BEHAVIORAL RESPONSES OF COMMON JUVENILE ESTUARINE FISHES TO DIEL-CYCLING HYPOXIA AND CORRESPONDING PH FLUCTUATIONS: A COMPARATIVE APPROACH

by

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ABSTRACT

Diel-cycling hypoxia and co-varying pH fluctuations can reduce the quality of shallow estuarine nursery habitat for juvenile fishes. Avoidance or compensatory behaviors allow fish to mitigate stress associated with low dissolved oxygen (DO). Aquatic surface respiration (ASR), or irrigating gills with the thin, air-saturated, surface layer, is widespread among teleosts and provides an advantage to fish routinely exposed to hypoxia. ASR begins when oxygen becomes physiologically limiting, and serves as an indicator for hypoxia tolerance in a species. 24-hour video observations were conducted under controlled laboratory conditions using computer-monitored recirculating aquarium systems. Behavioral responses of juvenile striped bass (*Morone saxatilis*), Atlantic silversides (*Menidia menidia*), striped killifish (*Fundulus majalis*), and mummichog (*Fundulus heteroclitus*) were observed during exposure to different severities and combinations of diel-cycling DO (3-9 mg O_2 l^{-1} or 1-11 mg O_2 l^{-1}) and corresponding pH (7.2-7.8 or 6.8-8.1), compared with responses at static normoxia (7.5 mg O_2 l^{-1}) and pH (7.5). Trials ran from 11:00 – 11:00 each day; DO decreased overnight (6 hours) to the lowest DO level in both diel-cycles, with the hypoxic period lasting through the morning (4.5 hours) before trials ended and oxygen increased again. The incidence of ASR, activity level, and position of each individual was recorded at pre-determined time intervals. A second set of observations was conducted under conditions where lowest DO levels (1.0 - 1.5 mg O_2 l^{-1}) were prolonged throughout the day; DO was decreased

overnight from super-saturation (~10 mg O_2 l^{-1}) to the oxygen minima (6 hours), low levels were extended (18 hours) then DO was increased back to saturation (6 hours) during the second night. Based on results of the first set of trials, another set of video observations was conducted on *F. heteroclitus* that were acclimated to moderate levels of diel-cycling hypoxia and pH (3-9 mg O_2 l^{-1}; 7.2-7.8) for ≥10 days.

ASR was initiated in *M. menidia* and *F. majalis* only when diel-cycling DO fell to (1.40 – 1.60 mg O_2 l^{-1}) and (1.31 – 1.46 mg O_2 l^{-1}), respectively, with both cycling and static pH. In *M. menidia*, under extended low DO, all individuals exhibited ASR and mortality occurred in all fish shortly after onset, suggesting that ASR is a last-ditch effort to prolong survival. Onset of ASR among *F. majalis* under diel-cycling conditions varied among individuals; once initiated the behavior was intermittent with variable duration until the end of the trial, with no mortality observed. At corresponding DO levels, *M. saxatilis* reduced swimming activity and exhibited positive rheotaxis, but no ASR was observed. ASR was also not observed in *F. heteroclitus* in diel-cycling treatments, or in acclimated fish, but when low DO (1.0 mg O_2 l^{-1}) was prolonged, fish performed ASR after approximately 5 hours exposure. This suggests that in *F. heteroclitus*, the incidence of ASR is a combination of dissolved oxygen concentration and duration of exposure. Given trade-offs associated with repeated surfacing and skimming, mummichogs appear to delay engaging in ASR under short term low DO exposure occurring during diel-cycles. Utilization of ASR as an adaptive strategy is dependent upon species-specific hypoxia tolerance, and the temporal scale over which low oxygen occurs.

Chapter 1

BEHAVIORAL RESPONSES OF COMMON JUVENILE ESTUARINE FISHES TO DIEL-CYCLING HYPOXIA AND PH FLUCTUATIONS

Introduction

Estuaries are highly productive, dynamic and ecologically complex ecosystems that serve as a transition zone between freshwater rivers and streams and the marine environment (Peterson & Ross, 1991). The complexity of estuarine habitats is due in part to spatial and temporal heterogeneity in temperature, salinity, and dissolved oxygen (DO) conditions that provide a wide range of physiochemical habitats for benthic and nektonic organisms (Peterson & Ross, 1991; Hanks & Secor, 2011). In addition to full-time estuarine residents, many species of marine fish utilize shallow-water coastal ecosystems as nursery habitat during their larval and juvenile stages (Breitburg, 2002; Ross, 2003; Brady et al. 2009). "Nurseries" are defined as a subset of juvenile fish habitat that contributes disproportionately to recruitment to the adult population (Beck & Bruland, 2000). Estuarine nurseries promote growth and survival in early ontogenetic stages of juvenile fishes due to a combination of high nutrients, abundant prey resources, reduced predation risk, and physiologically optimal temperature and salinity regimes (Able, 1999). The behavioral and physiological responses of fishes to spatial and temporal estuarine variability help define the boundaries of potential nursery habitats (Hanks & Secor, 2011). Research on the effects of physiochemical fluctuations in the environment

on fish ecology will help develop an understanding of how future shifts in estuarine habitat quality may impact juvenile growth, recruitment to adult populations, and general fishery health.

Estuaries can undergo sudden, daily, and seasonal shifts in dissolved oxygen concentration, pH, salinity, and temperature on multiple spatial and temporal scales as a function of both biotic and abiotic processes (Boynton et al. 1996; Stierhoff et al. 2009**a**; Tyler et al. 2009; Howarth et al. 2011). For instance, seasonal changes in temperature alter the solubility of oxygen in water, resulting in seasonal variability in DO concentration (Diaz, 2001; Tyler et al. 2009). DO is also dependent on the biological oxygen demand (BOD); higher respiration rates in aerobic organisms at higher temperatures also lower dissolved oxygen levels (Buzzelli et al. 2002).

The susceptibility of a water body to hypoxia – oxygen concentrations below saturation – is dependent upon the physical characteristics of the system, the amount of productivity and nutrient enrichment, and water temperature (Cloern, 2001; Diaz & Rosenberg, 2008; Rabalais et al. 2009). Hypoxia and anoxia has become increasingly prevalent in estuarine and temperate coastal marine ecosystems worldwide since the 1960's, particularly driven by anthropogenic nutrient loading and coastal development (Diaz, 2001; Bell, 2005; Diaz & Rosenberg, 2008).

Estuaries along the east coast of the U.S. commonly experience some degree of severe hypoxia (≤ 2.0 mg O_2 l^{-1}) and anoxia (0 mg O_2 l^{-1}) during the summer months, due to higher average water temperatures and increased productivity (Eby & Crowder, 2002; Breitburg, 2002; Bell, 2005). The U.S. Environmental Protection Agency has established

"growth protective criteria" (≥ 4.8 mg O_2 l^{-1}) and a "survival protective criteria" (≥ 2.3 mg O_2 l^{-1}) for the more sensitive species within a saltwater community in the U.S. Mid-Atlantic Bight (USEPA, 2000).

Hypoxia occurs in three general temporal categories: seasonal/chronic (months to weeks); episodic (weeks to days) and diel-cycling (hours to minutes) (Tyler et al. 2009). Diel-cycling hypoxia occurs primarily in the photic zone above the pycnocline in deep-water environments, but can extend to the benthos in shallow water habitats. The day/night cycles of photosynthesis and respiration of phytoplankton and macrophyte communities are the driving forces behind diel-cycling hypoxia and determine the duration and amplitude of the fluctuations (Kemp & Boynton, 1980; Beck & Bruland, 2000; Grecay & Stierhoff, 2002; Tyler et al. 2009). The high sustained productivity in estuarine environments, especially in conjunction with warm temperatures, generates ideal conditions for diel-cycling hypoxia (Tyler et al. 2009). Over a single 24-hour period, DO may range from anoxia at night to hyperoxia (≥15 mg O_2 l^{-1}) during the day (Beck & Bruland, 2000), with the highest concentrations occurring in the late afternoon following daytime photosynthesis and the lowest concentrations immediately following dawn, after aerobic respiration consumes oxygen throughout the night (Tyler et al. 2007).

The pH of a given body of water has the potential to fluctuate on short and long-term scales resembling those of dissolved oxygen that contribute to hypoxic events. As the concentration of atmospheric carbon dioxide is rising due to human activities and climate change, the amount of CO_2 dissolving into seawater is increasing, resulting in an elevated CO_2 partial pressure (pCO_2). In local environments, pH swings associated with

fluctuating CO_2 concentrations and warming temperatures contribute to the high daily variability observed in estuaries. pH fluctuates on shorter scales (respiration-driven) in addition to the long-term pH reductions (atmospheric-driven) that contribute to global ocean acidification (Denman et al. 2011). Therefore, pH can co-vary with diel-cycling hypoxia. The day/night cyclic nature of net production and consumption drives diel fluctuation of aqueous carbon dioxide concentration. In highly productive shallow-water estuaries and coastal areas, a reduction in DO at night corresponds with a drop in ambient pH from metabolically-produced CO_2 (Howarth et al. 2011). During the day, CO_2 is taken up in solution by photosynthetic organisms, allowing pH to rise (Pörtner, 2008). The integrated effect of both DO and pH fluctuating within shallow estuarine ecosystems has the potential to cause negative biological effects, particularly on juvenile fishes utilizing these as nurseries during summer months. Fish are exposed to substantial fluctuations in dissolved oxygen, and the availability of DO is one physiochemical factor that limits habitat quality, distribution, growth, reproduction, and survival. In addition, it is relevant to observe the combined and integrated effects of pH and hypoxia, as co-varying pH carries the potential to exacerbate the impacts of hypoxia, particularly in organisms with a narrower range of pH tolerance, relative to effects of either stressor alone.

While physiological and biochemical responses provide regulatory mechanisms allowing fish to respond to variable environmental oxygen levels, behavioral responses provide additional flexibility to mitigate exposure to hypoxia (Bell, 2005; Richards et al. 2009). Many behavioral responses to low DO occur at oxygen levels higher than lethal

limits, and may be categorized into one of two main groupings (Richards et al. 2009) (1) avoidance of hypoxic areas; or (2) compensation for decreased oxygen availability. Since the effects of hypoxia on fish are ultimately related to the ability to detect and avoid potentially harmful environments, many fish utilize and engage in avoidance behaviors (Bell, 2005; Stierhoff et al. 2009**b**). Species exhibit differences in movement responses to hypoxia, so determining avoidance responses is integral for identifying species directly and indirectly at risk (Bell, 2005). Bell (2005) states that mobile organisms generally move to shallower refuge habitats that contain greater DO concentrations and higher prey abundance to avoid hypoxia. However, juveniles in particular must consider a trade-off between avoiding predation and seeking more suitable habitat for optimizing growth. Given species-specific tolerance and avoidance responses, estuarine finfish were found to be less likely to avoid episodic or diel-cycling hypoxia even when avoidance of chronic deep-water hypoxia is demonstrated (Bell, 2005).

When low levels of DO cannot be avoided, some of these sub-lethal hypoxia effects can also manifest themselves in juvenile fishes through reduced feeding and growth rates, and habitat compression (McNatt & Rice, 2004; Bell, 2005; Stierhoff et al. 2006; Hanks & Secor, 2011). Some fishes are known to increase their gill ventilation rates or ventilation stroke volume when confronted with severe hypoxia (≤ 4.0 mg O_2 l^{-1}) (Wannamaker & Rice, 2000; Richards et al. 2009). Fish also adjust spontaneous swimming speed; reducing their speed in order to conserve energy or ultimately increasing speed to avoid hypoxia exposure (Brady et al. 2009). These adaptations may

prove useful to fish in chronic or persistent hypoxia, but could have long-term metabolic consequences.

One of the major behavioral mechanisms for compensating for reduced oxygen availability and increasing oxygen uptake during intermittent hypoxia is aquatic surface respiration (ASR). Distinct from air-breathing, ASR involves rising to the surface and irrigating the gills in the thin, air-saturated surface layer that is richer in dissolved oxygen than the underlying bulk water (Kramer & McClure, 1982). Occasionally referred to as 'skimming' or 'gulping' behavior, ASR is widespread among teleosts (Richards et al. 2009). Several species known to engage in ASR possess specific morphological adaptations such as upturned dorsally-oriented mouths and dorso-ventrally flattened heads that increase the efficiency of the behavior (Lewis, 1970; Kramer & McClure, 1982; Stierhoff et al. 2003; Richards et al. 2009).

Among species that engage in ASR, there is a high degree of variability in the hypoxic oxygen partial pressure (pO_2) threshold for the behavior. A higher ASR threshold (i.e., higher DO level at which ASR initiates) generally indicates a lower hypoxia tolerance, corresponding to a higher threshold for the critical oxygen tension pO_2 (P_{crit}) - or the minimum amount of dissolved oxygen necessary to sustain basal aerobic metabolic rate. Described in the majority of species that exhibit ASR, the behavior typically follows a discrete profile where the fish does not initiate ASR until a low ambient pO_2, beyond which it becomes the dominant behavior (Kramer & McClure, 1982). The pO_2 threshold for ASR has been shown to increase with environmental temperature and fish body mass, and decrease with prior acclimation to hypoxia (Yang et

al. 2013). Thresholds can also lower in association with other physiological traits corresponding with improved hypoxia tolerance, such as increased gill surface area and increased hematocrit (Richards et al. 2009). ASR is a clear behavioral response, but it is a reflex driven by oxygen-sensitive chemoreceptors (Shingles et al. 2005). These chemosensory modalities, along with the relationship between the behavioral threshold and P_{crit}, indicates that ASR begins when oxygen becomes physiologically limiting. Several studies have illustrated that the widespread prevalence of ASR among teleosts, and the evolution of specific morphological adaptations, indicates that it provides a distinct advantage to fishes in hypoxic environments (Kramer & McClure, 1982; Kramer, 1987; Wannamaker & Rice, 2000). ASR has been shown to reduce mortality and mitigate the effects of hypoxia on growth when fish are allowed access to the surface (Secor & Gunderson, 1998; Stierhoff et al. 2003). Kramer (1987) argues some of the physiological and ecological trade-offs of rising to the surface, suggesting the increased locomotor activity required for repeated surfacing and skimming could have negative physiological effects on the fish's energy budget. Furthermore, increased time at the surface significantly increases the risk of aerial predation and may have different effects on predator-prey encounter rates (Kramer & McClure, 1982; Kramer, 1983; Richards et al. 2009). While the ability to perform ASR has clear costs and benefits in previously studied species, it remains unknown how widespread this adaptation is in other estuary-dependent fishes that are routinely exposed to diel-cycling hypoxia.

Striped bass (*Morone saxatilis*) are in the temperate sea bass family Moronidae, found along the Atlantic coast of North America from the St. Lawrence River into the

Gulf of Mexico (Brandt et al. 2009). Coutant (1985) stated that striped bass become physiologically stressed as DO decreases to 3 mg O_2 l^{-1}. Further studies emphasize this approximate threshold; stating that below 3.5 mg O_2 l^{-1}, growth is depressed as fish reduce active swimming and begin to appear agitated (Cech et al. 1984; Wannamaker & Rice, 2000; Brandt et al. 2009). In addition, the lower lethal limit for striped bass has been reported between 2 mg O_2 l^{-1} and 1 mg O_2 l^{-1}, but individuals exhibit a "range of responses" below 3 mg O_2 l^{-1}including inactivity, loss of equilibrium and mortality (Coutant, 1985). Previous studies have emphasized indirect hypoxia effects on striped bass, such as decreased prey encounter rates, reduced consumption and growth (Brandt et al. 2009). ASR has not previously been reported in striped bass, however, recently the behavior has been observed among species of the temperate perch family (Percichthyidae), so there is the potential for the behavior to extend to the closely related temperate basses (Moronidae) (McNeil & Closs, 2007).

The Atlantic silverside (*Menidia menidia*) is a small species of forage fish from the Northwest Atlantic, ranging from the Gulf of St. Lawrence to northeastern Florida (Conover & Ross, 1982). Common in the Chesapeake and Delaware Bays, Atlantic silversides are frequently studied due to their sensitivity to environmental changes and importance as a dominant prey source for higher trophic level organisms. Smith & Able (2003) studied the dynamics of several salt marsh fishes in response to hypoxic conditions. ASR was the dominant behavior exhibited by silversides during 5h exposure to dissolved oxygen declining from ~7 to ~1 mg O_2 l^{-1}, and they were found to be overall less tolerant of hypoxia than other species of Atherinidae, Cyprinodontidae and

Fundulidae. In their work, silversides initiated ASR at 2.6 mg O_2 l^{-1}, and did not survive longer than 30 minutes at DO levels below 1 mg O_2 l^{-1}, with mortality beginning around 1.9 mg O_2 l^{-1}.

Killifish in the Family Fundulidae have been extensively studied in their ability to engage in ASR at low oxygen levels and hypoxic conditions. The mummichog (*Fundulus heteroclitus*) is a euryhaline fish that inhabits shallow coastal waters from Newfoundland to Florida (Able & Fahay, 1998). With dorsally oriented mouths and dorso-ventrally flattened heads, mummichogs possess the key morphological adaptations to supplement their oxygen supply by using ASR, a well-studied behavior in this species (Lewis, 1970; Wannamaker & Rice, 2000; Stierhoff et al. 2003; Smith & Able, 2003; Richards et al. 2009). The use of ASR has been found to mitigate the effects of hypoxia on growth in this species (Stierhoff et al. 2003). However, at 25°C ASR was only found to mitigate growth effects under extreme constant hypoxia (1 mg O_2 l^{-1}) whereas there was no effect of ASR on growth under diel-cycling conditions (1-11 mg O_2 l^{-1}) when comparing growth rates of fish with and without access to the surface (Stierhoff et al. 2003). Furthermore, when exposed to constant severe hypoxia (1 mg O_2 l^{-1}) for 28 days, they have exhibited a capacity to acclimate to treatment and recover growth rate to near normoxic levels after the initial two weeks of exposure (Rees et al. 2012). The striped killifish (*Fundulus majalis*) also possesses morphological adaptations of a flattened head and upturned mouth, and inhabit many of the same regions prone to hypoxia as mummichogs. Striped killifish engage in ASR, but exhibit an overall lower tolerance to

prolonged hypoxia compared to their conspecifics (Woodley & Peterson, 2003; Nordlie, 2006).

As evidenced, a fish's behavioral response to hypoxia can have significant ecological consequences by altering other behavioral components including habitat selection and usage patterns, predator-prey interactions, parental care, competition, and social aggregation/schooling. By determining which species are impacted the greatest by hypoxia - and how they respond behaviorally - we may gain an understanding for the community-level impacts of hypoxia, particularly if specific trophic levels (predators vs. prey) are unequally affected. Understanding hypoxia's role in modulating ecological interactions can help predict cascading effects on aquatic communities, again particularly relevant to economically and ecologically important fisheries.

This study investigated the behavioral responses to dynamic DO conditions in several fishes common to the U.S. Mid-Atlantic Bight. A comparative approach was taken by examining swimming and ASR behavior in relation to the relative hypoxia tolerance of these species. The objectives of this study were to (1) examine swimming behaviors and whether or not *M. saxatilis, M. menidia, F. majalis,* and *F. heteroclitus* perform ASR under variable diel-cycling hypoxia regimes, (2) to determine the threshold for ASR in these species, when present, and (3) to determine the effects of prior acclimation and prolonged hypoxia on the frequency and extent of ASR behavior.

Materials and Methods

Fish collection and acclimation

Striped killifish and Atlantic silversides were collected by seine from lower Delaware Bay. Mummichogs were collected in minnow traps from Canary Creek adjacent to the University of Delaware's Hugh R. Sharp campus in Lewes, DE. Juvenile striped bass were obtained from aquaculture facilities at the University of Maryland's Aquaculture Restoration and Ecology Laboratory in Cambridge, MD. All fish were young of the year (YOY) based on length (Table 1) (Able & Fahay 1998) and were held in 350 liter re-circulating trays in the laboratory at 14h light: 10h dark photoperiod, 25 °C, a salinity of 12 ppt (striped bass) or 25 ppt (Atlantic silversides, striped killifish, and mummichogs), pH ~7 and normoxia for ≥14 days prior to experiments. With the exception of DO and pH, these were the conditions during subsequent experiments. Water temperature (25 °C) was chosen to be representative of conditions encountered by juveniles during summer months in periods of exacerbated hypoxia (Stierhoff et al. 2009**a**; Tyler et al. 2009). Fish were fed frozen mysid shrimp (*Mysis relicta*), with the exception of striped bass which were fed Ziegler Finfish Silver® 3 mm sinking pellets, twice daily at 09:00 and 17:00. All fish were fed *ad libitum* to encourage maximum consumption and uneaten food was removed 30 minutes after each feeding. Ammonia, nitrates, and nitrites were monitored, and 50% water changes were conducted as necessary to maintain water quality (0 ppm NH_4^+, 0 ppm NO_2^-, ≤ 35ppm NO_3^-).

Laboratory Set-Up

Behavioral experiments were conducted in computer-controlled recirculating aquarium systems in a temperature- and photoperiod-controlled laboratory (see Appendix A for full details). The apparatus was a redesign of the computer-interfaced DO monitoring and control device (Grecay & Stierhoff, 2002) previously developed for experiments analyzing hypoxia-effects on estuarine fishes (Stierhoff et al. 2006, Stierhoff et al. 2009**a,b**). The redesign allowed for monitoring and manipulation of both DO and pH simultaneously within each of five individual recirculating treatment systems (~415 liters each). At pre-determined intervals, the computer-sensor interface measured and recorded the DO and pH level within an aquarium system through solenoid-controlled seawater flow past the DO and pH probes. Following each reading, the software actuated corresponding gas solenoids to inject the appropriate compressed gases (air, CO_2, N_2, and O_2) to regulate DO and pH to achieve or maintain the desired levels. CO_2 and air are bubbled in to lower and raise the pH while N_2 and O_2 are added to lower and raise the DO, respectively.

To observe behavioral responses to DO and pH fluctuations, two observation tanks were installed, each within the circuitry of two of the existing aquarium systems (Figure A1). Each tank (56 x 22.86 x 44.45 cm; volume approximately 56.8 l) was positioned on top of the system; water was pulled from the sump into the bottom of the tank, which then overflowed through a standpipe back into the overflow tray which served as a reservoir for the observation tank. A mesh divider/diffuser was installed to prevent fish from moving behind the standpipe out of view. The tanks were airtight

through the use of glass lids sealed with rubber gaskets to ensure that DO conditions within the tank were the same as that controlled through the computer/sensor interface in the surrounding system. A handheld YSI meter (YSI 556 MultiProbe System) was used to validate DO and pH in the tanks daily during experiments.

Behavioral trials

DO and pH treatments were established to simulate the periodicity of diel-cycles observed in shallow estuarine tributaries from the Maryland Department of Natural Resources' "Eyes on the Bay" monitoring program (http://mddnr.chesapeakebay.net/eyesonthebay/index.cfm, Accessed June 10, 2013). Treatments were chosen to be representative of conditions encountered by juveniles during the first late spring, summer, and early fall growth seasons. Three severities and combinations of fluctuating DO and pH were used. Two diel-cycling DO treatments ("Mid-Range DO"=3-9 mg O_2 l^{-1}; "Extreme-Range DO"= 1-11 mg O_2 l^{-1}) and the corresponding diel-cycling pH levels ("Mid-Range pH"=7.2-7.8; "Extreme-Range pH"=6.8-8.1) were used, along with a static normoxic control (DO=7.5 mg O_2 l^{-1}) with static pH (pH=7.5) for comparison (Table 2). Each species was exposed to a total of five treatments **[A-E]**: each diel-cycling DO treatment with a static pH level and the corresponding diel-cycling pH treatment, as well as a normoxic/static pH control (Table 2). This approach allowed for the assessment of the effects of DO on fish behavior, as well as whether the addition of cycling pH had any additional effect. Two treatments could be run simultaneously, and the order of treatments was randomized, with four

replicate trials run for each treatment. This design produced a total of 20 trials (5 treatment levels x 4 replicates), run over a period of 10 days, for each species.

24-hour diel-cycling DO and pH trials

Continuous video observation was recorded using a single camcorder (Sony Handycam DCR-SR88) per tank. Camcorders were positioned approximately 75 cm from the front of the tank and 25 cm from the tank base. This position was as close as possible while ensuring the entire tank was in the field of view. Trials lasted 24 hours and were conducted from 11:00 – 11:00 each day, beginning immediately following the end of the lowest DO level, such that each trial began as DO was increasing.

Fish were randomly selected from acclimation trays, weighed (± 0.01 g) and measured (± 0.1 mm) and introduced to ASR observation tanks in groups of 3 for each trial. Observing fish in small groups allowed a closer representation of natural conditions, as preliminary trials indicated that single fish did not perform normal behaviors regardless of DO level (personal obs.). Food was withheld for 12 hours prior to being introduced into the observation tanks and fish were allowed to adjust to the tanks for 10-15 minutes prior to recording (as per Wannamaker & Rice, 2000). Observation tanks and camcorders were surrounded by black plastic tarp to reduce disturbance by the observer, and white posterboard served as a backdrop to more easily detect fish on recordings under low light conditions. Recording commenced after the adjustment period and continued until the following day. Camcorders were capable of 13 h continuous monitoring, so approximately half way through the trial video files were downloaded

from the camcorder and a new file began to ensure non-stop recording overnight during the critical period as DO decreased.

A 14h light: 10h dark photoperiod was maintained during all behavioral trials. Minimum DO concentrations were reached 30 minutes prior to the start of the light cycle (07:00) coinciding with lowest DO levels in diel-cycles around dawn. Red LED light (630 nm peak; full-width half-max 620-640 nm) placed approximately 38 cm from the front of the tank was used to illuminate tanks during dark hours. As DO decreased overnight to a minimum near dawn, it was imperative to adequately illuminate fish on the video recordings during this critical period for observing behaviors. Horodysky et al. (2010) compared visual function in piscivorous fishes in Chesapeake Bay, and striped bass are shown to have a normalized response of 10-15% of V_{max} to spectral signatures similar to those emitted by the LED bulbs. While this allows for a distinct "dark" photoperiod, minimal light levels may have been detectable, so lights were covered with a paper screen to minimize light intensity and still ensure detection by the camcorders.

Extended low dissolved oxygen trials

Based on results of initial experiments, a second set of video trials were conducted to observe behavior under circumstances where low levels of dissolved oxygen were prolonged and extended throughout the day (Tyler et al. 2009). Trials began at midnight (0:00) as DO was decreasing from super-saturation (~10 mg O_2 l^{-1}) to the oxygen minima over 6 hours, as under diel-cycling conditions tested previously. Low levels of static DO were then extended for 18 hours, and then DO increased back to saturation over the next 6 hours. This treatment was designed to elucidate the effects of

constant hypoxia on swimming behavior and the prevalence and threshold of ASR behavior (if present), and to determine the pO_2 where ASR ceases as oxygen availability increases. For each of the four species, the target DO minimum during constant hypoxia was determined based on results from diel-cycling trials. DO levels chosen were below any observed ASR thresholds to encourage behavioral responses to hypoxia, but above mortality thresholds to allow fish to survive under prolonged low oxygen (striped bass and Atlantic silversides: 1.5 mg O_2 l^{-1}; striped killifish and mummichogs: 1 mg O_2 l^{-1}). pH remained constant at 7.5 for the entire trial. In cases where mortality occurred in all three fish, the trial was ended when no individuals remained. Temperature, salinity, and photoperiod were as previously described for the diel-cycling hypoxia trials. With striped bass, due to elapsed time between running diel-cycling trials and the extended low DO trials, experimental fish for the latter were larger ($\bar{x}$=173 mm) and as a result the group size for each trial was reduced to two fish to avoid crowding in the observation tanks.

Prior acclimation to diel-cycling hypoxia in Fundulus heteroclitus

A third set of trials were conducted to test the effects of prior acclimation to diel-cycling fluctuations in DO and pH on swimming and ASR behavior of mummichogs. Previous research has shown that this species uses ASR and the potential role of prior acclimation in hypoxia tolerance (Greaney et al. 1980; Wannamaker & Rice, 2000; Stierhoff et al. 2003; Rees et al. 2012; Yang et al. 2013; Dan et al. 2014). Mummichogs were exposed to moderate levels of diel-cycling hypoxia and pH ("Mid-Range": 3-9 mg O_2 l^{-1}; pH 7.2-7.8) for ≥10 days. Following acclimation, 24-hour video trials were

conducted using the same initial five diel-cycling treatments **[A-E]** (Table 2). Fish were fed frozen mysid shrimp (*Mysis relicta*) *ad libitum* twice daily at 09:00 and 17:00.

Video and data analysis

Video recordings were analyzed by focusing on critical periods for hypoxia-induced behaviors. In order to maximize time and efficiency, observations were conducted on an "inversely protracted time series", so that recordings were analyzed more frequently as DO decreased and therefore the probability of response behaviors increased. Videos were watched for 1 minute every hour during DO incline (11:00-0:00) and subsequent overnight decline (0:00-06:30) then increased to 1 minute every 15 minutes at the lowest DO level in the specified treatment (1 or 3 mg O_2 l^{-1}). In total, 42 discrete 1 minute observations were made for each 24-hour recording during diel-cycling trials, and 87 discrete 1 minute observations were made for extended low-DO trials. Measured response variables (as per Smith & Able, 2003) were (1) aquatic surface respiration; (2) surface swimming; (3) mid-water swimming; (4) swimming near the bottom; and (5) resting on the bottom. ASR was distinguished from surface swimming by behaviors that caused the fish to be in direct contact with the water surface coupled with ventilation at the air-water interface. Surface or mid-water swimming was defined as swimming in either the top or middle third of the tank, whereas swimming in the lower third of the tank was distinguished from resting directly on the bottom. Visually dividing the tank into three horizontal sections provided an approximation of fish position in the water column relative to DO concentration at a given point during treatment. In addition to these four response variables, any additional notable behaviors were recorded

during each trial. Time intervals, watch duration, and response variables were chosen by pre-screening recordings for each species.

During each watch interval, the incidence of a behavior was recorded when at least one individual exhibited the response. When ASR was present, thresholds were reported as the DO and pH level where at least one individual first engaged in the behavior. Once ASR was observed in a particular fish, watch continued until ASR ceased, and duration of each ASR event was recorded for each individual. In the event that a watch interval contained fish already engaging in ASR, video was rewound to determine the point where the behavior began. The ranges of DO/pH thresholds for ASR onset for individuals were consolidated across trials, and were reported along with the range of duration times for discrete ASR bouts exhibited by individuals (Table 1). On occasions where mortality occurred, it was noted if that individual had been previously performing ASR, and the time and DO/pH level at loss of equilibrium (LOE) was noted.

Results

Variability in DO and pH regulation

Raising the pH of a system required bubbling compressed air, which prevented DO from reaching supersaturated concentrations. Therefore, in treatments where both DO and pH fluctuated on diel cycles, pH had to first reach the target level before DO could supersaturate (see Appendix A for full details). As a result, variability in pH regulation during each experiment generated fine scale differences in DO levels achieved during a given treatment. This variability made it difficult to maintain constant DO

levels at the high point of a diel cycle (the longer pH took to reach its target level, the amount of time fish were at supersaturated levels was reduced). In addition, at the low point of the diel cycle DO was not absolutely constant, as irregularities in pH prevented DO from maintaining minimum values (e.g. 1 mg O_2 l^{-1}). Therefore, "replicate" trials were not exact replicates in terms of minimum DO achieved. This variability allowed for a fine-scale assessment of responses during this critical period for hypoxia-induced behaviors at low DO levels.

24-hour diel-cycling DO and pH trials

<u>Morone saxatilis</u>

During treatment **[A]** ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; static pH=7.5), juvenile striped bass exhibited routine swimming and predominantly maintained position in the mid-water column at high DO levels (Figure 1). Individuals displayed group behavior, generally related to the direction of flow in the tank; fish swam in a counter-clockwise ellipse from the bottom right corner of the tank toward the top left corner. As DO decreased the group disbanded as individual fish reduced apparent swimming speed and erratically changed position in the tank. Below 2.0 mg O_2 l^{-1}, fish positioned directly in front of the mesh diffuser over the water inflow, exhibiting strong rheotaxis into the inflow current, and occasionally resting on the bottom of the tank. Occasionally an individual would maintain steady position at the top of the tank near the water surface, but was not in direct contact with the surface. No ASR was observed. Mortality occurred (n=1) in a single trial at approximately 09:30, DO=1.42 and pH=7.51 (Figure 1).

During treatment **[B]** ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; "Extreme-Range pH"=6.8-8.1) fish swam routinely and individuals formed close groups in the mid-water column or moved in counter-clockwise formation during high DO periods. As DO decreased, all individuals spent more time resting on the bottom, displaying strong rheotaxis into the inflow after approximately 30 minutes of exposure to DO levels $\leq$ 2.0 mg O_2 l^{-1}. No ASR was observed, and no mortality occurred in any trial (Figure 2).

In treatments **[C]** ("Mid-Range DO"=3-9 mg O_2 l^{-1}; static pH=7.5) and **[D]** ("Mid-Range DO"=3-9 mg O_2 l^{-1}; "Mid-Range pH"=7.2-7.8), fish performed routine swimming and aggregated in the mid-water throughout the entire trial. No ASR was observed, and no mortality occurred in any trial. Observed behavior was indistinguishable from fish exposed to treatment **[E]** (Control= 7.5 mg O_2 l^{-1}; pH=7.5).

Menidia menidia

Atlantic silversides exposed to treatment **[A]** ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; static pH=7.5) performed routine swimming and aggregated in the mid-water column during the day when DO levels were high (Figure 3). As DO decreased fish tended to orient toward the inflow, but did not maintain steady rheotaxis or rest on the bottom. At 06:30, once DO reached the low point in the diel cycle, fish rested on the bottom with occasional rapid surfacing. ASR was observed in the single trial with the lowest minimum DO, and began at 08:23, DO=1.49 mg/L and pH=7.1 (Figure 3). The threshold for ASR was varied among individuals, but once all three fish were engaged, each continued ASR until the end of the trial. No mortalities occurred.

During treatment **[B]** ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; "Extreme-Range pH"=6.8-8.1), fish aggregated in the mid-water column and exhibited routine swimming while DO was high (Figure 4). Once DO dropped below saturation (7.5 mg O_2 l^{-1}) fish rested on the bottom with occasional rapid surfacing. At minimum DO levels, fish initiated ASR behavior at 07:12, DO=1.40 mg O_2 l^{-1}, pH=6.95 in one trial and at 07:14, DO=1.42 mg O_2 l^{-1}, pH=6.94 in a second (Figure 4). In the trial with ASR initiating at 07:12, mortality occurred (n=1) at 08:20, DO=1.31 mg O_2 l^{-1}, pH=6.88 (Figure 4). At lethal limits, all individuals were engaging in ASR, and duration lasted until the end of the trial or mortality occurred.

In treatments **[C]** ("Mid-Range DO"=3-9 mg O_2 l^{-1}; static pH=7.5) and **[D]** ("Mid-Range DO"=3-9 mg O_2 l^{-1}; "Mid-Range pH"=7.2-7.8), fish performed routine swimming and grouping behavior and maintained position in the mid-water column. Behavior was indistinguishable from fish exposed to treatment **[E]** (Control= 7.5 mg O_2 l^{-1}; pH=7.5). No ASR was observed and no mortality occurred in either the Mid-Range or Control treatments.

Fundulus majalis

During treatment **[A]** ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; static pH=7.5), striped killifish aggregated in the mid-water column or near the bottom during the day at high DO levels; fish did not rest on the bottom nor did they orient in relation to the water inflow (Figure 5). Fish displayed strong group behavior, remaining in close contact throughout the trials. As DO decreased overnight, fish moved toward the top of the tank, remained in close groups and exhibited routine swimming near the surface. At minimum

DO levels, fish 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=1.4 mg O_2 l^{-1}, pH=7.5 (Figure 5). Individuals began ASR intermittently, and once it commenced individuals exhibited discrete bouts lasting between 1-18 minutes, increasing in duration as the trial continued. No mortality occurred.

During treatment **[B]** ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; "Extreme-Range pH"=6.8-8.1), fish again displayed strong group behavior and engaged in routine swimming in the mid-water column or near the bottom of the tank during high DO levels, moving vertically toward the water surface as DO decreased (Figure 6). ASR was observed in all four trials, fish initiated the behavior at (1) 7:10, DO=1.46 mg O_2 l^{-1}, pH=6.93, (2) 7:40, DO=1.4 mg O_2 l^{-1}, pH=6.93, (3) 7:50, DO=1.31 mg O_2 l^{-1}, pH=6.87, and (4) 8:01, DO=1.39 mg O_2 l^{-1}, pH=6.88 (Figure 6). Once fish first initiated ASR, individuals performed the behavior intermittently for the remainder of the trial, starting and stopping for variable lengths of time. No mortality occurred.

Fish did not perform ASR and no mortalities occurred during treatments **[C]** ("Mid-Range DO"=3-9 mg O_2 l^{-1}; static pH=7.5) or **[D]** ("Mid-Range DO"=3-9 mg O_2 l^{-1}; "Mid-Range pH"=7.2-7.8). All individuals exhibited routine group swimming in the mid-water column or near the bottom of the tank for the entire trial duration. Behavior was indistinguishable from fish exposed to treatment **[E]** (Control= 7.5 mg O_2 l^{-1}; pH=7.5).

Fundulus heteroclitus

During treatment **[A]** ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; static pH=7.5), fish aggregated in the bottom corners of the tank and around the mesh divider in front of the standpipe. Fish displayed strong group behavior, remaining in close contact throughout the trials. This position was maintained throughout, punctuated by short (minutes) bursts of routine swimming in the mid-water column, regardless of decreasing DO concentrations overnight. ASR was initiated by 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 (Figure 7). This bout of ASR lasted 5 minutes until the end of the trial. No mortalities occurred.

During treatment **[B]** ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; "Extreme-Range pH"=6.8-8.1), fish again displayed strong group behavior and aggregated in the bottom corners of the tank, favoring remaining near the mesh divider (Figure 8). Behavior was unchanged throughout the duration of the trial, even as DO decreased overnight. No individuals engaged in ASR, and no mortalities occurred (Figure 8).

Fish also did not perform ASR and no mortalities occurred during treatments **[C]** ("Mid-Range DO"=3-9 mg O_2 l^{-1}; static pH=7.5) **[D]** ("Mid-Range DO"=3-9 mg O_2 l^{-1}; "Mid-Range pH"=7.2-7.8). All individuals aggregated near the bottom corners of the tank or exhibited routine swimming in the mid-water column for the entire trials. Behavior was indistinguishable from fish exposed to treatment **[E]** (Control= 7.5 mg O_2 l^{-1}; pH=7.5).

Extended low dissolved oxygen trials

Morone saxatilis

For trials where the low DO period was extended throughout the following day (18 hours), the DO minimum was initially set at 1.5 mg O_2 l^{-1} for striped bass (Figure 9). Fish exhibited routine swimming during the entire trial, maintaining position in the mid-water. Once the DO minimum was reached, fish moved upward and maintained stationary position near the surface for several minutes at a time, but were not in direct contact with the water surface. No rheotaxis into the inflow was displayed and fish did not rest on the bottom of the tank. Fish did not exhibit ASR and no mortalities occurred during 18 hours of exposure to 1.5 mg O_2 l^{-1}. The DO minimum was changed to 1.0 mg O_2 l^{-1} for the second set of two trials to further elucidate hypoxia-induced behavior and mortality, and none were observed (Figure 9).

Menidia menidia

The DO minimum was set at 1.5 mg O_2 l^{-1} in Atlantic silversides. As DO decreased, fish generally engaged in routine swimming, with occasional surfacing and increased rheotaxis into the inflow as minimum DO values were approached. Fish engaged in ASR in all four trials: (1) 7:58, DO=1.59 mg O_2 l^{-1}, pH=7.52; (2) 12:37, DO=1.59 mg O_2 l^{-1}, pH=7.52, (3) 14:49, DO=1.6 mg O_2 l^{-1}, pH=7.51, and (4) 15:02, DO=1.59 mg O_2 l^{-1}, pH=7.52 (Figure 10). Although individual fish initiated ASR independently at slightly different DO levels, all three were engaged near their lethal limit, and ASR continued until mortality. Consequently, mortality of all three individuals occurred in each trial within 30 to 120 minutes following initiation of ASR: (1) 8:29,

DO=1.57 mg O_2 l^{-1}, pH=7.51, (2) 14:46, DO=1.57 mg O_2 l^{-1}, pH=7.51, (3) 15:35, DO=1.59 mg O_2 l^{-1}, pH=7.52, and (4) 16:33, DO=1.62 mg O_2 l^{-1}, pH=7.52 (Figure 10). Trials were ended once full mortality occurred, after ≤ 10 hours of low DO exposure.

Fundulus majalis

DO minimum was set at 1.0 mg O_2 l^{-1} in *F. majalis* and due to restricted numbers of experimental fish available of comparable sizes to those used in the diel-cycling trials, only two extended low-DO trials were conducted (Figure 11). As DO values decreased, fish aggregated in the mid-water column, occasionally moving upward towards the water surface as the DO minimum was reached. Individuals exhibited ASR intermittently, starting and stopping discrete bouts lasting between approximately 1 – 20 minutes each throughout the remainder of the trial. In both trials fish first engaged in ASR (1) 7:31, DO=1.08 mg O_2 l^{-1}, pH=7.51, and (2) 12:33, DO=1.2 mg O_2 l^{-1}, pH=7.51 (Figure 11). No mortalities occurred.

Fundulus heteroclitus

The DO minimum during extended trials was set at 1.0 mg O_2 l^{-1} for *F. heteroclitus*. As DO decreased overnight, fish aggregated in the bottom corners of the tank, occasionally swimming in the mid-water column but predominantly favoring the structure of the mesh divider. In three of the four trials, fish initiated ASR approximately 5 hours after the DO minimum was reached, or 30 minutes past the end of the low DO period in the previously prescribed diel-cycling treatments. Mummichogs 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 (Figure 12). In the fourth trial, fish initiated

ASR earlier, within the low DO period of the diel-cycle, at 8:06, DO=1.06 mg O_2 l^{-1}, pH=7.52 (Figure 12). Individuals exhibited ASR intermittently, starting and stopping for durations ranging between approximately 1 – 30 minutes throughout the remainder of the trial. No mortalities occurred in any trials.

Prior acclimation to diel-cycling hypoxia in Fundulus heteroclitus

Mummichogs that had been acclimated to "Mid-Range": 3-9 mg O_2 l^{-1}; pH 7.2-7.8 (Treatment **[D]** conditions) for ≥10 days, exhibited the same behavior as those fish exposed to treatments following normoxia. Fish displayed strong group behavior, aggregating in the bottom corners of the tank, showing affinity for the mesh divider adjacent to the standpipe. Occasional routine swimming in the mid-water column occurred, but under all diel-cycling treatments **[A-D]** behavior was indistinguishable from mummichogs under treatment **[E]** (Control= 7.5 mg O_2 l^{-1}; pH=7.5) and non-acclimated fish. No fish engaged in ASR and no mortalities occurred.

Discussion

The four Mid-Atlantic species observed in this study represent a continuum of potential to engage in aquatic surface respiration as a compensatory avoidance behavior under diel-cycling fluctuations. YOY of all species are exposed to diel-cycling hypoxia and pH fluctuations in shallow-water estuarine nursery grounds (Breitburg, 2002). They represent a spectrum of relative hardiness and tolerance to hypoxia, from the mummichog that is very tolerant of oxygen variability and high temperatures (Steirhoff et al. 2003; Rees et al. 2012), to the striped bass that is much less tolerant of low levels of dissolved

oxygen and lack morphological characteristics specific to the ASR response (Coutant, 1985; Brandt et al. 2009). Both *Fundulus* species possess unique morphological adaptations such as dorso-ventrally flattened heads and upturned mouths that greatly increase the efficiency of ASR. The incidence of ASR in these four species under diel-cycling fluctuations in DO and pH is a reflection of species-specific utilization of behavior as an adaptive strategy, and is contingent upon the extent and duration of exposure to low dissolved oxygen.

No ASR was observed in striped bass during diel-cycling DO and pH treatments, even at lethal levels (Table 3). In general, decreasing DO, particularly under high amplitude fluctuations ("Extreme-Range"), resulted in fish abandoning group behavior and routine swimming in favor of reducing activity level, resting on the bottom of the tank, and maintaining a strong positive rheotaxis into the direction of the inflow once DO dropped below 2.0 mg O_2 l^{-1}. It should be emphasized that there was no DO gradient present within the tanks, and any advantage of orienting into the water inflow would have been to directly increase water flow over the gills (Freadman, 1979) (see Appendix A for full details). The ability to ram ventilate in this experimental apparatus could have led to increased survivorship of striped bass at such low DO levels, and the observation of rheotaxis as DO decreased. Across all diel-cycling trials, even under extreme hypoxia for 6.5 hours, there was very low mortality (n=1). This demonstrates that even at lethal limits striped bass do not attempt to engage in ASR behavior. In the extended static low-DO level experiments, fish did not display ASR, and no mortality was observed over 18 hours at 1.5 and 1.0 mg O_2 l^{-1}. These results show that YOY striped bass are tolerant of

DO levels as low as 1.0 mg O_2 l^{-1} even in the absence of ASR behavior. However, striped bass used in the extended trials were larger ($\bar{x}$=173 mm) than those used in diel-cycling trials ($\bar{x}$=115 mm). Observed behavior of these fish seemed to indicate a higher tolerance to extended periods of low dissolved oxygen; fish maintained position in the mid-water column through the duration of the experiment, with no resting on the bottom or strong rheotaxis evident under decreasing or low DO. Thus, larger body mass may have contributed to increased hypoxia tolerance in these fish. Although respiratory surface area generally matches metabolic rate over a wide size range, differences in hypoxia tolerance can be seen across fish of variable sizes due to adaptation to different lifestyle or habitat choice (Nilsson et al. 2008). During severe hypoxia or anoxia, fish rely more heavily on anaerobic ATP production (glycolysis) for survival. Larger individuals have an advantage over smaller ones, due to the fact that small fish will a) run out of glycogen faster, or b) reach lethal levels of anaerobic end-products (i.e. lactate) sooner due to higher mass-specific metabolic rates (Burleson et al. 2001; Nilsson et al. 2008). In studies analyzing scaling effects on hypoxia tolerance, hypoxia survivorship is shown to increase as fish grow due to increasing "anaerobic power", or ability to survive with high levels of anaerobic end-products (Almedia-Val et al. 2000). However, in the context of this study, there still exists the possibility that fish smaller than those tested in extended trials may be capable of engaging in ASR under prolonged exposure.

In Atlantic silversides, ASR was observed only under extreme DO cycles, both with static and cycling pH. Across all trials, fish initiated ASR between 1.40 and 1.60 mg O_2 l^{-1}. This species also possesses a flattened head and upturned mouth, which helps

facilitate ASR behavior. Incidence of ASR was not uniform among trials, due to the slight variability in DO and pH regulation during the critical low-DO period. Due to regulation of DO and pH simultaneously, slight differences in minimum DO achieved among trials allowed for a fine-scale assessment of responses, and a greater precision for determining exact DO levels when ASR initiated (Table 3). During the extended low DO trials, variability in the minimum DO levels maintained was evident in the irregularities in the time until ASR thresholds were met (Table 4). Although the first occurrence of ASR ranged between 1.5 – 8.5 hours after the DO minimum was reached, the DO threshold was almost identical (1.59 mg O_2 l^{-1}) across extended low-DO trials (Figure 10). The ASR thresholds observed in Atlantic silversides in the present study are lower than those previously reported. Smith and Able (2003) reported that silversides initiated ASR at 2.6 mg O_2 l^{-1}, and did not survive longer than 30 minutes at DO levels below 1 mg O_2 l^{-1}, with mortality beginning around 1.9 mg O_2 l^{-1}. In the present work, once ASR thresholds were reached and at least one individual initiated ASR, individuals would start ASR at slightly variable DO levels, which appeared to be independent of fish size or whether other individuals were performing the behavior. Fish performed the behavior for the remainder of the trials or until mortality occurred, and at either the end of the trial or at lethal limits all three fish were engaged.

ASR thresholds in Atlantic silversides are the result of both the extent of low dissolved oxygen, and the duration of exposure. In diel-cycling trials, fish first engaged in ASR (~1.4 mg O_2 l^{-1}) 1-2 hours after the DO minimum was reached. In the extended low-DO trials, the DO minimum was increased to 1.5 mg O_2 l^{-1} in order to ensure

survival for the duration of the experiment, and all fish still engaged in ASR, albeit at higher thresholds (1.59 mg O_2 l^{-1}), 1.5-8.5 hours after the DO minimum was reached.

In the case of Atlantic silversides, the utilization of ASR appears to be a "last ditch" effort to prolong survival on a magnitude of minutes under conditions of extreme diel hypoxia or prolonged low oxygen. The DO range of initial mortality was lower than that reported in Smith and Able (2003), and much closer to the DO threshold for initiation of ASR. In all trials, at lethal limits (1.31 – 1.62 mg O_2 l^{-1}) all individuals were engaged in ASR. LOE, when present, occurred approximately 60 minutes after ASR began in diel-cycling treatments, and under extended trials full mortality occurred 30 to 120 minutes following initiation of ASR. Furthermore, once fish initiated ASR it was continuous until the end of the trial (or until mortality), without any start/stop of discrete ASR bouts as seen in *Fundulus* species. In addition, in preliminary trials (results not shown) where the low DO period for diel-cycling treatment (1.0 mg O_2 l^{-1}) was extended for 1 hour, mortality occurred in all three fish. This "last ditch" ASR behavior by Atlantic silversides may be a result of survival trade-offs associated with ventilating at the surface. It may be that the predation risk associated with ASR in this species precludes such behavior until threatened by hypoxia-induced mortality. Further emphasizing this point, during extended trials for Atlantic silversides, the DO threshold at initial mortality (~1.57 – 1.62 mg O_2 l^{-1}) was very close to that where ASR commenced (1.59 mg O_2 l^{-1}) in individuals, highlighting that these fish are engaging in ASR at DO levels very close to lethal limits, apparently in a final attempt to prolong survival.

In striped killifish, ASR was also observed only under the extreme DO diel cycle, both with static and cycling pH (Table 3). Fish initiated ASR between 1.31 and 1.46 mg O_2 l^{-1} under diel-cycling treatments. Previous research has been conducted on ASR in this species, although morphological adaptations of the head and mouth suggest it engages in ASR under hypoxic conditions (Woodley & Peterson, 2003). Again, minor differences in minimum DO achieved among trials allowed for an assessment of responses to fine scale differences in low DO levels. ASR began intermittently in striped killifish, with individual bouts of ASR varying in length. Initially, fish moved to the surface and engaged in ASR in short bursts, on average only 1 – 2 minutes in duration. As the trial continued, the duration of these bursts lengthened, lasting up to ~ 18 minutes, between which individuals would return position to the mid-water column for seconds to minutes before returning to the surface.

In extended low-DO treatments, striped killifish initiated ASR at levels below thresholds observed in diel-cycling treatments (1.08 and 1.20 mg O_2 l^{-1}) (Table 4). ASR thresholds again appear to be the result of both the extent of low dissolved oxygen and duration of exposure. In the treatment where ASR was initiated at 1.08 mg O_2 l^{-1}, the behavior started very early, only an hour after the DO reached the minimum value in a typical diel-cycle. ASR thresholds in the present study were reported as the DO when at least one individual engaged in ASR, and due to the intermittent starting and stopping of ASR bouts of variable duration, there may be a degree of individual variability in the precise DO threshold and duration of low DO exposure that triggers an individual to engage in ASR.

In contrast, in mummichogs ASR did not occur under any diel-cycling treatment conditions except for a single trial, at the very end (1.19 mg O_2 l^{-1}) (Table 3). Variability in minimum DO achieved among trials allowed for a precise fine-scale assessment of responses at critically low levels. However, all fish engaged in ASR during the extended low-DO treatments (Table 4). Fish initiated ASR between 1.06 and 1.19 mg O_2 l^{-1}, approximately the same as the 1.09 mg O_2 l^{-1} level reported by Smith and Able (2003). This species did not engage in ASR until after prolonged exposure (≥ 5 hours), except in the single case where DO was extremely low (1.06 mg O_2 l^{-1}), showing that under extreme hypoxia mummichogs will surface and exhibit ASR after shorter exposure. These results suggest that under diel-cycling hypoxia, mummichogs defer engaging in ASR, unless low DO conditions extend beyond ≥ 5 hours, in which case they have the capability of occasionally utilizing ASR as a way to mitigate negative effects of prolonged hypoxia.

In a study by Stierhoff et al. (2003), growth rate of mummichogs exposed to diel-cycling treatments 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}), ASR clearly mitigated negative growth effects; growth was negative in fish restricted access to the surface and prevented from engaging in ASR. Furthermore, in a study by Rees et al. (2012), growth rate in mummichogs was significantly reduced during an initial two week exposure to static severe hypoxia (1.0 mg O_2 l^{-1}), but during subsequent two-week exposure, growth rates no longer differed from that at normoxia. This demonstrates that mummichogs have the capacity to

acclimate to pronounced hypoxia when conditions persist beyond 14 days. In addition, research has shown that after 3 weeks of acclimation to hypoxic exposure, mummichogs cease performing ASR altogether, as physiological and enzymatic adaptations have had time to develop in white skeletal muscle (Greaney et al. 1980). By being able to delay ASR past the temporal scale of low DO during diel-cycling hypoxia, mummichogs avoid any negative trade-offs of performing ASR (such as increased risk of predation).

If fish routinely exposed to hypoxia are able to adjust aspects of their physiology after prolonged exposure, then hypoxia-induced fish behavior can differ between fish that have acclimated to extended hypoxic periods and those that have not. Yang et al. (2013) found that when juvenile southern catfish (*Silurus meridionalis*) were acclimated to diel-cycling hypoxia (7:00-21:00, DO=7.0±0.2 mg O_2 l^{-1}; 21:00-7:00, DO=3.0±0.2 mg O_2 l^{-1}) for 15 days, the oxygen thresholds for P_{crit}, ASR, and LOE were significantly lower than those of non-acclimated fish. Interestingly, growth rate decreased in the acclimated group as compared to the non-acclimated group. Acclimation to hypoxic conditions, as fish in shallow water estuaries naturally undergo, may adjust maintenance energy expenditure and metabolic scope, contributing to a species overall hypoxia tolerance. In the trials conducted on acclimated mummichogs in the present study, there was no observed change in ASR, avoidance behaviors, or mortality, as expected.

ASR or other avoidance behaviors indicating stress (erratic movement, repeated surfacing) did not occur under exposure to any moderate ("Mid-Range") diel cycles **[C]** and **[D]**. Observed behavior in these trials was indistinguishable from behavior of fish under control, normoxia conditions **[E]**. Circadian behaviors did not seem to influence

ASR incidence or duration in any species, as ASR occurred during both the day and night phase. Although experimental pH fluctuations were designed to mimic naturally co-varying DO and pH swings in the environment, no effect of pH was discernable. When present, ASR occurred under extreme DO conditions regardless of whether pH was static or cycling. This corroborates that ASR is initiated when oxygen becomes physiologically limiting, a behavior driven by oxygen-sensitive chemoreceptors (Richards et al. 2009).

ASR is a behavioral component of a complex chemosensory response, but it is relatively plastic. Results from the present research demonstrate that it is utilized for different strategies by different species. ASR serves as a behavioral strategy to prolong survival in Atlantic silversides for minutes to hours, and serves as a means to ameliorate negative growth effects in mummichogs on a scale of days to weeks, with striped killifish intermediary between the two.

Several studies have shown that ASR is an alternate behavior to lateral emigration from hypoxic habitat, and may have different effects on prey susceptibility and predator-prey encounter rates (Kramer & McClure, 1982; Domenici et al. 2007). In the case of predation risk for example, fish have been shown to modulate ASR behavior in response to the perception of risk. In a study by Shingles et al. (2005), fish came to the surface to perform ASR more frequently under a sheltered area or increased surfacing behavior in turbid water, indicating that the behavioral component of the ASR "reflex" is "relatively plastic" (Woodley & Peterson, 2003; Richards et al. 2009). These behaviors give juvenile fish greater flexibility in shallow water estuaries in coping with wide daily swings in DO availability.

As a result, a fish's behavioral response to hypoxia can have ecological consequences for how it interacts in its environment, by altering other behavioral components including habitat selection and usage patterns, predator-prey interactions, parental care, competition, and social aggregation/schooling. By determining which species are impacted the greatest by hypoxia - and how they respond behaviorally - we gain an understanding for the community-level impacts of hypoxia, particularly when specific trophic levels (predators vs. prey) are unequally affected. In addition, as the ability to perform ASR is shown to be species-specific, this behavior may allow some species to inhabit hypoxic regions that less tolerant species cannot. Understanding hypoxia's role in modulating ecological interactions can help predict cascading effects on aquatic communities.

Table 1. Fish size, summary of dissolved oxygen (DO) level at onset of aquatic surface respiration (ASR) behavior, ASR duration, and DO at loss of equilibrium (LOE) across diel-cycling and static DO and pH regimes. Mean size refers to fish used in diel-cycling trials. Range of values for DO level at ASR onset and DO at LOE are reported as the DO where at least one individual first exhibited the response. Duration range refers to duration of discrete ASR events collectively exhibited by individuals.

Species	**Mean Size (mm ±SE)**	**DO Range (mg O_2 l^{-1}) of ASR onset**	**Duration Range of Discrete ASR event (min)**	**DO Range (mg O_2 l^{-1}) of LOE**
Morone saxatilis	115 ± 0.97	--	--	1.42
Menidia menidia	79 ± 0.88	1.40-1.60	36-228	1.31-1.62
Fundulus majalis	67 ± 1.32	1.08-1.46	1-18	--
Fundulus heteroclitus	60 ± 0.92	1.06-1.19	1-30	--

Table 2. Summary of diel-cycling DO/pH treatments. (n=4 replicates; groups of 3 fish)

	DO=1-11 mg O_2 l^{-1}	**DO=3-9 mg O_2 l^{-1}**	**DO=7.5 mg O_2 l^{-1}**
pH = 7.5	Extreme-range DO, static pH **A**	Mid-range DO, static pH **C**	[Control] Normoxia, static pH **E**
pH= 7.2-7.8 **pH= 6.8-8.1**	Extreme-range DO and pH **B**	Mid-range DO and pH **D**	

Table 3. Overview of "Extreme" (1-11 mg O_2 l^{-1}) diel-cycling treatments with static and cycling (6.8-8.1) pH for all species. Minimum dissolved oxygen (DO) value attained in each trial, with DO level at onset of aquatic surface respiration (ASR) behavior, ASR duration, and DO at mortality (LOE), reported for each individual.

	Treatment Description	DO Minimum	DO Range (mg O_2 l^{-1}) of ASR Onset	Duration (mins)	DO Range (mg O_2 l^{-1}) of LOE
Morone saxatilis	Extreme DO; static pH – Rep 1	1.22	--	--	--
	Extreme DO; static pH – Rep 2	1.39	--	--	1.42
	Extreme DO; static pH – Rep 3	1.36	--	--	--
	Extreme DO; static pH – Rep 4	1.42	--	--	--
	Extreme DO; cycling pH – Rep 1	1.19	--	--	--
	Extreme DO; cycling pH – Rep 2	1.09	--	--	--
	Extreme DO; cycling pH – Rep 3	1.14	--	--	--
	Extreme DO; cycling pH – Rep 4	1.40	--	--	--
Menidia menidia	Extreme DO; static pH – Rep 1	1.84	--	--	--
	Extreme DO; static pH – Rep 2	1.55	--	--	--
	Extreme DO; static pH – Rep 3	1.55	--	--	--
	Extreme DO; static pH – Rep 4	1.17	1.49, 1.46, 1.45	157, 138, 136	--
	Extreme DO; cycling pH – Rep 1	1.34	--	--	--
	Extreme DO; cycling pH – Rep 2	1.59	--	--	--
	Extreme DO; cycling pH – Rep 3	1.29	1.42, 1.41, 1.31	226, 224, 187	--
	Extreme DO; cycling pH – Rep 4	1.23	1.40, 1.41, 1.36	228, 223, 17	--, --, 1.31
Fundulus majalis	Extreme DO; static pH – Rep 1	1.86	--	--	--
	Extreme DO; static pH – Rep 2	1.42	--	--	--
	Extreme DO; static pH – Rep 3	1.41	1.40, 1.40, 1.41	40, 37, 10	--
	Extreme DO; static pH – Rep 4	1.37	1.46, 1.41, 1.40	135, 120, 116	--
	Extreme DO; cycling pH – Rep 1	1.34	1.46, 1.44, 1.41	230, 221, 217	--
	Extreme DO; cycling pH – Rep 2	1.34	1.40, 1.40, 1.38	200, 198, 195	--
	Extreme DO; cycling pH – Rep 3	1.28	1.31, 1.30, 1.30	190, 188, 187	--
	Extreme DO; cycling pH – Rep 4	1.38	1.39, 1.39, 1.38	179, 175, 169	--

Table 3. cont.

Fundulus heteroclitus	Extreme DO; static pH – Rep 1	1.49	--	--	--
	Extreme DO; static pH – Rep 2	1.42	--	--	--
	Extreme DO; static pH – Rep 3	1.24	--	--	--
	Extreme DO; static pH – Rep 4	1.19	1.19, --, --	5, --, --	--
	Extreme DO; cycling pH – Rep 1	1.56	--	--	--
	Extreme DO; cycling pH – Rep 2	1.61	--	--	--
	Extreme DO; cycling pH – Rep 3	1.19	--	--	--
	Extreme DO; cycling pH – Rep 4	1.15	--	--	--

Table 4. Overview of extended low-level treatments for all species. DO level was determined by results of diel-cycling trials; pH static (7.5). Minimum dissolved oxygen (DO) value attained in each trial, with DO level at onset of aquatic surface respiration (ASR) behavior, ASR duration, and DO at mortality (LOE), reported for each individual.

	Treatment Description	DO Minimum	DO Range (mg O_2 l^{-1}) of ASR Onset	Duration (mins)	DO Range (mg O_2 l^{-1}) of LOE
Morone saxatilis	Rep 1 – DO 1.5 mg O_2 l^{-1}	1.42	--	--	--
	Rep 2 – DO 1.5 mg O_2 l^{-1}	1.41	--	--	--
	Rep 3 – DO 1.0 mg O_2 l^{-1}	1.18	--	--	--
	Rep 4 – DO 1.0 mg O_2 l^{-1}	1.17	--	--	--
Menidia menidia	Rep 1 – DO 1.5 mg O_2 l^{-1}	1.57	1.59, 1.58, 1.59	61, 54, 47	1.57, 1.58, 1.57
	Rep 2 – DO 1.5 mg O_2 l^{-1}	1.53	1.60, 1.61, 1.60	55, 46, 69	1.57 ,1.59, 1.59
	Rep 3 – DO 1.5 mg O_2 l^{-1}	1.59	1.59, 1.61, 1.63	91, 98, 112	1.62, 1.62, 1.61
	Rep 4 – DO 1.5 mg O_2 l^{-1}	1.53	1.59, 1.59, 1.57	31, 115, 118	1.57, 1.60, 1.55
Fundulus majalis	Rep 1 – DO 1.0 mg O_2 l^{-1}	1.01	1.08, 1.05, 1.06	990, 975, 964	--
	Rep 2 – DO 1.0 mg O_2 l^{-1}	1.15	1.20, 1.18, 1.19	387, 379, 372	--
Fundulus heteroclitus	Rep 1 – DO 1.0 mg O_2 l^{-1}	1.14	1.16, 1.16, 1.15	758, 753, 746	--
	Rep 2 – DO 1.0 mg O_2 l^{-1}	1.15	1.18, 1.16, 1.17	640, 635, 633	--
	Rep 3 – DO 1.0 mg O_2 l^{-1}	1.13	1.14, 1.14, 1.13	587, 584, 583	--
	Rep 4 – DO 1.0 mg O_2 l^{-1}	1.02	1.06, 1.05, 1.05	960, 945, 941	--

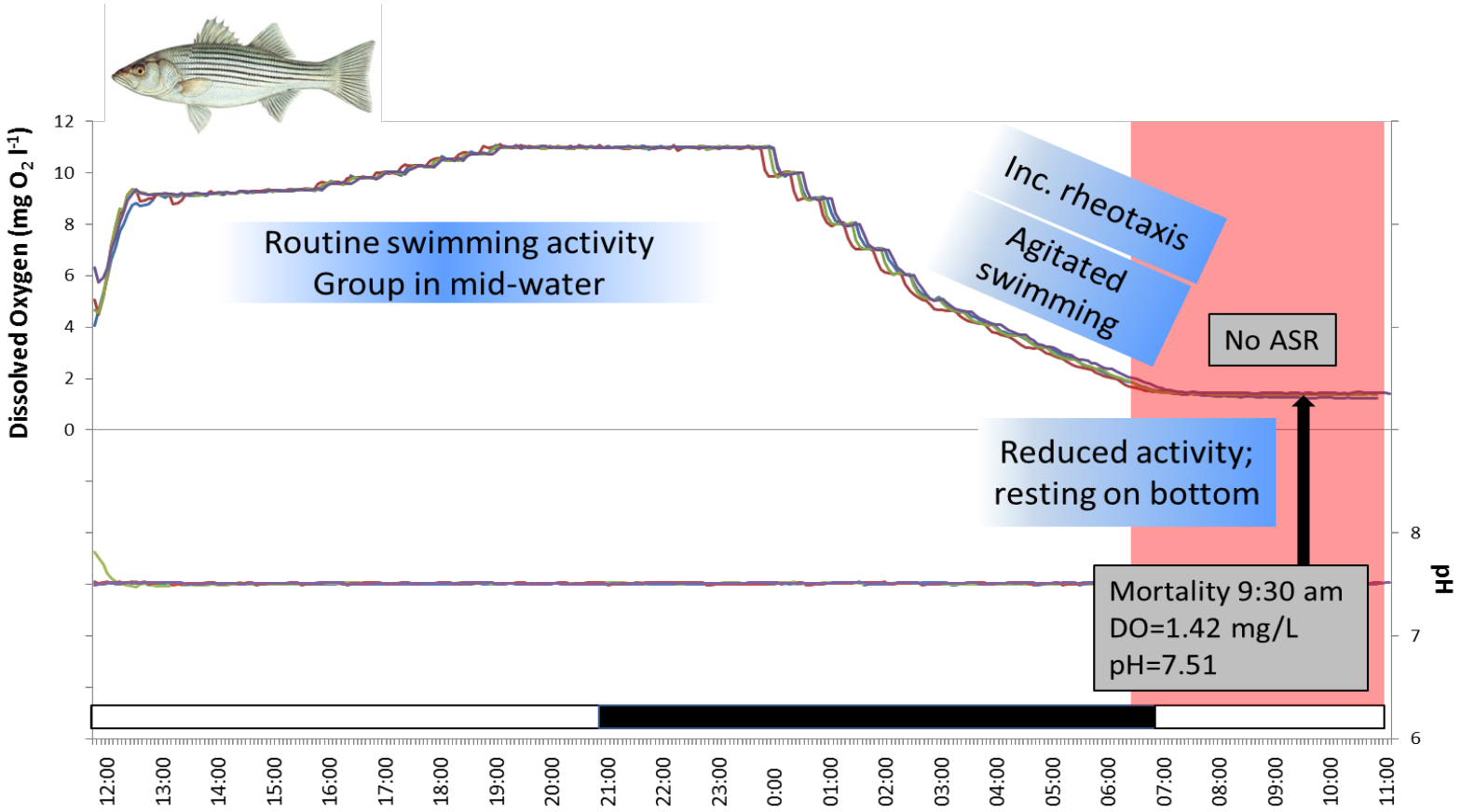


Figure 1: Diel-cycling treatment [A] ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; static pH=7.5) for *Morone saxatilis*. Each line indicates DO/pH levels for each replicate. Arrow indicates DO/pH threshold where at least one individual in one replicate exhibited the response. Shaded/unshaded horizontal bar on bottom indicates light (07:00-21:00) and dark (21:00-7:00) photoperiods, respectively. Red shaded area marks period of minimum DO values in diel-cycle.

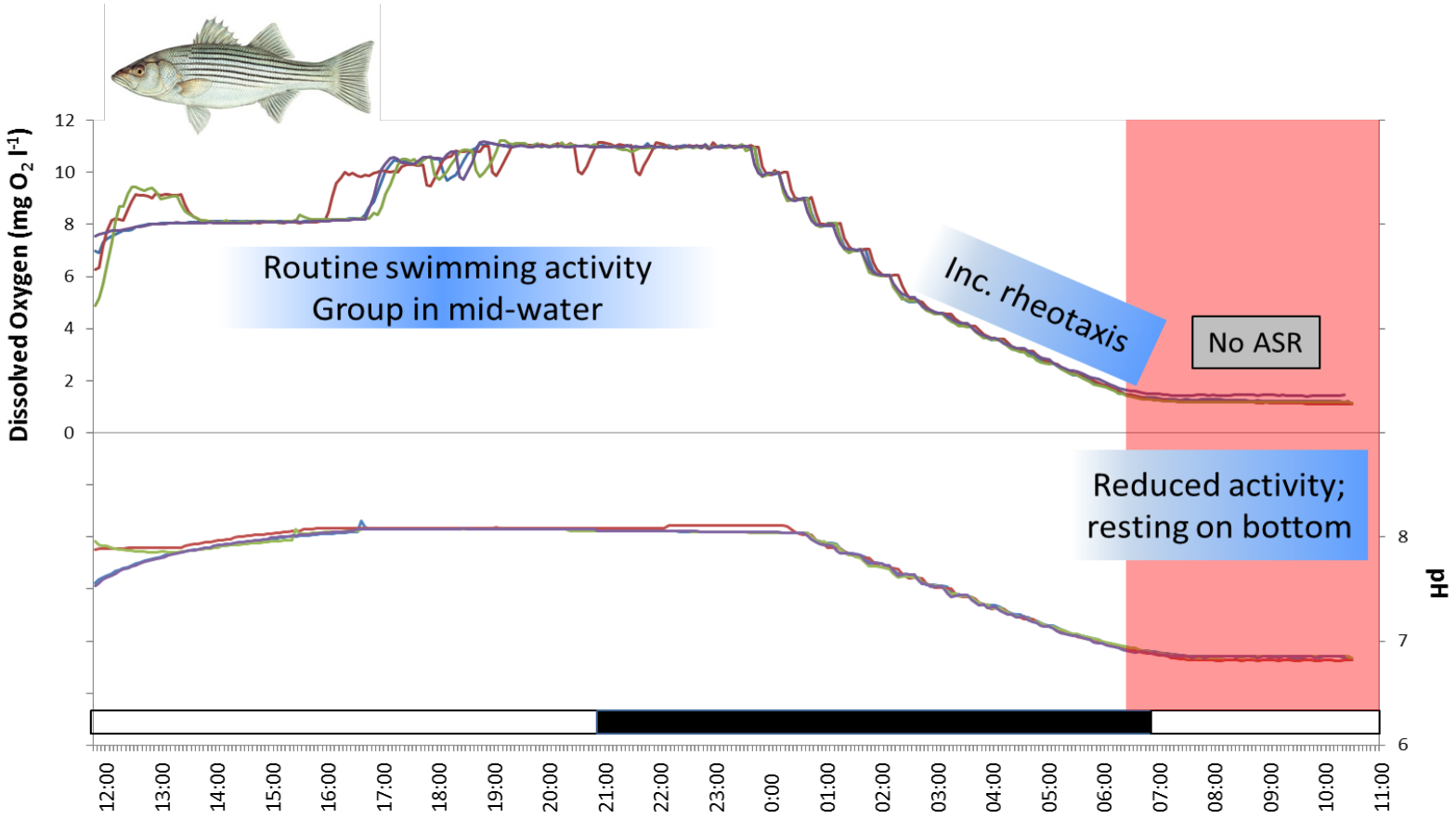


Figure 2: Diel-cycling treatment [B] ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; "Extreme-Range pH"=6.8-8.1) for *Morone saxatilis*. Each line indicates DO/pH levels for each replicate. Arrow indicates DO/pH threshold where at least one individual in one replicate exhibited the response. Shaded/unshaded horizontal bar on bottom indicates light (07:00-21:00) and dark (21:00-7:00) photoperiods, respectively. Red shaded area marks period of minimum DO values in diel-cycle.

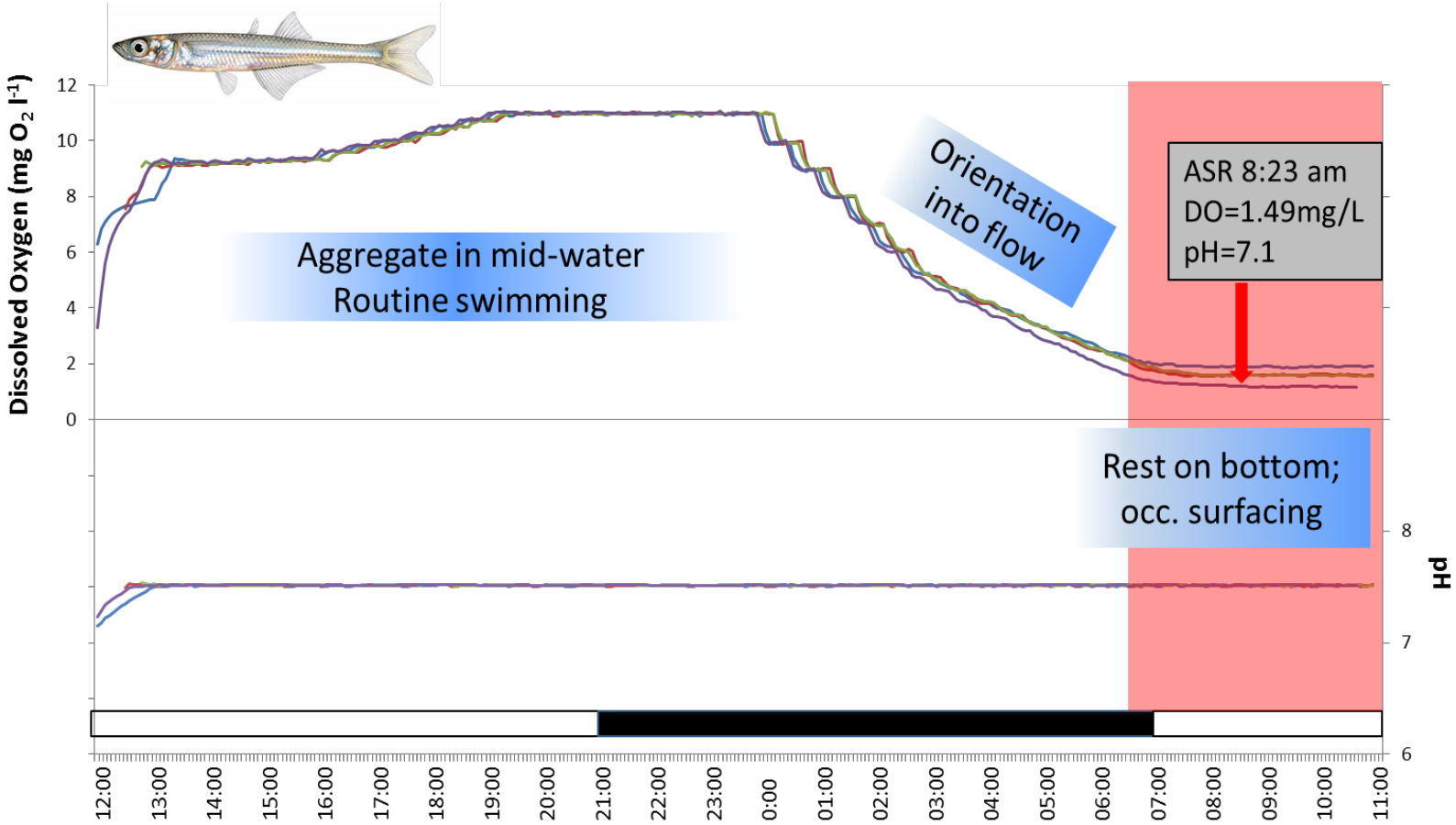


Figure 3: Diel-cycling treatment [A] ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; static pH=7.5) for *Menidia menidia*. Each line indicates DO/pH levels for each replicate. Arrow indicates DO/pH threshold where at least one individual in one replicate exhibited the response. Shaded/unshaded horizontal bar on bottom indicates light (07:00-21:00) and dark (21:00-7:00) photoperiods, respectively. Red shaded area marks period of minimum DO values in diel-cycle.

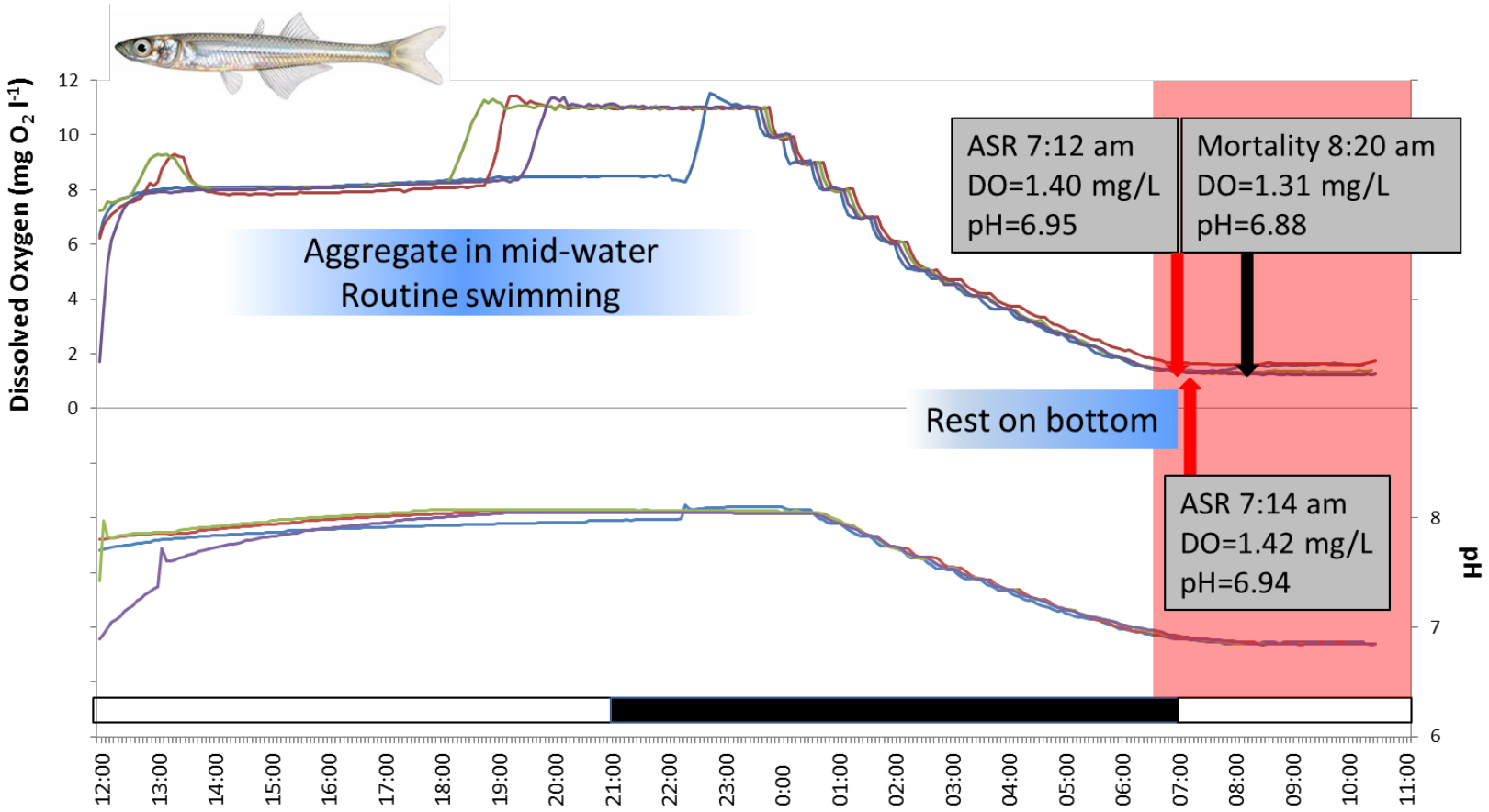


Figure 4: Diel-cycling treatment [B] ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; "Extreme-Range pH"=6.8-8.1) for *Menidia menidia*. Each line indicates DO/pH levels for each replicate. Arrow indicates DO/pH threshold where at least one individual in each replicate exhibited the response. Shaded/unshaded horizontal bar on bottom indicates light (07:00-21:00) and dark (21:00-7:00) photoperiods, respectively. Red shaded area marks period of minimum DO values in diel-cycle.

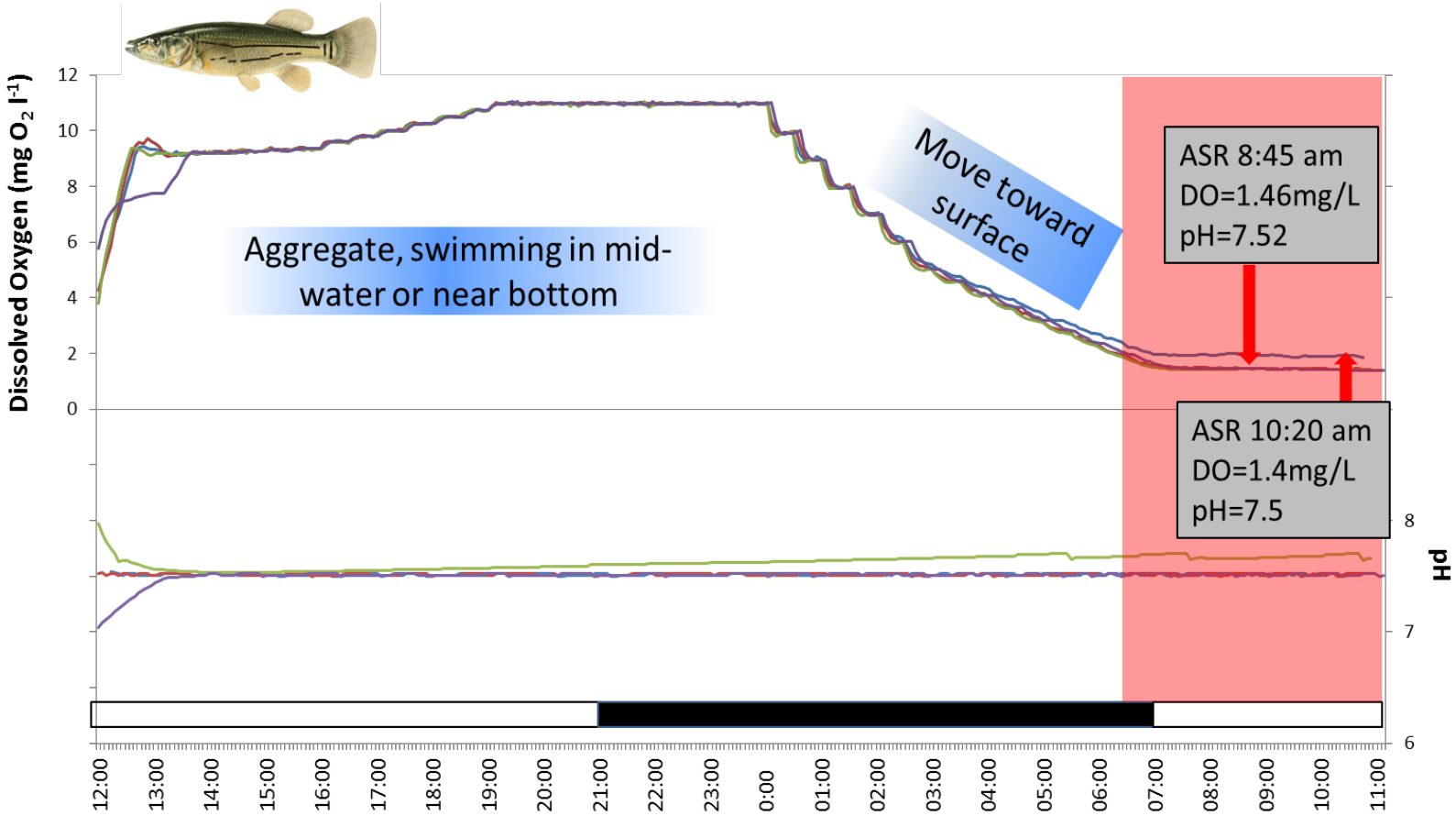


Figure 5: Diel-cycling treatment [A] ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; static pH=7.5) for *Fundulus majalis*. Each line indicates DO/pH levels for each replicate. Arrow indicates DO/pH threshold where at least one individual in one replicate exhibited the response. Shaded/unshaded horizontal bar on bottom indicates light (07:00-21:00) and dark (21:00-7:00) photoperiods, respectively. Red shaded area marks period of minimum DO values in diel-cycle.

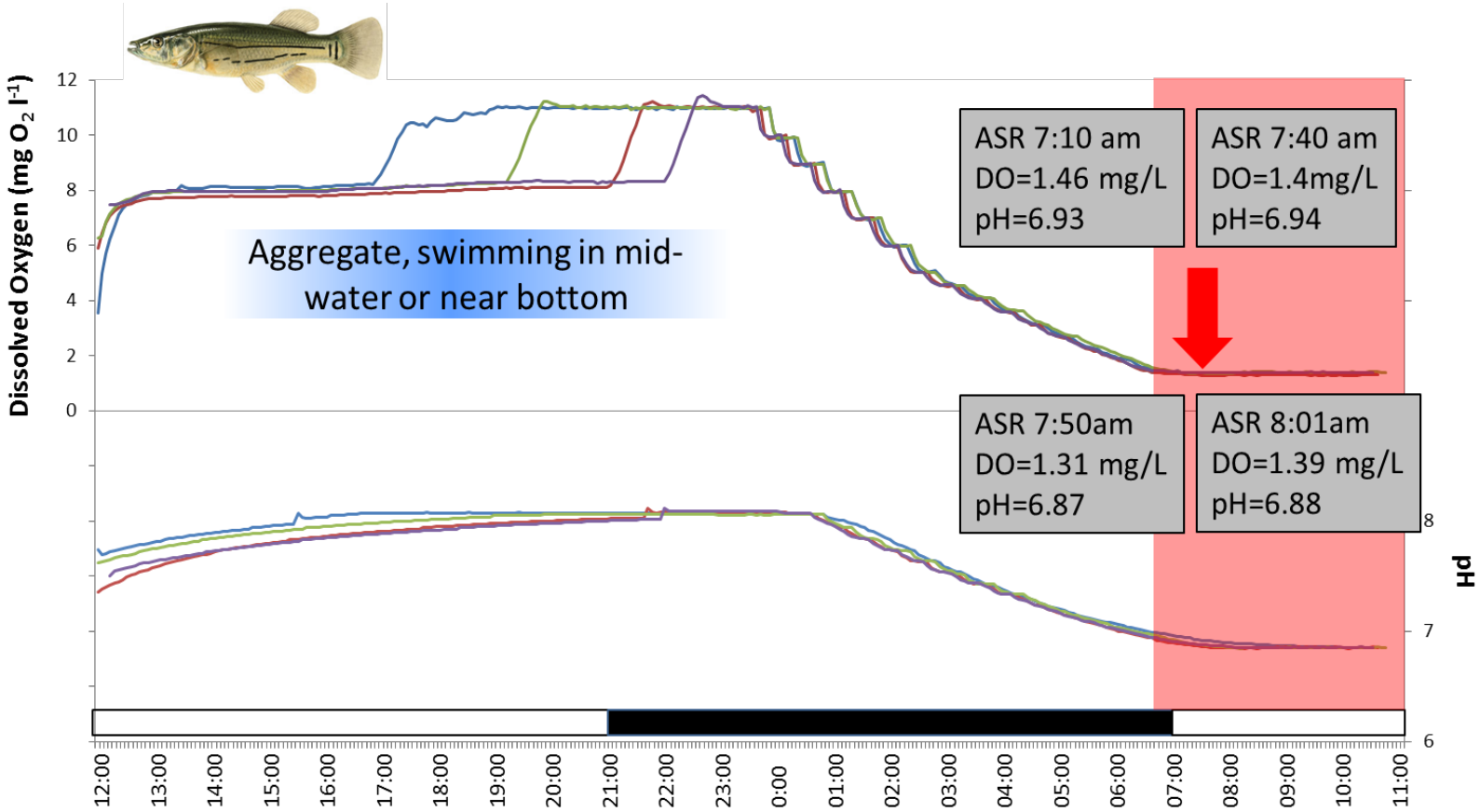


Figure 6: Diel-cycling treatment [B] ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; "Extreme-Range pH"=6.8-8.1) for *Fundulus majalis*. Each line indicates DO/pH levels for each replicate. Single arrow indicates DO/pH threshold where at least one individual exhibited the response in each of the four replicates. Shaded/unshaded horizontal bar on bottom indicates light (07:00-21:00) and dark (21:00-7:00) photoperiods, respectively. Red shaded area marks period of minimum DO values in diel-cycle.

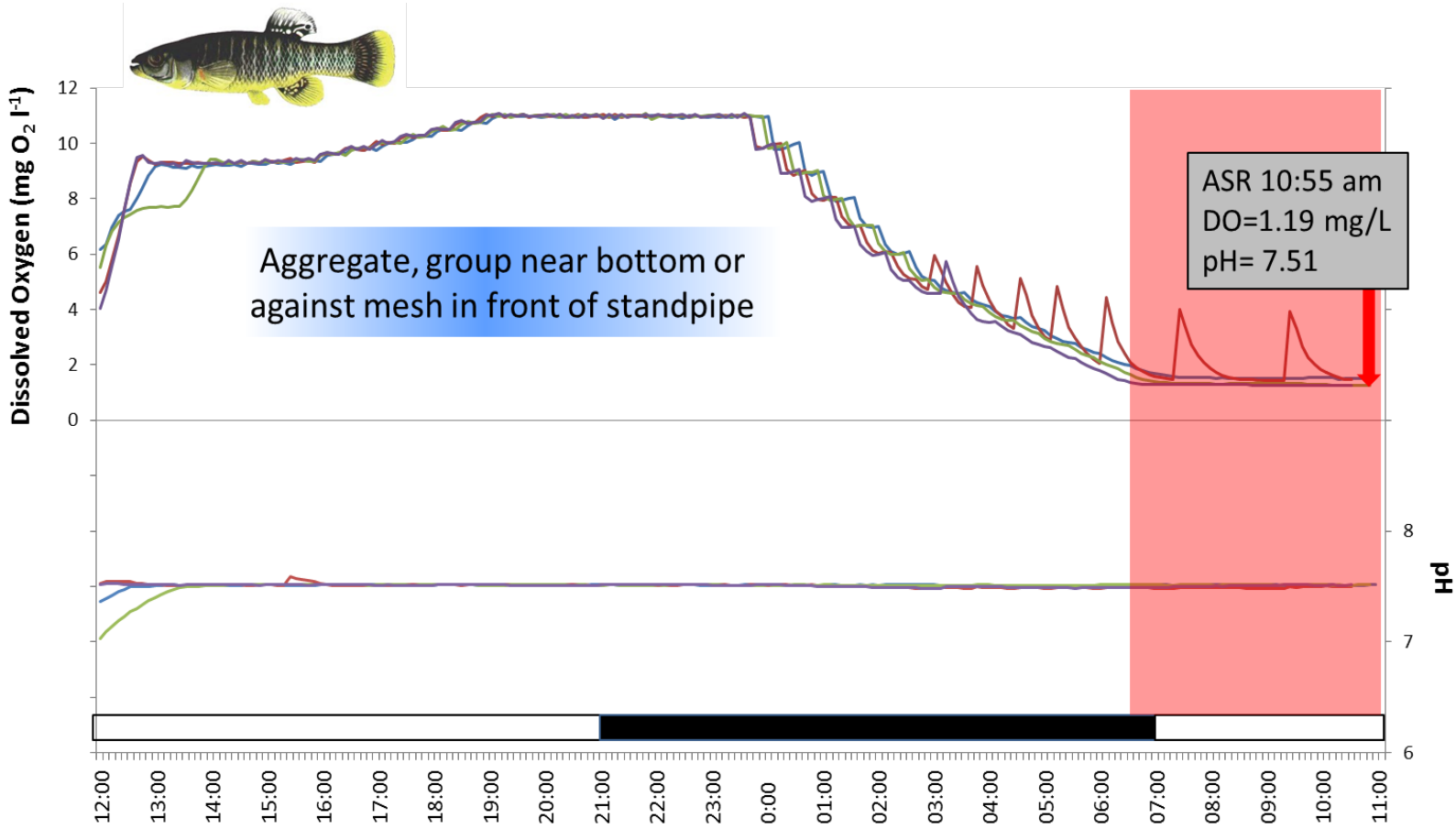


Figure 7: Diel-cycling treatment [A] ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; static pH=7.5) for *Fundulus heteroclitus*. Each line indicates DO/pH levels for each replicate. Arrow indicates DO/pH threshold where at least one individual in one replicate exhibited the response. Shaded/unshaded horizontal bar on bottom indicates light (07:00-21:00) and dark (21:00-7:00) photoperiods, respectively. Red shaded area marks period of minimum DO values in diel-cycle.

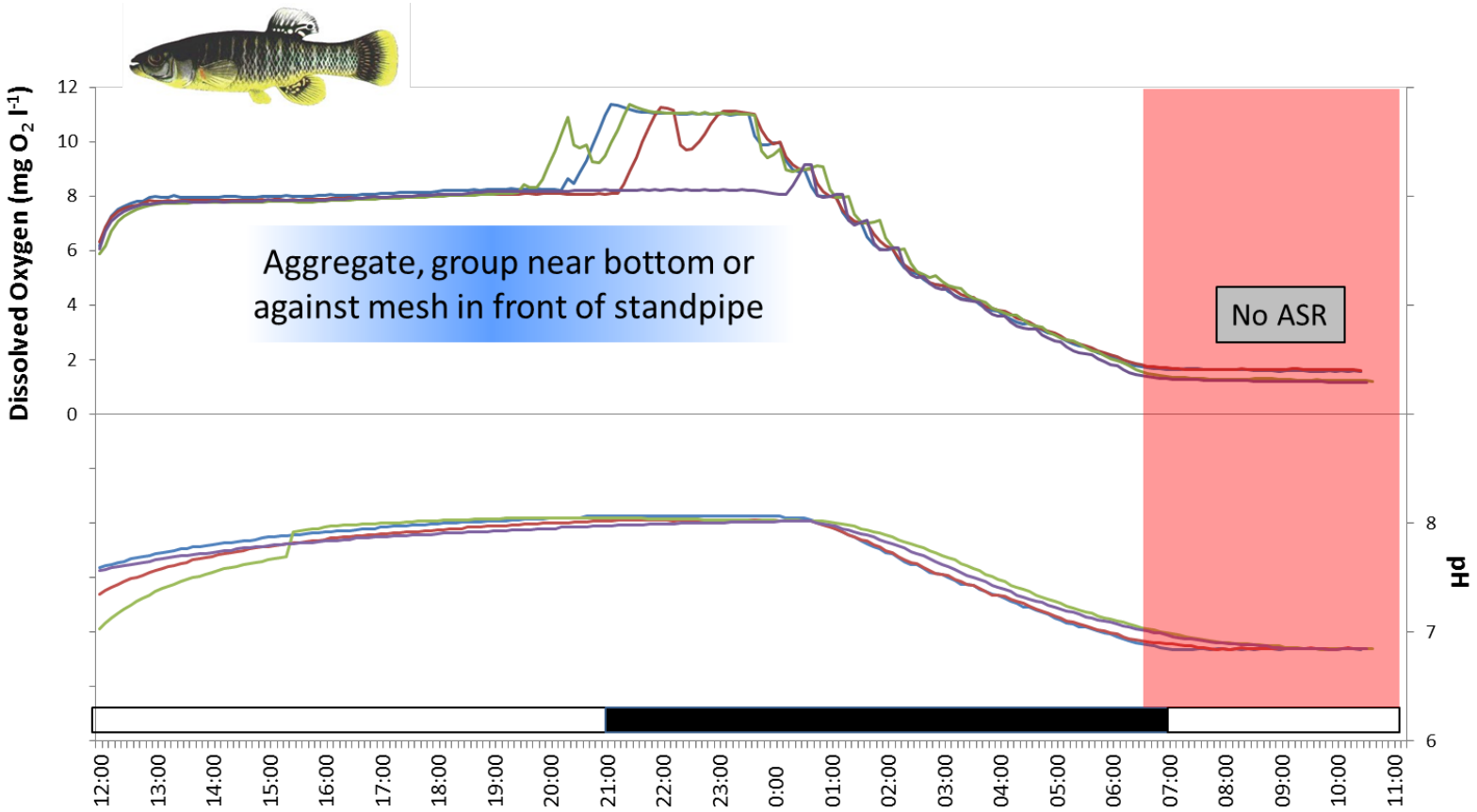


Figure 8: Diel-cycling treatment [B] ("Extreme-Range DO"= 1-11 mg O_2 l^{-1}; "Extreme-Range pH"=6.8-8.1) for *Fundulus heteroclitus*. Each line indicates DO/pH levels for each replicate. Arrow indicates DO/pH threshold where at least one individual in one replicate exhibited the response. Shaded/unshaded horizontal bar on bottom indicates light (07:00-21:00) and dark (21:00-7:00) photoperiods, respectively. Red shaded area marks period of minimum DO values in diel-cycle.

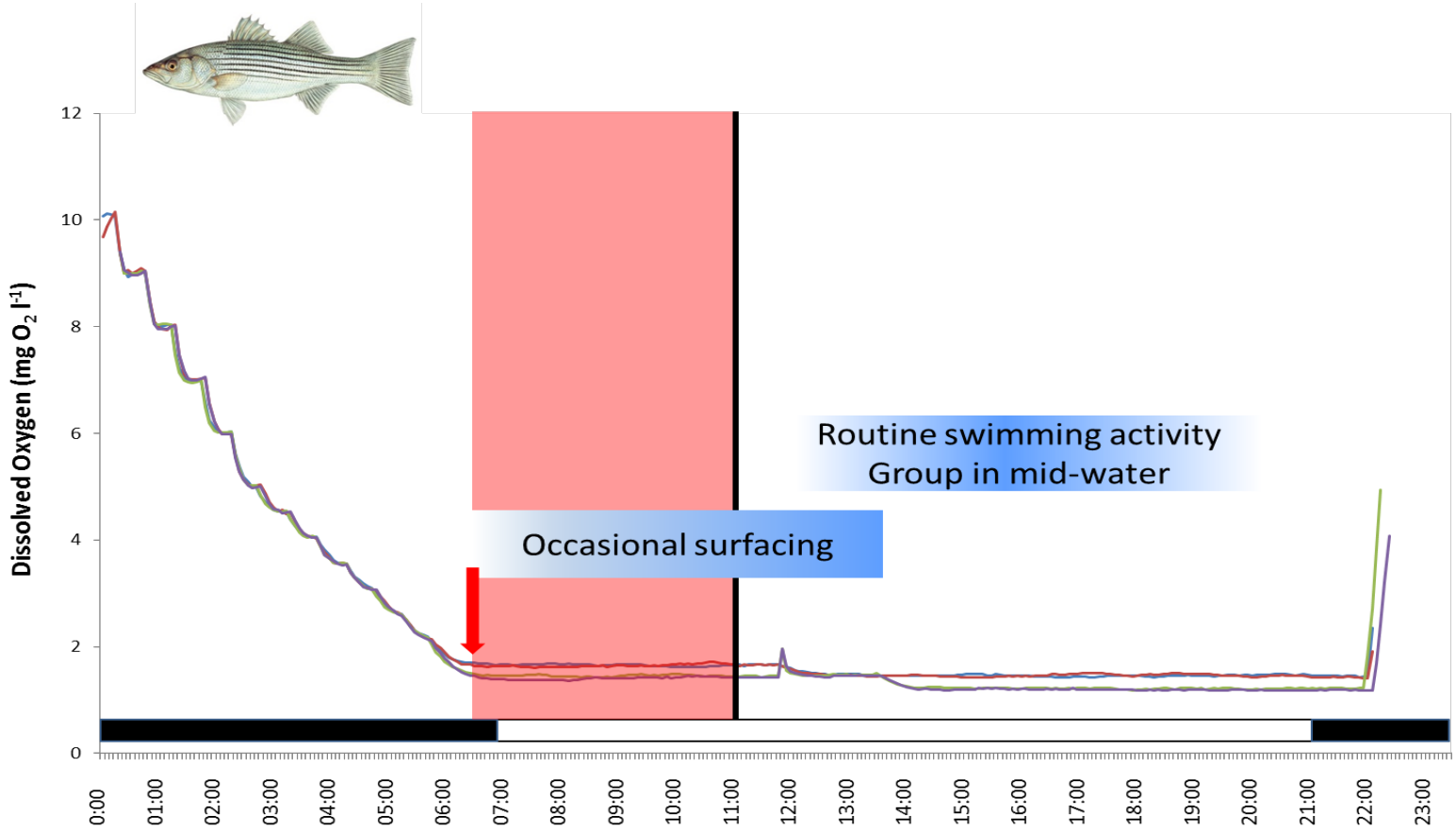


Figure 9: Extended low-dissolved oxygen trial for *Morone saxatilis.* DO minimum (1.5 mg O_2 l^{-1} to 1.0 mg O_2 l^{-1}), static pH (7.5). Each line indicates DO/pH levels for each replicate. Arrow indicates DO/pH threshold where at least one individual in one replicate exhibited the response. Shaded/unshaded horizontal bar on bottom indicates light (07:00-21:00) and dark (21:00-7:00) photoperiods, respectively. Red shaded area marks period of minimum DO values under diel-cycling conditions; black line indicating end of previous diel-cycling trials.

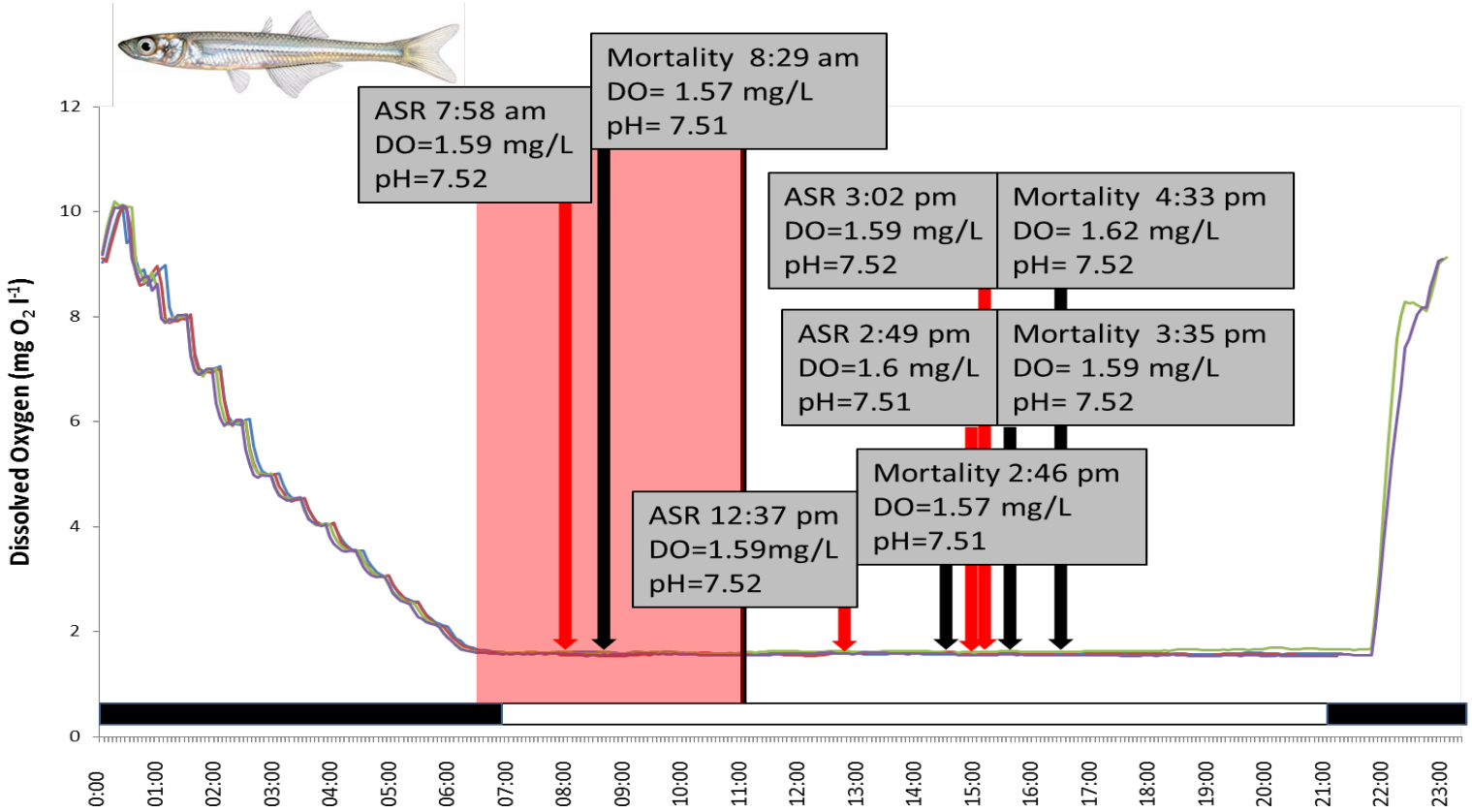


Figure 10: Extended low dissolved oxygen trial for *Menidia menidia*. DO minimum (1.5 mg O_2 l^{-1}), static pH (7.5). Each line indicates DO/pH levels for each replicate. Arrow indicates DO/pH threshold where at least one individual exhibited the response. Shaded/unshaded horizontal bar on bottom indicates light (07:00-21:00) and dark (21:00-7:00) photoperiods, respectively. Red shaded area marks period of minimum DO values under diel-cycling conditions; black line indicating end of previous diel-cycling trials. ASR and mortality events are paired for each trial; when offset, first individual to reach lethal limit was not the first to initiate ASR. At lethal limits all three fish were engaged.

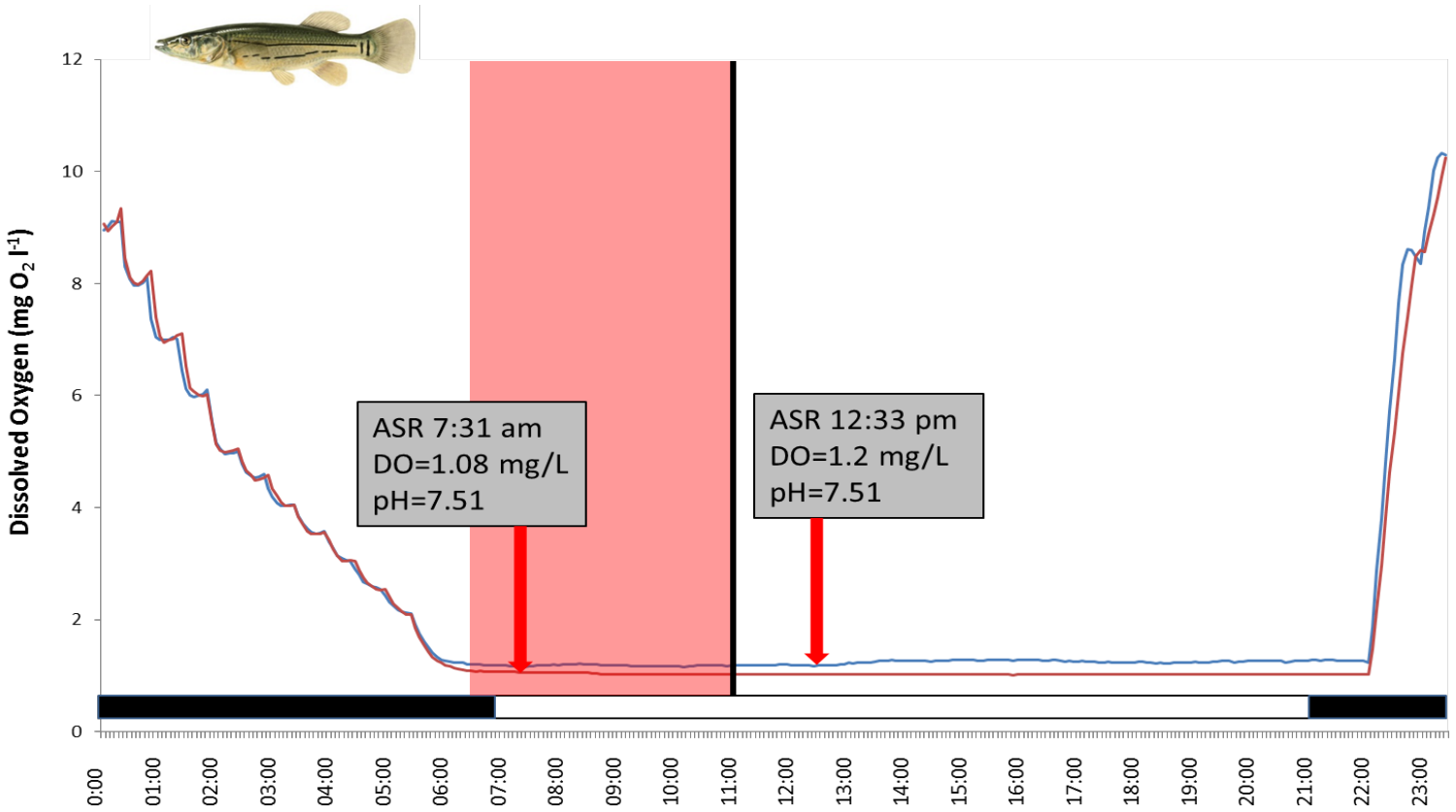


Figure 11: Extended low dissolved oxygen trial for *Fundulus majalis*. DO minimum (1.0 mg O_2 l^{-1}), static pH (7.5). Each line indicates DO/pH levels for each replicate. Arrow indicates DO/pH threshold where at least one individual exhibited the response. Shaded/unshaded horizontal bar on bottom indicates light (07:00-21:00) and dark (21:00-7:00) photoperiods, respectively. Red shaded area marks period of minimum DO values under diel-cycling conditions; black line indicating end of previous diel-cycling trials.

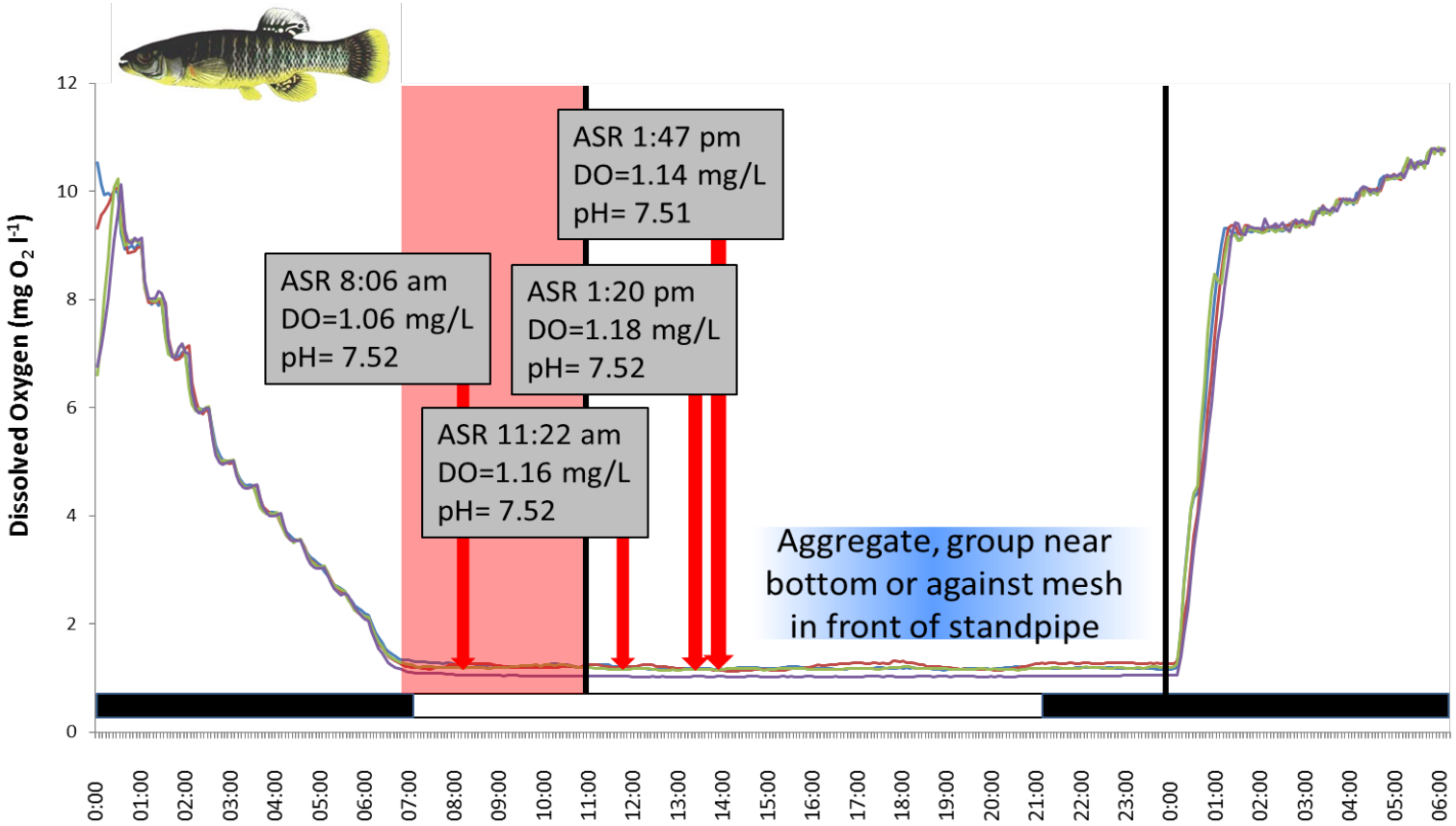


Figure 12: Extended low dissolved oxygen trail for *Fundulus heteroclitus*. DO minimum (1.0 mg O_2 l^{-1}), static pH (7.5). Each line indicates DO/pH levels for each replicate. Arrow indicates DO/pH threshold where at least one ind ividual exhibited the response. Shaded/unshaded horizontal bar on bottom indicates light (07:00-21:00) and dark (21:00-7:00) photoperiods, respectively. Red shaded area marks period of minimum DO values under diel-cycling conditions; black line indicating end of previous diel-cycling trials.

REFERENCES

Able K.W., M.P. Fahay. 1998. *The First Year in the Life of Estuarine Fishes in the Middle Atlantic Bight.* New Brunswick, NJ: Rutgers University Press.

Able K.W. 1999. Measures of juvenile fish habitat quality: examples from a National Estuarine Research Reserve. *Am Fish Soc Symp*. 22:134-147.

Almeida-Val V.M.F., A.L. Val, W.P. Duncan, F.C.A. Souza, M.N. Paula-Silva, S. Land. 2000. Scaling effects on hypoxia tolerance in the Amazon fish *Astronotus ocellatus* (Perciformes: Cichlidae): contribution of tissue enzyme levels. *Comp Biochem Phys B*. 125:219-226.

Beck N.G., K.W. Bruland. 2000. Diel biogeochemical cycling in a hyperventilating shallow estuarine environment. *Estuaries.* 23:177-187.

Bell G.W, D.B. Eggleston. 2005. Species-specific avoidance responses by blue crabs and fish to chronic and episodic hypoxia. *Marine Biology*. 146:761-770.

Boynton W.R., J.D. Hagy, L. Murray, C. Stokes, W.M. Kemp. 1996. A comparative analysis of eutrophication patterns in a temperate coastal lagoon. *Estuaries*. 19:408-421.

Brady D.C., T.E. Targett, D.M. Tuzzolino. 2009. Behavioral response of juvenile weakfish (*Cynoscion regalis*) to diel-cycling hypoxia: swimming speed, angular correlation, expected displacement, and effects of hypoxia acclimation. *Can J Fish Aquatic Sci.* 66:415-424.

Brandt S.B., M. Gerken, K.J. Hartman, E. Demers. 2009. Effects of hypoxia on food consumption and growth of juvenile striped bass (*Morone saxatilis*). *J. Exp Mar Biol and Ecol.* 381:S143-149.

Breitburg D.L. 1994. Behavioral response of fish larvae to low dissolved oxygen concentrations in a stratified water column. *Marine Biology* 120:615-625.

Breitburg D. 2002. Effects of hypoxia, and the balance between hypoxia and enrichment, on coastal fishes and fisheries. *Estuaries.* 25:767-781.

Burleson M.L., D.R. Wilhelm, N.J. Smatresk. 2001. The influence of fish size on the avoidance of hypoxia and oxygen selection by largemouth bass. *J Fish Biol*. 59:1336-1349.

Buzzelli C.P., R.A. Luettich Jr., S.P. Powers, C.H. Peterson, J.E. McNinch, J.L. Pinckney, H.W. Paerl. 2002. Estimating the spatial extent of bottom-water hypoxia and habitat degradation in a shallow estuary. *Mar Ecol Prog Ser.* 230:103-112.

Cech Jr. J.J., S.J. Mitchell, T.E. Wragg. 1984. Comparative growth of juvenile white sturgeon and striped bass: Effects of temperature and hypoxia. *Estuaries*. 7:12-18.

Cloern J.E. 2001. Our evolving conceptual model of the coastal eutrophication problem. *Mar Ecol Prog Ser* 210:223-253.

Conover D.O, M.R. Ross. 1982. Patterns in seasonal abundance, growth, and biomass of the Atlantic silverside, *Menidia menidia*, in a New England estuary. *Estuaries*. 5:275-286.

Coutant C.C. 1985. Striped bass, temperature, and dissolved oxygen: A speculative hypothesis for environmental risk. *Trans Am Fish Soc*. 114:31-61.

Dan X.M., G.J. Yan, A.J. Zhang, Z.D. Cao, S.J. Fu. 2014. Effects of stable and diel-cycling hypoxia on hypoxia tolerance, postprandial metabolic response, and growth performance in juvenile qingbo (*Spinibarbus sinensis*). *Aquaculture*. 428:21-28.

Denman K, J.R. Christian, N. Steiner, H.O. Pörtner, Y. Nojiri. 2011. Potential impacts of future ocean acidification on marine ecosystems and fisheries: current knowledge and recommendations for future research. *ICES J Mar Sci.* 68: 1019-1029.

Diaz R.J. 2001. Overview of hypoxia around the world. *J. Environ Qual*. 30:275-281.

Diaz R.J., R. Rosenberg. 2008. Spreading dead zones and consequences for marine ecosystems. *Science*. 321: 926-929.

Domenici P, C. Lefrançois, A. Shingles. 2007. Hypoxia and the antipredator behaviours of fishes. *Phil Trans R Soc B.* 362:2105-2121.

Eby L.A., L.B. Crowder. 2002. Hypoxia-based habitat compression in the Neuse river estuary: context-dependent shifts in behavioral avoidance thresholds. *Can J Fish Aquat Sci.* 59:952-965.

Freadman, M.A. 1979. Swimming energetics of striped bass (*Morone saxatilis*) and bluefish (*Pomatomus saltatrix*): Gill ventilation and swimming metabolism. *J Exp Biol*. 83:217-230.

Gee J.H., P.A. Gee. 1991. Reactions of Gobioid fishes to hypoxia: Buoyancy control and aquatic surface respiration. *Copeia*. 1991:17-28.

Greaney G.S., A.R. Place, R.E. Cashon, G. Smith, D.A. Powers. 1980. Time course of changes in enzyme activities and blood respiratory properties of killifish during long-term acclimation to hypoxia. *Phys Zool*. 53:136-144.

Grecay P.A., K.L. Stierhoff. 2002. A device for simultaneously controlling multiple treatment levels of dissolved oxygen in laboratory experiments. *J. Exp Mar Biol and Ecol*. 280: 53-62.

Hanks D.M., D.H. Secor. 2011. Bioenergetic responses of Chesapeake Bay white perch (*Morone americana*) to nursery conditions of temperature, dissolved oxygen, and salinity. *Mar Biol.* 158:805-815.

Horodysky A.J., R.W. Brill, E.J. Warrant, J.A. Musick, R.J. Latour. 2010. Comparative visual function in four piscivorous fishes inhabiting Chesapeake Bay. *J. Exp Biol*. 213:1751-1761.

Howarth R., F. Chan, D.J. Conley, J. Garnier, S.C. Doney, R. Marine, G. Billen. 2011. Coupled biogeochemical cycles: eutrophication and hypoxia in temperate estuaries and coastal marine ecosystems. *Frontiers in Ecol Enviro.* 9:18-26.

Kemp W.M., W.R. Boynton. 1980. Influence of biological and physical processes on dissolved oxygen dynamics in an estuarine system: implications for measurement of community metabolism. *Est Coast Mar Sci.* 11:407-431.

Kramer D.L., M. McClure. 1982. Aquatic surface respiration, a widespread adaptation to hypoxia in tropical freshwater fishes. *Env Biol Fishes.* 7:47-55.

Kramer D.L. 1983. Aquatic surface respiration in the fishes of Panama: distribution in relation to risk of hypoxia. *Env Biol Fishes*. 8:49-54.

Kramer D.L. 1987. Dissolved oxygen and fish behavior. *Env Biol Fishes*. 18:81-92.

Lewis W.M. 1970. Morphological adaptations of Cyprinodontoids for inhabiting oxygen deficient waters. *Copeia.* 2:319-326.

Maryland Department of Natural Resources. "Eyes on the Bay." June 10, 2013. <http://mddnr.chesapeakebay.net/eyesonthebay/index.cfm>

McNatt R.A., J.A. Rice. 2004. Hypoxia-induced growth rate reduction in two juvenile estuary-dependent fishes. *J. Exp Mar Biol and Ecol.* 311:147-156.

McNeil D.G., G.P. Closs. 2007. Behavioral responses of a south-east Australian floodplain fish community to gradual hypoxia. *Freshwater Biology*. 52:412-420.

Nilsson G.E., S. Östlund-Nilsson. 2008. Does size matter for hypoxia tolerance in fish? *Biol Reviews*. 83:173-189.

Nordlie F.G. 2006. Physicochemical environments and tolerances of Cyprinodontoid fishes found in estuaries and salt marshes of eastern North America. *Rev Fish Biol Fisheries.* 16:51-106.

Peterson G.W., S.T. Ross. 1991. Dynamics of littoral fishes and decapods along a coastal river-estuarine gradient. *Est Coast Shelf Sci.* 33: 467-483.

Pörtner H.O. 2008. Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Mar Ecol Prog Ser.* 373:203-217.

Rabalais N.N., R.J. Diaz, L.A. Levin, R.E. Turner, D. Gilbert, J. Zhang. 2009. Dynamics and distribution of natural and human-caused coastal hypoxia. *Biogeosci Discuss.* 6:9359-9453.

Rees B.B., T.E. Targett, B.J. Ciotti, C.A. Tolman, S.S. Akkina, A.M. Gallaty. 2012. Temporal dynamics in growth and white skeletal muscle composition of the mummichog *Fundulus heteroclitus* during chronic hypoxia and hyperoxia. *J Fish Biol.* 81:148-164.

Richards J.G., A.P. Farrell, C.J. Braumer. (eds) 2009. *Hypoxia*. Fish Physiology (vol 27). *Acad. Press.*

Ross S.W. 2003. The relative value of different estuarine nursery areas in North Carolina for transient juvenile marine fishes. *Fishery Bulletin*. 101:384-404.

Secor D.H., T.E. Gunderson. 1998. Effects of hypoxia and temperature on survival, growth, and respiration of juvenile Atlantic sturgeon, *Acipenser oxyrinchus*. *Fishery Bulletin*. 96:603-613.

Shingles A., D.J. McKenzie, G. Claireaux, P. Domenici. 2005. Reflex cardioventilatory responses to hypoxia in the flathead gray mullet (*Mugil cephalus*) and their behavioral modulation by perceived threat of predation and water turbidity. *Phys Biochem Zool.* 78:744-755.

Smith K.J., K.W. Able. 2003. Dissolved oxygen dynamics in salt marsh pools and its potential impacts on fish assemblages. *Mar Ecol Prog Series.* 258:223-232.

Stierhoff K.L., T.E. Targett, P.A. Grecay. 2003. Hypoxia tolerance of the mummichog: the role of access to the water surface. *J Fish Biol.* 63:580-592.

Stierhoff K.L., T.E. Targett, K. Miller. 2006. Ecophysiological responses of juvenile summer and winter flounder to hypoxia: experimental and modeling analyses of effects on estuarine nursery quality. *Mar Ecol Prog Series*. 325: 255-266.

Stierhoff K.L., T.E. Targett, J.H. Power. 2009**a**. Hypoxia-induced growth limitation of juvenile fishes in an estuarine nursery: assessment of small-scale temporal dynamics using RNA:DNA. *Can J Fish Aquat Sci.* 66:1033-1047.

Stierhoff K.L., R.M. Tyler, T.E. Targett. 2009**b**. Hypoxia tolerance of juvenile weakfish (*Cynoscion regalis*): laboratory assessment of growth and behavioral avoidance responses. *J. Exp Mar Biol and Ecol.* 381:S173-S179.

Tyler R.M., D.C. Brady, T.E. Targett. 2009. Temporal and spatial dynamics of diel-cycling hypoxia in estuarine tributaries. *Estuaries and Coasts.* 32:123-145.

Tyler R.M., T.E. Targett. 2007. Juvenile weakfish *Cynoscion regalis* distribution in relation to diel-cycling dissolved oxygen in an estuarine tributary. *Mar Ecol Prog Ser.* 333:257-269.

US EPA. 2000. Ambient water quality criteria for dissolved oxygen (saltwater) Cape Cod to Cape Hatteras. Report No. EPA-822-R-00-012, United States Environmental Protection Agency, Narrangansett, RI.

Wannamaker C.M., J.A. Rice. 2000. Effects of hypoxia on movements and behavior of selected estuarine organisms from the southeastern United States. *J Exp Mar Biol and Ecol.* 249:145-163.

Woodley C.M., M.S. Peterson. 2003. Measuring responses to simulated predation threat using behavioral and physiological metrics: the role of aquatic vegetation. *Oecologia*. 136:155-160.

Yang H, Z.D. Cao, S.J. Fu. 2013. The effects of diel-cycling hypoxia acclimation on the hypoxia tolerance, swimming capacity, and growth performance of southern catfish (*Silurus meridionalis*). *Comp Biochem Physiol A*. 165:131-138.

Appendix A

LABORATORY REGULATION OF DO AND PH

Below are details on the computer-controlled laboratory apparatus used to continuously monitor and regulate dissolved oxygen and pH during behavioral experiments (Figure A1).

A re-design of an existing DO-controlling apparatus (Grecay & Stierhoff, 2002), DO and pH can be regulated in up to five separate treatment systems. Each system consists of a rectangular tray (~208.25 x 68.5 x 31.75 cm) and holds approximately 415 liters seawater, each containing a single external sump tank. The trays are fitted with glass lids and sealed with gasket material to create an enclosed environment to ensure that the regulated gas mixture input within the system is isolated from the atmosphere. ASR observation tanks (56 x 22.86 x 44.45 cm; volume approximately 56.8 l) were designed and installed into the circuitry of two of these treatment systems, positioned on top of the tray. A pump (Pan World Magnetic Water Pump 50 PX-X) constantly drew water from an external polyethylene sump to supply the observation tank via an inflow line located inside the tray. Flow rate of the inflow supply into the tank could be regulated with a valve inside the tray; rate was determined to create even flow inside the tank and reduce the potential for the development of eddies and micro-habitats that could influence fish behavior. Flow directionality within the tank was traced with methylene

blue dye to ensure adequate turnover within the tank. In this manner the observation tank was constantly supplied with water, which overflowed into the rectangular tray beneath through a 2" PVC standpipe. The height of the standpipe was designed for a maximum water height that allowed potential ASR behavior to be readily visible on video recordings. In addition, black vinyl 'vexar' mesh was used to create a diffuser positioned directly in front of the inflow supply to the tank, and a barrier installed in front of the standpipe that spanned the entire width and height of the tank. This mesh diffuser/barrier served two purposes: (1) small fish used in trials had difficulty maintaining routine swimming with flow rates required for adequate turnover and oxygen regulation in observation tanks, and (2) some fish, particularly *Fundulus* spp., sought refuge and security by hiding behind the standpipe and in corners of the tank, where any ASR behavior was not detectable by the camcorder during trials. At the far end of the tray opposite the observation tank, a large bulkhead fitting allows water to overflow into the sump via a 4" PVC pipe, where it is again pumped into the observation tank. Therefore, constant re-circulation is achieved, with the rectangular tray and sump of the existing system serving as a single reservoir for the observation tank.

Using National Instrument's LabVIEW System Design software, the environment within a treatment system can be regulated through the use of a Hach® sc200™ Universal Controller in conjunction with sensors for DO and pH (Hach® LDO dissolved oxygen probe and Hach® Differential pH/ORP sensor, respectively). These probes are submersed in an independent reservoir at the same temperature as treatment systems. LabVIEW enables the researcher to specify desired values for DO and pH, either constant

levels for static treatments, or change in half hour intervals to create a desired pattern of diel fluctuation. DO and pH values and associated deviations are set before experimentation in a .csv file.

At pre-determined intervals, the program measures and records the current DO and pH level through the solenoid-controlled flow of seawater from each system past the probes. When DO and/or pH deviates from desired values, the software actuates corresponding gas solenoids to inject the appropriate compressed gases (air, CO_2, N_2, and O_2) in order to achieve or maintain the desired levels. CO_2 and air are bubbled in to lower and raise the pH while N_2 and O_2 are added to lower and raise the DO, respectively. Gases were injected into the system via a port positioned over the sump. Observation tanks were made airtight by clamping glass lids sealed with rubber gasket to the top of the tank to guarantee that the environment within the tank is the same as that regulated in the surrounding system – the overflow standpipe also allows the atmosphere within the ASR tanks to equilibrate with that of the surrounding system. Handheld YSI meters (YSI 556 MultiProbe System) confirmed that the DO/pH level in the observation tanks matched that of the surrounding system during experimentation.

Compressed air is added to raise the pH of an individual system, making it difficult to maintain supersaturated concentrations of DO. As a result, in diel-cycling treatments where both DO and pH cycle, the pH must first reach the desired level before attempting to supersaturate dissolved oxygen. In order to increase the stability of seawater pH within the system, tanks are treated with Kent Marine® Superbuffer d-KH bicarbonate salts to establish a higher carbonate alkalinity (8 dKH; equivalent to water

sampled from Canary Creek where *F.heteroclitus* were collected). This elevated buffering capacity prevents the rapid fluctuations of pH and allows for more precise and prolonged control of dissolved oxygen.

The process described above for a single treatment system was sequentially repeated to maintain DO and pH control in all systems running experiments. The program and apparatus operates continuously, therefore providing regulation throughout the duration of an experiment. During behavioral trials, only the two systems outfitted with ASR observation tanks were regulated for DO and pH, therefore water was sampled and adjusted every five minutes.

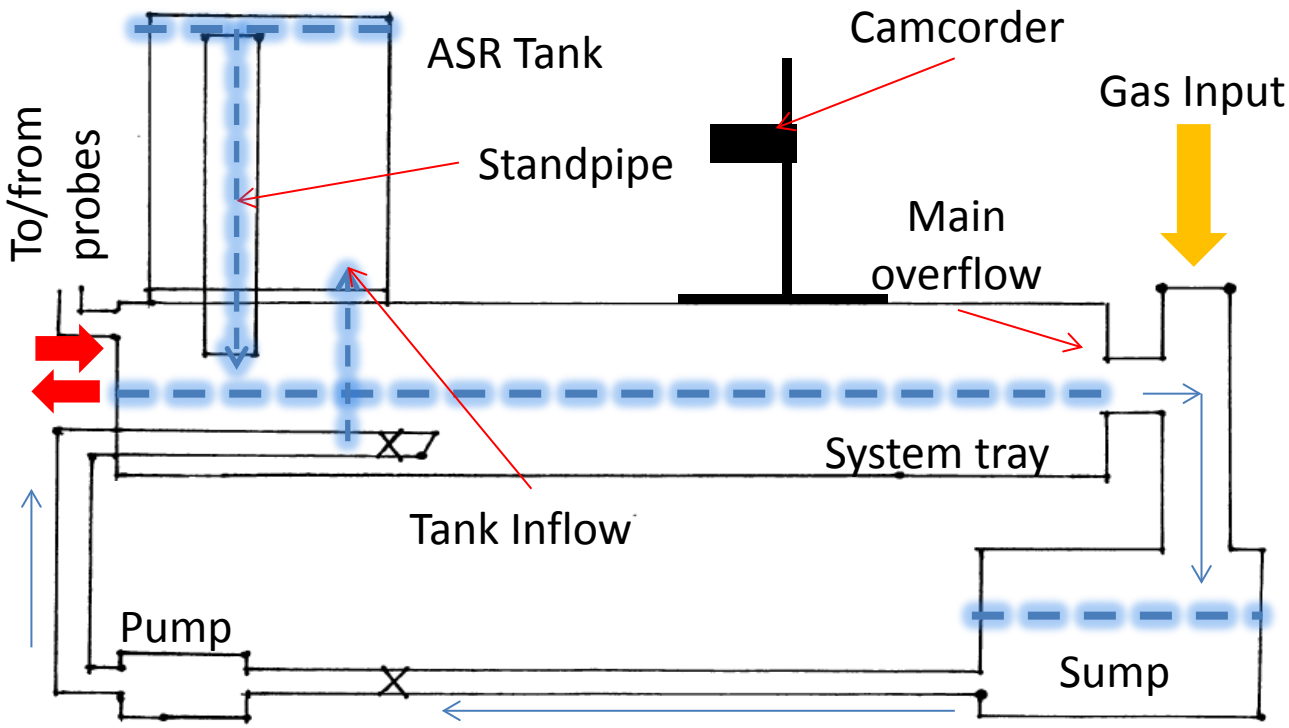


Figure A1: A side-view representation of a single treatment system within the computer-controlled laboratory apparatus used for continuous DO and pH regulation. ASR observation tanks are positioned on top of the tray. A pump draws water from an external sump to the observation tank via an inflow line located inside the tray, and overflows through a standpipe back into the reservoir and sump. At pre-determined intervals, LabVIEW software takes samples from each system through the solenoid-controlled flow of seawater past DO and pH probes, then actuating solenoids to inject the appropriate gases (air, CO_2, N_2, and O_2) to achieve or maintain desired levels. Both the ASR tank and the surrounding system tray are sealed with glass lids.

Appendix B

IACUC

The following contain the annual review from the University of Delaware Institutional Animal Care and Use Committee pertaining to the animals used in these behavioral experiments.

University of Delaware
Institutional Animal Care and Use Committee

Annual Review

(Please complete below using Arial, size 12 Font.)

RECEIVED
FEB 25 2014
IACUC CR

Title of Protocol: Ecology and Physiological Ecology of Estuarine and Coastal Marine Fishes	
AUP Number: 1131-2014-1	← **(4 digits only)**
Principal Investigator: Dr. Timothy Targett	
Common Name: Juvenile Weakfish, Juvenile Summer Flounder, Juvenile Striped Bass, Mummichog, Atlantic silverside, Bay anchovy, Silver perch, Atlantic Croaker, Spot, Mullet, Striped killifish, Atlantic menhaden, Halfbeak, Northern Kingfish **Genus Species: :** *Cynoscion regalis, Paralichthys dentatus, Morone saxatilis, Fundulus heteroclitus, Menidia menidia, Anchoa mitchilli, Bairdiella chrysoura, Micropognias undulatus Leiostomus xanthurus, Mugilidae sp., Fundulus majalis, Brevoortia tyrannus, Hemiramphus sp., Menticirrhus saxatilis*	

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: ***(Note change of categories from previous form)***	
Category	**Description**
☐ **B**	Breeding or holding where NO research is conducted
☐ **C**	Procedure involving momentary or no pain or distress
☐ **D**	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
X E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: _____

Date of Approval: 4-1-2014

Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist listed on this AUP. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
7. I assure that any modifications to the protocol will be submitted to the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.
10. I assure that the experimental design has been developed with **consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.**
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. *(Teaching Protocols Exempt)*
12. I understand that by signing, I agree to these assurances.

Timothy E. Taggett

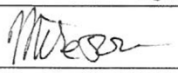
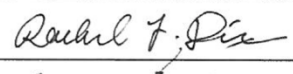
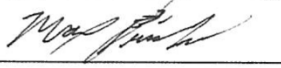
Signature of Principal Investigator

2-23-2014

Date

SIGNATURE(S) OF ALL PERSONS LISTED ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only the procedures that have been approved by the IACUC.

Name	Signature
1. Michael P. Torre	
2. Rachel L. Dixon	
3. Max Davidson	
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IACUC approval of animal protocols must be renewed on an annual basis.

1. **Previous Approval Date:** 4/1/2013

Is Funding Source the same as on original, approved AUP?

X Yes ☐ **No**

If no, please state Funding Source and Award Number:

2. **Record of Animal Use:**

Common Name	Genus Species	Total Number Previously Approved	Number Used To Date
1. Weakfish	Cynoscion regalis	350	20
2. Summer flounder	Paralichthys dentatus	220	50
3. Mummichog	Fundulus heteroclitus	900	400
4. Striped Bass	Morone saxatilis	400	200
5. Atlantic silverside	Menidia menidia	3600	1500
6. Silver Perch	Bairdiella chrysoura	1300	250
7. Striped killifish	Fundulus majalis	250	150
8. Atlantic croaker	Micropogonias undulatus	450	300
9. Atlantic menhaden	Brevoortia tyrannus	240	40
10. Bay anchovy	Anchoa mitchilli	1200	250
11. Spot	Leiostomus xanthurus	400	300

12. Mullet sp.	Mugil sp.	350	50
13 Northern kingfish	Menticirrhus saxitilis	180	40
14. Halfbeak	Hemiramphus sp.	220	40
12. Various species		500	100

3. Protocol Status: *(Please indicate by check mark the status of project.)*

Request for Protocol Continuance:

X A. Active: Project ongoing

☐ B. Currently inactive: Project was initiated but is presently inactive

☐ C. Inactive: Project never initiated but anticipated starting date is:

Request for Protocol Termination:

☐ D. Inactive: Project never initiated

☐ E. Completed: No further activities with animals will be done.

4. Project Personnel: Have there been any personnel changes since the last IACUC approval? X Yes ☐ No

If Yes, fill out the Amendment to Add/Delete Personnel form to "Add" Personnel.

Project Personnel Deletions:

Name	Effective Date
1. Katherine A. Bogue	Dec 1 2013
2.	

5. **Progress Report:** If the status of this project is 3.A or 3.B, please provide a brief update on the progress made in achieving the aims of the protocol.

For project (A): The fish assemblage was sampled biweekly during 2013 at 3 locations (Lewes Ferry Terminal, Mispillion, Port Mahon) from May-October. Sampling at each location occurred at least one hour after sunrise and one hour before sunset. Sampling took place at 2 sites for each stretch of shoreline. Sites are randomly chosen from the 5 possible sites along each stretch of shoreline. Fish were collected a single tow of a 36m bag-seine net deployed by boat. Captured fish are counted, and a subsample of up to 20 individuals per species is measured (fork length for fishes with forked tails, total length for everything else). Predatory fish are stunned with a blow to the head and immediately placed on dry ice and later stored in a -80c freezer for diet analysis. The rest of the catch is released alive adjacent to the shoreline where they were collected. Night sampling was also be conducted weekly, but only at the Lewes site. Sampling methodology was identical to that described above. The other component of our work in 2013 was tagging spot (256) and Atlantic croaker (137) with Visible Implant Elastomer (Northwest Marine Technology), using standard procedures, to study movement patterns. VIE tags are injected as a 1mmX2mm spot of liquid that soon cures into a pliable, biocompatible solid. Fish will be tagged just under the skin using an NMT syringe injector and immediately released alive at the capture location. None were recaptured.

For Project (B): Juvenile striped bass were obtained from aquaculture facilities at GenOn's Patuxent River Chalk Point Generating Station in MD. Planned growth experiments were attempted in the temperature- and photoperiod-controlled room, using the computer-controlled recirculating aquarium systems, described in the latest approved protocol #1131. Survivorship was poor, due largely to a disease issue in the aquacultured fish, and the experiment had to be abandoned. Instead, mummichogs, striped killifish, and Atlantic silversides were collected from the field and, along with the remaining striped bass, were used in a series aquatic surface respiration (ASR) experiments. These behavioral observations of ASR were conducted in the same temperature and photoperiod-controlled lab as used in growth experiments. Fish were exposed to replicate DO/pH treatment combinations over a period of 48 hours, as described in the latest approved protocol #1131. Fish were subsequently euthanized via cranial concussion and pithing for tissue analyses.

6. **Problems or Adverse Effects:** If the status of this project is 3.A or 3.B, please describe any unanticipated adverse events, morbidity, or mortality, the cause if known, and how these problems were resolved. If there were none, this should be indicated.

As noted above, survivorship of juvenile striped bass obtained from the aquaculture facilities at GenOn's Patuxent River Chalk Point Generating Station in MD was poor, due largely to a disease issue. The growth experiment had to be abandoned. .