.44, 1.41	–	1.34	1.40, 1.40, 1.38	–	1.28	1.31, 1.30, 1.30	–	1.38	1.39, 1.39, 1.38	–
Extended 16h low DO/static pH	6	1.01	1.05, 1.08, 1.06	–	1.15	1.20, 1.18, 1.19	–						
F. heteroclitus													
4h low DO/static pH	12	1.49	–	–	1.42	–	–	1.24	–	–	1.19	1.19	–
4h low DO/cycling pH	12	1.56	–	–	1.61	–	–	1.19	–	–	1.15	–	–
Extended 16h low DO/static pH	12	1.14	1.16, 1.16, 1.15	–	1.15	1.18, 1.16, 1.17	–	1.13	1.14, 1.14, 1.13	–	1.02	1.06, 1.05, 1.05	–
M. saxatilis													
4h low DO/static pH	12	1.22	–	–	1.39	–	1.42	1.36	–	–	1.42	–	–
4h low DO/cycling pH	12	1.19	–	–	1.09	–	–	1.14	–	–	1.4	–	–
Extended 16h low DO/static pH	8	1.42	–	–	1.41	–	–	1.18	–	–	1.17	–	–

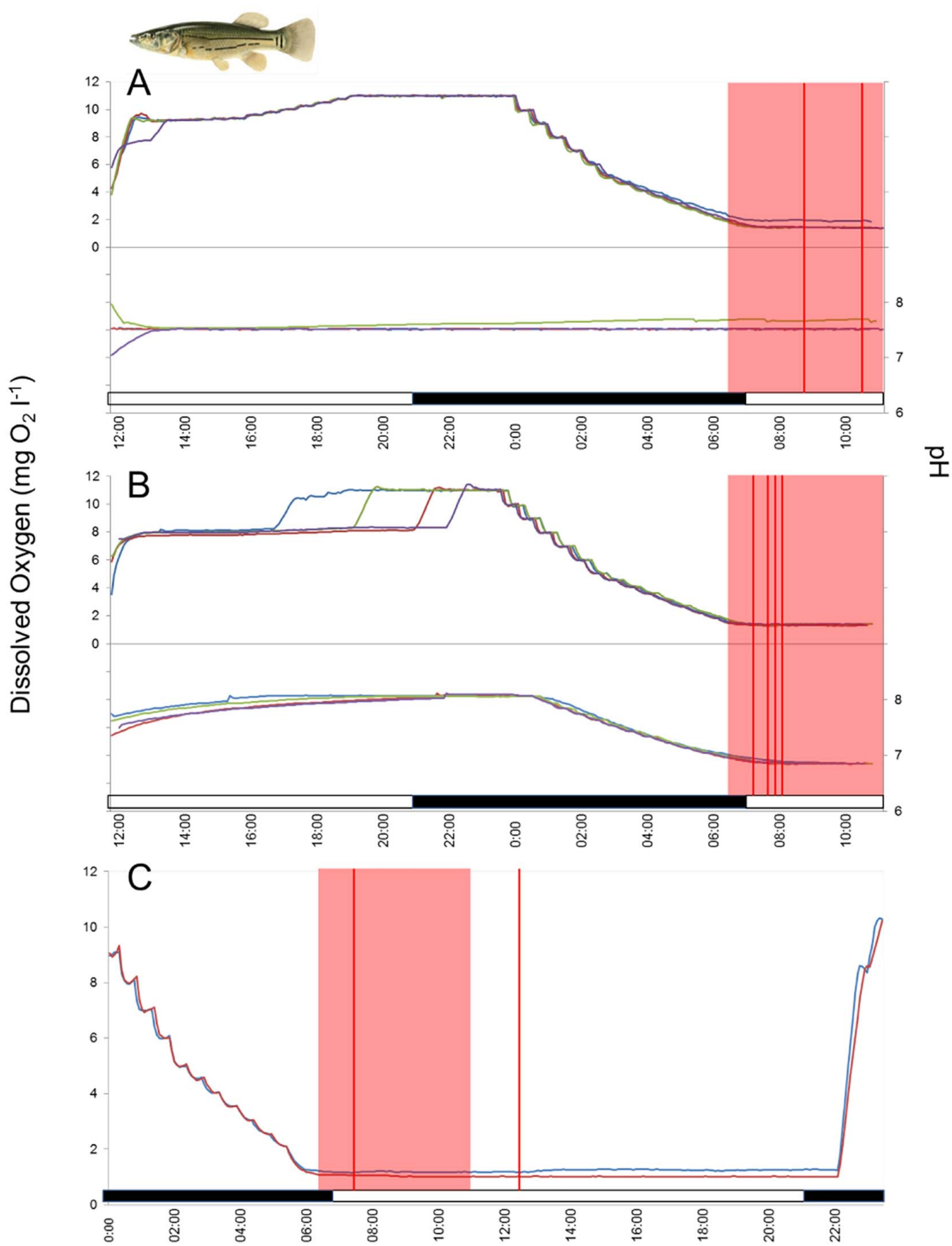

Fig. 2. *Fundulus majalis* behavioral responses to treatments: (a) 4-hour low DO with static pH (7.5), (b) 4-hour low DO with cycling pH (6.8–8.1), and (c) extended 16-hour low DO with static pH (7.5). DO/pH levels are shown for each replicate. Shaded/unshaded horizontal bar indicates light (07:00–21:00) and dark (21:00–7:00) photoperiods, respectively. Red shaded region indicates minimum DO in the diel cycle. Vertical red line indicates first ASR by an individual in a particular trial in (a) [1] (08:45, 1.46 mg O_2 l^{-1}, pH 7.52) and [2] (10:20, 1.40 mg O_2 l^{-1}, pH 7.5); (b) [1] (07:10, 1.46 mg O_2 l^{-1}, pH 6.93); [2] (07:40, 1.40 mg O_2 l^{-1}, pH 6.94); [3] (07:50, 1.31 mg O_2 l^{-1}, pH 6.87); [4] (08:01, 1.39 mg O_2 l^{-1}, pH 6.88) and (c) [1] (7:31, 1.05 mg O_2 l^{-1}, pH 7.51), and [2] (12:33, 1.2 mg O_2 l^{-1}, pH 7.51). No mortality occurred. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

aggregated on the tank bottom and remained in close contact with one another throughout the trials. ASR was initiated by a single fish in one trial at the very end of the low DO period at 10:55, DO = 1.19 mg O_2 l^{-1}, pH = 7.51 (Fig. 3a; Table 3). This period of ASR lasted 5 min and continued through end of the trial. During Extreme DO/cycling pH treatment, observed behavior remained unchanged from static pH treatments throughout the duration of the trial, even as DO decreased overnight. No individuals engaged in ASR, and no mortalities occurred (Fig. 3b; Table 3).

3.1.4. *Morone saxatilis*

No ASR was observed during either Extreme-Range DO treatment with static or cycling pH. *M. saxatilis* swam as a group and remained in mid-water at high DO levels, and after ~30 minutes exposure to DO levels below 2.0 mg O_2 l^{-1}, fish exhibited rheotaxis into the water inflow with occasional rests on the tank bottom. One mortality occurred in a single static pH trial at approximately 09:30, DO = 1.42 and pH = 7.51 (Table 3).

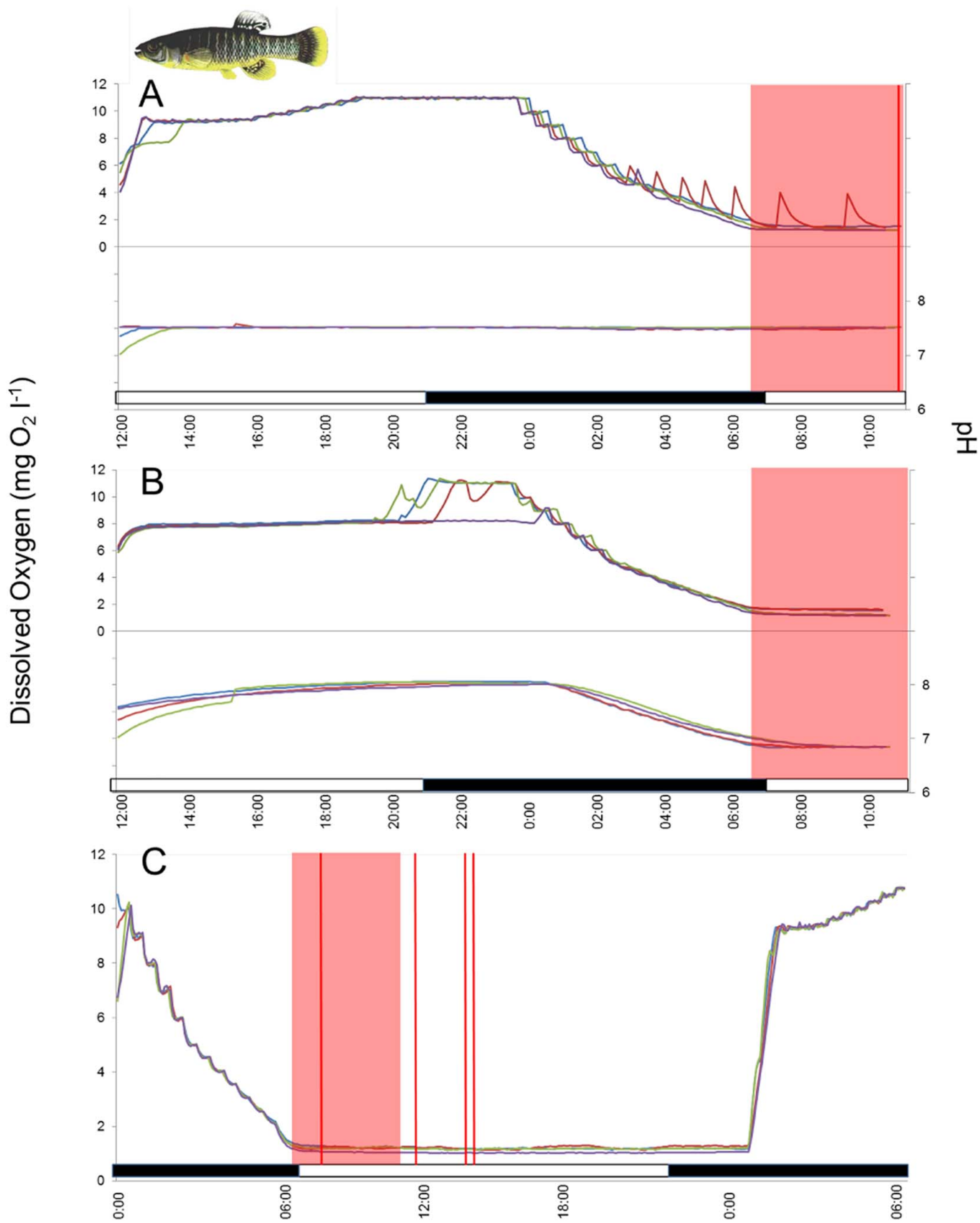

Fig. 3. *Fundulus heteroclitus* behavioral responses to treatments: (a) 4-hour low DO with static pH (7.5), (b) 4-hour low DO with cycling pH (6.8–8.1), and (c) extended 16-hour low DO with static pH (7.5). DO/pH levels are shown for each replicate. Shaded/unshaded horizontal bar indicates light (07:00–21:00) and dark (21:00–7:00) photoperiods, respectively. Red shaded region indicates minimum DO in the diel cycle. Vertical red line indicates first ASR by an individual in a particular trial in (a) [1] (10:55, 1.19 mg $O_2\,l^{-1}$, pH 7.51); and (c) [1] (8:06, 1.06 mg $O_2\,l^{-1}$, pH 7.52); [2] (11:22, 1.16 mg $O_2\,l^{-1}$, pH 7.52); [3] (13:20, 1.18 mg $O_2\,l^{-1}$, pH 7.52) and [4] (13:47, 1.14 mg $O_2\,l^{-1}$, pH 7.51). No mortality occurred. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Extended 16-hour low DO trials

For the extended 16-hour low DO trials, target DO minimum was set at 1.5 mg $O_2\,l^{-1}$ in *M. menidia*. In each of the four replicates, fish first engaged in ASR at the following levels: (1) 7:58, DO = 1.59 mg $O_2\,l^{-1}$, pH = 7.52; (2) 12:37, DO = 1.60 mg $O_2\,l^{-1}$, pH = 7.52; (3) 14:49, DO = 1.62 mg $O_2\,l^{-1}$, pH = 7.51; and (4) 15:02, DO = 1.59 mg $O_2\,l^{-1}$, pH = 7.52 (Fig. 1c; Table 3). DO levels where fish first initiated ASR were not necessarily the lowest observed in a given trial (Table 3); however, in each trial all three individuals engaged in ASR near their lethal limit, and ASR continued until mortality (Table 3). Mortality of all three fish in each trial occurred within 30 to 120 min following initiation of ASR by the first individual, at DO levels ranging from 1.55–1.62 mg $O_2\,l^{-1}$ (Fig. 1c; Table 3). All fish died ≤ 10 h of low DO (or ≤ 1.62 mg $O_2\,l^{-1}$) exposure. No detectable difference was found between the threshold for ASR and that for mortality ($F(1,3) = 1.67$, $p = 0.71$), but there was a significant effect of trial ($F(3,16) = 9.02$, $p = 0.00094$).

For *F. majalis,* target DO minimum was set at 1.0 mg $O_2\,l^{-1}$, and because availability of experimental fish of comparable sizes to those used in the 4-hour trials was restricted, only two extended 16-hour low DO trials were conducted (Fig. 2c; Table 3). As observed in 4-hour trials, once an individual began ASR the fish exhibited ASR intermittently, in discrete bouts of ~1–18 minutes duration (Table 2) throughout the remainder of the trial. Fish began engaging in ASR in the first trial at 7:31, DO = 1.05 mg O_2 l^{-1}, pH = 7.51; and in the second trial at 12:33, DO = 1.20 mg O_2 l^{-1}, pH = 7.51 (Fig. 2c; Table 3). In the second trial, the DO level where fish first initiated ASR was not the lowest observed threshold for the behavior. No mortality occurred.

The target DO minimum was set at 1.0 mg O_2 l^{-1} for *F. heteroclitus*. In three of the four trials, fish initiated ASR approximately 5 h after the DO minimum was reached, which equates to 30 min past the end of the low DO period in the 4-hour low DO and pH treatments. These fish first engaged in ASR at (1) 11:22, DO = 1.16 mg O_2 l^{-1}, pH = 7.52; (2) 13:20, DO = 1.18 mg O_2 l^{-1}, pH = 7.52; and (3) 13:47, DO = 1.14 mg O_2 l^{-1}, pH = 7.51 (Fig. 3c; Table 3). In the fourth trial fish initiated ASR earlier, coinciding with the low DO period of the diel cycle at 8:06, DO = 1.06 mg O_2 l^{-1}, pH = 7.52 (Fig. 3c). Individuals exhibited ASR intermittently, with single bout durations ranging from ~5–30 min (Table 2) and continuing throughout the remainder of the trial. No mortality occurred.

M. saxatilis did not exhibit ASR and no mortalities occurred during 16 hour exposure to 1.5 mg O_2 l^{-1}. The DO minimum was lowered to 1.0 mg O_2 l^{-1} for a second set of two trials and again no ASR or mortality occurred (Table 3).

Post hoc tests on a mixed effects model shows that mean DO thresholds at ASR initiation do not differ between *F. majalis* and *F. heteroclitus* ($p = 0.87$), but the threshold for *Menidia* differs from both *Fundulus* species ($p \leq$ 1e-07) (Fig. 4).

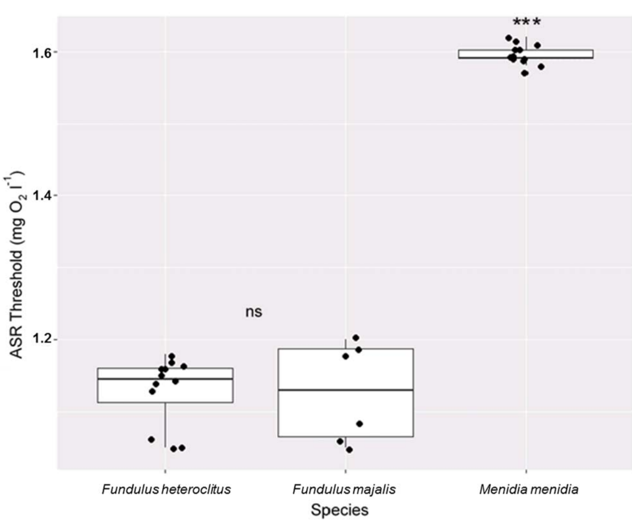

Fig. 4. Dissolved oxygen thresholds (mg O_2 l^{-1}) at initiation of aquatic surface respiration (ASR) in *Fundulus heteroclitus, Fundulus majalis,* and *Menidia menidia* during extended 16 hour treatments. Box plots show medians as horizontal lines inside the boxes, which enclose the middle 50% of the data (25th to 75th percentile), and individual dots are the data points. Tukey's HSD pairwise comparisons of ASR threshold between species showed no significant difference (ns; $p = 0.87$) between the two *Fundulus* species whereas the ASR threshold of *M. menidia* differs significantly ($p < 0.00000005$) from those of both *Fundulus* species (***).

3.3. pCO_2 vs pH in the laboratory and Pepper Creek.

Across treatments, pH ranged from 6.70–7.82 and pCO_2 from ~14,000–800 µatm (Fig. 5). Water samples from Pepper Creek showed a similar relationship between pCO_2 and pH. The pH and mean pCO_2 values from the field were 7.35 and 2084 µatm at 08:09, 7.60 and 1081 µatm at 12:03, and 7.79 and 684 µatm at 16:41, respectively.

4. Discussion

Diel fluctuations in dissolved oxygen (DO) and pH create episodic hypoxia conditions that alter the quality of shallow estuarine nursery habitats for juvenile fishes. In Moderate-Range treatments in which DO declined to ~3 mg O_2 l^{-1}, none of the species examined exhibited ASR behavior either with or without the associated moderate-range decline in pH and increase in pCO_2. Low pH and high pCO_2 values in these cases were either pH = 7.5; pCO_2 ~ 2000 µatm (static) or pH = 7.2; pCO_2 ~ 4300 (with pH and pCO_2 decline). No mortality occurred in these treatments. However, in Extreme-Range treatments where DO declined to ~1.0–1.6 mg O_2 l^{-1} (Table 3) ASR was performed by *M. menidia* and both *Fundulus* species, but not *M. saxatilis.* ASR occurred in treatments both with and without the associated extreme-range decline in pH and increase in pCO_2; low pH and high pCO_2 values in these cases were either pH = 7.5; pCO_2 ~ 2000 µatm (static) or pH = 6.8; pCO_2 ~ 10,200 (with pH and pCO_2 decline). In these treatments, ASR onset was observed in *M. menidia* at or near lethal levels (1.31–1.62 mg O_2 l^{-1}).

No evidence of a pH (pCO_2) effect on ASR or survival was found in any species in response to naturally co-varying DO and pH swings, despite pH as low as 6.8 and high pCO_2 levels of ≥ ~12,000 µatm. In *F. majalis*, there was no statistically detectable effect of treatment (static vs. cycling pH) on the DO threshold for ASR ($p = 0.47$). *M. menidia* initiated ASR between 1.31 and 1.62 mg O_2 l^{-1} under both 'Extreme-range' 4-hour low DO/pH treatments, and the first instances of individuals performing ASR occurred 1–2 h after the DO minimum was reached, regardless of whether pH was co-varying or not.

Recent work by Miller et al., 2016 reported that *M. menidia* initiated ASR at ~1.5 mg O_2 l^{-1} in response to declining DO only, but at higher DO levels (~2.2–2.5 mg O_2 l^{-1}) when exposed to low DO and low pH combined. The low pH (6.3–6.8) and high pCO_2 (~7400–22,800 µatm) levels used in their treatments were generally more extreme than those used in the present work. However, our results on *M. menidia* showed no evidence of a pH (pCO_2) effect on ASR and survival even at pCO_2 as high as ~12,000 µatm. It is probable there is a threshold level of pH/ pCO_2 above which diel acidification cycles begin to exert an interactive effect, with hypoxia, on ASR and survival in this species. Our work indicates that this level is at pH < 6.8 and pCO_2 > ~10,200 µatm. More research on such thresholds is warranted.

Very few studies have examined the independent and synergistic effects of co-varying hypoxia and pH on growth and behavior of estuarine fishes (DePasquale et al., 2015; Gobler and Baumann, 2016; Lifavi et al., 2017). The present research has shown that the occurrence of ASR in *M. menidia, F. majalis* and *F. heteroclitus* is not affected by diel cycles of low pH and high pCO_2 lasting at least 4 h. Our ASR results are consistent with the few recent studies available on growth effects of diel acidification cycles on young fishes; studies showing substantial tolerance to diel pH/pCO_2 cycles of similar magnitude (Davidson et al., 2016; Lifavi et al., 2017) and that the thresholds for negative pCO_2 effects are species-specific (DePasquale et al., 2015). CO_2 levels relevant to ocean acidification are generally considered to be < ~2000 µatm CO_2 (Heuer and Grosell, 2014), a value predicted for mean oceanic CO_2 by the year 2300 (Caldeira and Wickett, 2003). Studies of the potential impacts of chronic ocean acidification levels in this range and higher have reported variable impacts on juvenile and adult fishes (see supplemental Table 1 in Heuer and Grosell, 2014).

The ASR thresholds (1.31–1.62 mg O_2 l^{-1}) observed in *Menidia menidia* are lower than those previously reported by Smith and Able (2003) who state that *M. menidia* initiates ASR at 2.6 mg O_2 l^{-1}, and did not survive > 30 min at DO levels below 1.0 mg O_2 l^{-1}. Our results suggest that ASR thresholds in *M. menidia* are related to both the magnitude of hypoxia and duration of exposure: in each 4-hour treatment, the first instances of individuals performing ASR (at ~1.40 mg O_2 l^{-1}) occurred 1–2 h after the DO minimum was reached. However, in the extended 16-hour low DO treatments, individual fish first engaged in ASR between 1.5 and 8.5 h after the DO minimum was reached, but at slightly higher thresholds (~1.60 mg O_2 l^{-1}).

The DO range of initial mortality (LOE) observed in *M. menidia* was also lower than that reported in Smith and Able (2003) at

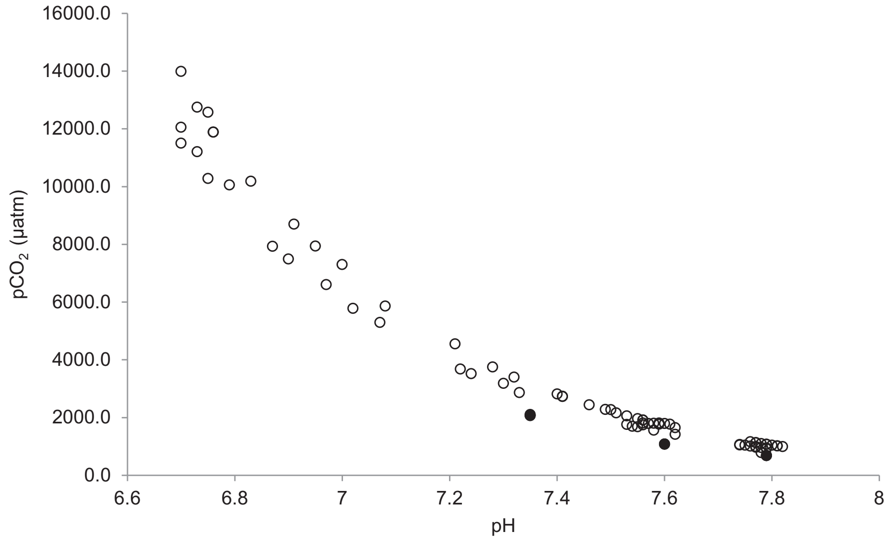

Fig. 5. pCO_2 and pH of water samples collected under laboratory conditions (open circles) and the water samples taken from Pepper Creek, Delaware (black circles).

$\sim$1.90 mg O_2 l^{-1}, but considerably closer to DO levels at ASR onset. Throughout extended 16-hour treatments, the DO threshold at which ASR commenced ($\sim$1.60 mg O_2 l^{-1}) was approximately that at which initial mortality occurred ($\sim$1.57–1.62 mg O_2 l^{-1}); there was no detectable difference between DO thresholds for ASR and morality (F (1,3) = 1.67, p = 0.71), reiterating that these fish are initiating the behavior very close to their lethal limits. Across all trials, the DO range over which onset of ASR occurs and that at mortality are equivalent, and at lethal DO limits all individuals were observed to be engaged in ASR. Moreover, once individual *M. menidia* initiated ASR, that fish engaged in ASR continuously until the end of the trial (or mortality), without starting and stopping discrete ASR bouts as was the case in both *Fundulus* species. This implies that *M. menidia* resort to using ASR in a "last-ditch" effort to prolong survival for minutes to hours at or near lethal DO levels. Delaying ASR until facing the threat of imminent hypoxia-induced mortality may be a reaction by *M. menidia* to potential predation risk associated with rising to the surface.

F. majalis initiated ASR at DO ranging from 1.05 to 1.20 mg O_2 l^{-1} in extended 16-hour low DO treatments – lower thresholds than those observed in 4-hour treatments (between 1.30 and 1.46 mg O_2 l^{-1}) with both static and cycling pH. *F. majalis* exposed to lower DO in 16-hour treatments (minimum 1.01–1.15 mg O_2 l^{-1} attained) also engaged in ASR much earlier than those in 4-hour treatments (minimum 1.28–1.36 mg O_2 l^{-1} attained), equal to approximately an hour after the DO low is reached in a typical diel cycle. Across all trials, ASR in *F. majalis* was observed to occur intermittently in short (averaging 1–2 min) discrete bouts that lengthened as trials progressed and often started and stopped over the course of several ($\geq$ 2) hours without mortality. ASR bout duration increased during periods when low DO was stable as well as when DO continued to decline, suggesting that ASR frequency and the initial DO threshold for ASR in *F. majalis* appear to be the function of both magnitude and duration of low DO exposure, as in *M. menidia.* There is no previously published evidence of ASR in this species.

In contrast, *Fundulus heteroclitus* did not engage in ASR during any 4-hour low DO/pH treatment, except for one individual at the very end of a trial (at 1.19 mg O_2 l^{-1}), 4 h and 25 min after reaching a minimum of 1.0 mg O_2 l^{-1}. This would suggest that when experiencing diel-cycling hypoxia, *F. heteroclitus* defer ASR for several hours unless low DO conditions are severe ($< \sim$1.10 mg O_2 l^{-1}). However, all fish engaged in ASR during the extended 16-hour DO treatments. Fish in these trials initiated ASR between 1.05 and 1.18 mg O_2 l^{-1}, comparable to the threshold of 1.09 mg O_2 l^{-1} reported by Smith and Able (2003), and generally not until after prolonged exposure ($\geq$ 4 h). ASR response in *F. heteroclitus* again appears to result from the extent of low DO exposure. This species may use ASR to reduce the growth limiting effects of chronic severe hypoxia on a scale of days to weeks (Stierhoff et al., 2003; Rees et al., 2012). Stierhoff et al. (2003) showed that growth rate of *F. heteroclitus* exposed to diel-cycling conditions (1.2–11 mg O_2 l^{-1}) was not statistically different than at normoxia, regardless of whether fish had access to the surface to perform ASR. However, at chronically low DO levels (3–9 days at 1.0–1.5 mg O_2 l^{-1}, 25 °C), ASR partially mitigated the impact of hypoxia on growth; fish performing ASR experienced a $\sim$ 60% reduction in growth compared to normoxia, but fish deprived of access to the surface did not grow at all. *F. heteroclitus* has also demonstrated a capacity to acclimate to pronounced (1.0 mg O_2 l^{-1}) and persistent hypoxia (Rees et al., 2012; Greaney et al., 1980), with reduced growth during an initial two-week exposure recovering to that at normoxia during a subsequent two weeks of hypoxia exposure.

ASR was not observed in juvenile *M. saxatilis* during 4-hour low DO/pH treatments, even at lethal DO levels, or during 16-hour exposure to 1.5–1.0 mg O_2 l^{-1}. Several studies have shown that growth is depressed below 4 mg O_2 l^{-1}, as juveniles reduce feeding and active swimming, and experience loss of equilibrium (Coutant, 1985; Cech et al., 1984; Breitburg et al., 1994; Brandt et al., 2009). In the current work, decreasing DO to below 2 mg O_2 l^{-1}, particularly under high amplitude fluctuations ("Extreme-Range" treatments), resulted in cessation of schooling, reduced activity such as resting on the tank bottom, and rheotaxis into the water inflow. Considering the rapid rate of mixing, it is unlikely that fish were responding to a diffusion gradient (Dixon, 2014); orienting into the inflow was likely an attempt to increase water flow over the gills (Freadman, 1979). It is likely that the ability to ram ventilate in our experimental apparatus enabled increased survivorship of *M. saxatilis* at DO levels that may typically be lethal in this species, illustrated by very low mortality (n = 1) after 6.5 h at $\leq$ 1.42 mg O_2 l^{-1} in 4-hour treatments, and no mortality over longer exposures at 1.5 and 1.0 mg O_2 l^{-1} without ASR. The lower lethal limit for juvenile *M. saxatilis* has been reported to be between 1 and 2 mg O_2 l^{-1} (Miller et al., 2002; Coutant, 1985). However, fish used in the 16-hour treatments were significantly larger ($\bar{x}$ = 173 mm) than those in 4-hour trials ($\bar{x}$ = 115 mm) and did not perform rheotaxis. It is possible therefore that fish smaller than those tested in the 16-hour treatments may undertake ASR under similar prolonged ($>$ 4 h) exposure.

Small fluctuations in the DO environment, evident in minor differences in minimum DO levels achieved among all trials, generated differences in the threshold at which an individual fish engages in ASR behavior. Comparing ASR thresholds with survival in individual fish raises the question of whether fish that initiate ASR earlier than their conspecifics ultimately exhibit more resistance to hypoxia-induced mortality, or whether early ASR indicates greater individual sensitivity. In *M. menidia*, 4-hour low DO/pH treatments, mortality (when observed) occurred approximately 70 min after ASR began, while in extended 16-hour treatments complete mortality of all subjects occurred between 30 and 120 min after ASR. Across all species, initiation of ASR was not simultaneous by all three individuals in a trial, and for *Menidia*, the first fish to undertake ASR were not consistently either the first or last to experience mortality, thus neither relationship is seen here to be evident.

We hypothesized that the tendency to perform ASR will be observed in *F. heteroclitus* > *F. majalis* > *M. menidia* > *M. saxatilis*, based on the presence of specific morphological adaptations (i.e., upturned mouths and dorso-ventrally flattened heads) and the current literature. However, based on our results – particularly DO range (mg $O_2\,l^{-1}$) at ASR onset – the potential to exhibit ASR under ~4-hour low DO/pH conditions occurring during diel cycles appears highest in *M. menidia*, followed by *F. majalis* and *F. heteroclitus*. Additionally, DO range at mortality (LOE) would suggest that *M. menidia* displays the lowest tolerance to similar exposures, regardless of static or cycling pH. In summary, these results illustrate the nuanced differences in a species' tendency to engage in ASR and its role in survival and adaptation to hypoxia. A species' behavioral response to hypoxia can have significant ecological consequences by influencing other behaviors such as habitat selection and predator-prey interactions. The use of compensatory behaviors like ASR rather than lateral emigration from hypoxic habitat represents important trade-offs. The relationship between fish behavior and a changing environment is especially relevant for these and other estuarine species that utilize habitats where stressors like low DO and acidification are expected to increase in severity due to climate change. Additional research determining species-specific ASR responses, and the associated role of ASR in improving hypoxia survival, in estuarine fishes will further characterize hypoxia's role as a modulator of structure and ecology in aquatic communities.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted, IACUC (AUP#: 1131-2014-1) from the University of Delaware.

Acknowledgments

The authors thank J. Cohen for advice and assistance with videography methodology and M. Davidson, M. Torre, and D. Lifavi for assisting with fish collection and care, lab maintenance, and behavioral observations. We are grateful to E. Markin, University of Maryland's Aquaculture Restoration and Ecology Laboratory, and D. Secor, University of Maryland Center for Environmental Science, for providing *M. saxatilis* specimens and assistance with care and rearing. We thank P. Gaffney for advice on statistical analyses. Additional thanks to W.-J. Cai and N. Hussain for conducting dissolved inorganic carbon analyses. We thank D. Secor for helpful comments on an early draft of this manuscript. The present work was supported financially and logistically by NOAA National Centers for Coastal Ocean Science, Center for Sponsored Coastal Ocean Research, through the Coastal Hypoxia Research Program (CHRP Grant number NA10NOS4780156 to T. Targett). This is CHRP Contribution No. 223. Mentioning commercial products does not imply endorsement. Experiments conducted in this study comply with IACUC (AUP#: 1131-2014-1) from the University of Delaware.

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