

Evaluating the effects of diel-cycling hypoxia and pH on growth and survival of juvenile summer flounder *Paralichthys dentatus*

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ABSTRACT: Effects of diel-cycling dissolved oxygen (DO) and pH on young-of-the-year summer flounder *Paralichthys dentatus* were examined in laboratory experiments. Flounder were exposed to 2 cycling DO levels (extreme = 1–11 mg O₂ l⁻¹; moderate = 3–9 mg O₂ l⁻¹), 2 cycling pH levels (extreme = 6.8–8.1; moderate = 7.2–7.8), and static normoxia (7.5 mg O₂ l⁻¹) and pH (7.5) in a fully crossed 3 × 3 experimental design for 20 d. Cycling conditions reflected summertime DO and pH fluctuations in flounder nursery habitat. Experiments were conducted over 3 partial factorial trials. Growth was significantly reduced in fish exposed to the most extreme diel-cycling DO, across all pH treatments, with no consistent growth reduction in other treatments. Cycling treatments with mean daily low pH (~6.87) and high pCO₂ (~10 000 µatm) had neither an independent nor interactive effect, with hypoxia, on growth. Flounder exhibited growth rate recovery. Following initial growth reduction when exposed to extreme diel-cycling hypoxia and pH over Days 1–10, growth increased ~2-fold under static DO (7.5 mg O₂ l⁻¹) and pH (7.5) conditions over the following 10 d. Flounder did not exhibit growth rate acclimation, defined as increased growth during prolonged exposure, under extreme diel-cycling DO and pH for 20 d. Flounder experienced mortality (>90 % of individuals) after 2–3 wk exposure to extreme diel-cycling DO and pH. These experimental results demonstrate that extreme diel-cycling DO and pH can significantly impact summer flounder growth and survival and that the growth rate reduction is driven by DO.

KEY WORDS: Diel-cycling hypoxia · Diel-cycling pH · Eutrophication · Flounder · Hypoxia · Ocean acidification

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INTRODUCTION

A variety of fishes utilize estuaries as nursery habitat (Beck et al. 2001, Able & Fahay 2010). Such fishes benefit from the physicochemical conditions in these habitats, including shelter, increased food availability, and optimal water temperatures conducive to increased growth rates and survival (Weinstein 1979, Lubbers et al. 1990, Able 1999, Minello 1999). The suitability of estuarine habitats positively correlates with adult population recruitment (Fogarty et al. 1991, Gibson 1994, Beck et al. 2001). Furthermore, increased growth early in ontogeny decreases the

risk of predation, increasing the likelihood that young fishes will reach adulthood (Houde 1987, Vaquer-Sunyer & Duarte 2008).

The physicochemical conditions of estuaries, including dissolved oxygen (DO), pH, temperature, and salinity, vary daily, seasonally, and annually (Boynton et al. 1996, Stierhoff et al. 2009a, Tyler et al. 2009, Howarth et al. 2011). During the summer, algal photosynthesis during the day and subsequent respiration during the night drives shallow estuarine waters to exhibit diel-cycling DO and pH (Tyler & Targett 2007, MD DNR 2012, Baumann et al. 2015). During summer, summer flounder nursery habitat

may experience DO values ranging from anoxia just after dawn to hyperoxia $>15 \text{ mg O}_2 \text{ l}^{-1}$ just before sunset (Tyler & Targett 2007, Tyler et al. 2009) and pH values ranging from 6.5 to 8.5 (MD DNR 2012). These diel cycles have the potential to expose estuarine organisms to multiple potential stressors simultaneously (Orr et al. 2005, Denman et al. 2011). Such diel cycles can be amplified by anthropogenic influences such as nutrient loading and impacts of fossil fuel combustion, including increasing sea surface temperatures and ocean acidification (Diaz 2001, Cai et al. 2011).

Diel-cycling hypoxia can have sub-lethal impacts on fishes, such as reduction in growth, feeding, and fecundity (McNatt & Rice 2004, Bell & Eggleston 2005, Stierhoff et al. 2006, 2009a). Exposure to chronic and diel-cycling hypoxia may lead to reductions in food intake (Stierhoff et al. 2003, 2006, Brandt et al. 2009, Wang et al. 2009), and slower swimming speeds (Schurmann & Steffensen 1994, Brady et al. 2009, Chapman & McKenzie 2009, Stierhoff et al. 2009b) potentially leading to decreased foraging efficiency, resulting in growth reduction (Stierhoff et al. 2003, 2006, Wang et al. 2009, Brandt et al. 2009). Other behavioral responses to hypoxia, such as increased gill ventilation rate and stroke volume (Breitburg et al. 1994, Wannamaker & Rice 2000, Taylor & Miller 2001, Perry et al. 2009), are energy intensive, forcing fish to allocate less energy toward growth and reproduction (Steffensen et al. 1982, Herbert & Steffensen 2006).

Juvenile fishes subjected to extreme diel-cycling hypoxia exhibit avoidance behaviors, when DO approaches $\sim 1 \text{ mg l}^{-1}$, to search for sufficiently oxygenated water (Eby & Crowder 2002, Tyler & Targett 2007, Stierhoff et al. 2009b, Brady & Targett 2010, 2013). Although avoidance behavior increases survival of fishes subjected to severe hypoxia or anoxia, growth reduction is often experienced before fish actively avoid hypoxic environments (Stierhoff et al. 2006, 2009a,b). Several juvenile estuarine species do not exhibit avoidance behavior until DO approaches $\sim 1 \text{ mg O}_2 \text{ l}^{-1}$, well below the EPA growth protective criterion of $>4.8 \text{ mg O}_2 \text{ l}^{-1}$ (Wannamaker & Rice 2000, Stierhoff et al. 2006, 2009b). Furthermore, if fish are able to find normoxic waters, overcrowding may occur, increasing competition for prey and causing density dependent growth impacts (Eby et al. 2005, Campbell & Rice 2014).

In laboratory experiments juvenile summer flounder exhibit growth rate reductions of $\sim 25\%$ at static levels of $3.5 \text{ mg O}_2 \text{ l}^{-1}$, $50\text{--}60\%$ at $2.0 \text{ mg O}_2 \text{ l}^{-1}$ and $20\text{--}25^\circ\text{C}$, and $\sim 90\%$ at $2.0 \text{ mg O}_2 \text{ l}^{-1}$ and 30°C (Stier-

hoff et al. 2006). Diel-cycling hypoxia, with DO minima of $2\text{--}3 \text{ mg O}_2 \text{ l}^{-1}$ caused growth reductions of $\sim 35\%$ at both 25 and 30°C (Stierhoff et al. 2006). Juvenile summer flounder exhibit active avoidance responses to diel-cycling hypoxia in the laboratory, with swimming speed peaking at $\sim 1.4\text{--}2.8 \text{ mg O}_2 \text{ l}^{-1}$ (Brady & Targett 2010). During summertime, diel-cycling hypoxia can cause DO levels below the static LC_{50} for juvenile summer flounder ($1.1\text{--}1.6 \text{ mg O}_2 \text{ l}^{-1}$; Poucher & Coiro 1997, Miller et al. 2002).

There is a growing literature on the effects of ocean acidification on marine organisms (Melzner et al. 2009, Kroeker et al. 2013). Heuer & Grosell (2014) reviewed the impacts of acidification on marine teleost fishes across a range of physiological processes including development, metabolism, growth, reproduction, and other vital functions. Furthermore, anthropogenic influences are exacerbating biogeochemical cycles in estuaries and coastal waters, leading to localized pH reductions, compounding pH reductions caused by ocean acidification (Duarte et al. 2013, Heuer & Grosell 2014, Baumann et al. 2015). Few studies have investigated the independent and interactive impacts of diel-cycling hypoxia and pH on young fishes that utilize estuarine nursery habitats impacted by present-day hypoxia and low pH (Melzner et al. 2009, Bogue 2013, Borowiec et al. 2015). The combined impacts of hypoxia and decreased pH have been shown to increase mortality and decrease cellular metabolic efficiency compared to hypoxia alone in tilapia *Oreochromis mossambicus* (van Ginneken et al. 1996). No studies have investigated the synergistic impacts of diel-cycling hypoxia and co-varying pH on growth and mortality of juvenile summer flounder.

The objectives of this study were to investigate the individual and interactive effects of moderate and severe diel-cycling DO and pH on growth and mortality of juvenile summer flounder. To understand longer term growth impacts of diel hypoxia and pH cycles this study investigated whether these fish exhibit either growth rate recovery or residual negative effects on growth following 10 d exposure to diel-cycling DO and pH. Recovery would be manifested in increased growth rate during a subsequent 10 d period of normoxia and normal pH conditions, whereas residual effects would be evident by continued growth detriment. In addition, the study investigated whether juvenile summer flounder are capable of growth rate acclimation, by increasing growth rate during prolonged exposure to diel-cycling hypoxia and pH, relative to initial rates.

MATERIALS AND METHODS

Summer flounder collection and acclimation

Juvenile summer flounder were collected using seine and otter trawl from Pepper Creek and Indian River, tributaries of the Delaware Coastal Bays, during June and July 2014. Fish were held in ~450 l recirculating aquaria at 25°C, salinity of 25, and normoxia ($7.5 \text{ mg O}_2 \text{ l}^{-1}$) for 14 to ~60 d to acclimate to laboratory conditions. Photoperiod was 14 h light: 10 h dark and fish were fed mysid shrimp (*Mysis relicta*) ad libitum twice daily, at 09:00 h and 17:00 h. After acclimation, fish were transferred to recirculating aquarium systems with computer-controlled DO and pH.

Experimental aquarium systems

Growth experiments took place at the University of Delaware (Lewes, Delaware) in 5 recirculating aquarium systems originally described by Greccay & Stierhoff (2002) and subsequently used in studies of diel-cycling and chronic hypoxia impacts on growth and behavior of juvenile estuarine fishes (Stierhoff et al. 2006, 2009b). These computer-controlled aquarium

systems were modified (Bogue 2013) to allow independent control of DO and pH. Each system was airtight ensuring minimal atmospheric contamination (Fig. 1). Water constantly flowed into each experimental chamber and overflowed into the surrounding tray, ensuring that the fish's metabolism did not alter DO and pH exposure within the chambers.

DO and pH of each aquarium system was measured and adjusted every ~10 min, by a loop containing probes, controllers, gases, and valves connected to and controlled by LabVIEW (Greccay & Stierhoff 2002, Bogue 2013). A Hach sc200 Universal Controller, Hach LDO DO probe and Hach Differential pH/ORP sensor regulated DO and pH in each system by adding necessary amounts of compressed N_2 , CO_2 , O_2 , and air into the sump (Fig. 1) via solenoid-controlled gas manifolds. DO levels were controlled by N_2 and O_2 infusion and pH levels were controlled by CO_2 and air. Both DO and pH could be manipulated in each aquarium system independently, allowing 5 treatment combinations to be run concurrently. Water was buffered and alkalinity adjusted using Kent Marine Superbuffer-dkH™ to allow precise and prolonged pH control. Continuous DO and pH data during experiments were recorded and analyzed to ensure accuracy of the treatments.

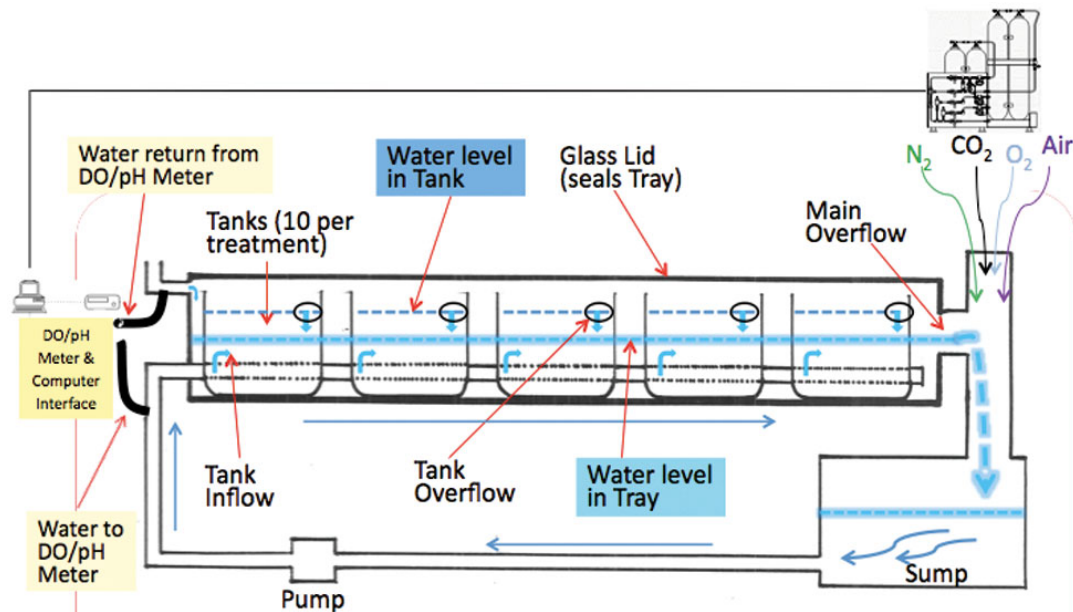


Fig. 1. One of the 5 aquarium systems used for DO and pH control. The DO/pH meter and computer interface dictated the amount of N_2 , CO_2 , O_2 , and air that was bubbled into the sump via solenoid-controlled gas manifolds. A portion of the water in the vertical supply line was diverted to the DO and pH probes to measure DO and pH levels. The water was then returned to the supply line. The computer received DO and pH data from the Hach sc200 meter and determined the correct amount of gas to be released through the gas solenoids to ensure DO and pH levels were maintained for each particular treatment regime. Each of the 5 systems had ten 18 l experimental chambers, holding 1 summer flounder per chamber (Note: only 5 chambers are visible in this side view). Water flowed into each chamber inflow and overflowed back into the tray where it flowed back into the sump. The DO and pH were again adjusted as necessary, and the water was pumped back through the supply line

Each aquarium system had a volume ~415 l and held 10 juvenile summer flounder in 10 individual 18 l polyethylene experimental chambers. Individual aquarium systems could be controlled independently allowing 5 separate and concurrent DO and pH treatments. Aquaria were maintained at 25°C, a salinity of 25, and photoperiod of 14 h light: 10 h dark, to mimic conditions in the field; these conditions were maintained throughout all experiments. Minimum and maximum DO and/or pH in the diel-cycling treatments coincided with the beginning of the light portion of the photoperiod (07:00 h) and the dark portion of the photoperiod (21:00 h), respectively. Summer flounder were fed mysid shrimp (*Mysis relicta*) ad libitum twice daily at 09:00 h and 17:00 h during experiments.

Water in the aquarium systems was seeded with appropriate bacterial communities via the addition of filter media from the aquaria where flounder were held prior to experiments to ensure adequate microbial control of ammonia and nitrite levels. Ammonia and nitrite levels were monitored 3–4 times a week using API test kits.

Experimental design

Experimental regimes of diel-cycling hypoxia and pH were selected based on observed conditions in shallow-water estuarine nursery habitats for summer flounder (Tyler et al. 2009, MD DNR 2012, Baumann et al. 2015). Flounder were exposed to 2 cycling DO levels (extreme = 1–11 mg O₂ l⁻¹; moderate = 3–9 mg O₂ l⁻¹), 2 cycling pH levels (extreme = 6.8–8.1; moderate = 7.2–7.8), and a constant normoxia (7.5 mg O₂ l⁻¹) and pH (7.5) control treatment. This produced a 3 × 3 matrix (n = 10 per cell), yielding 8 different 20 d diel-cycling hypoxia and pH treatments and 1 control treatment (Table 1). A series of partial factorial experiments was conducted over 3 time intervals, or trials, during the summer of 2014; a control treatment

was run in each trial to provide a concurrent control for treatment comparisons (Table 1). Trial 1 tested the following treatments: Extreme DO Cycle & Moderate pH Cycle, Static DO & Moderate pH Cycle, Extreme DO Cycle & Static pH, and Static DO & Extreme pH Cycle. Trial 2 tested the Extreme DO & Extreme pH Cycles and the Moderate DO & Moderate pH Cycles treatments. Trial 3 tested the Moderate DO Cycle & Extreme pH Cycle and the Moderate DO Cycle & Static pH treatments.

Growth rate recovery, residual effects, and acclimation were investigated with additional treatments outside the 3 × 3 matrix. These treatments were conducted during the same trial (Trial 2) as the moderate diel-cycling DO and pH group and the extreme diel-cycling DO and pH group. The other treatments were randomly assigned to the other 2 trials. Daily DO and pH minima and maxima were recorded during experimentation to ensure accurate reporting of DO and pH treatment means.

Growth rate recovery or residual negative effects following 10 d exposure to diel-cycling hypoxia and pH were also investigated, outside the 3 × 3 matrix, by exposing juvenile summer flounder to either moderate (3–9 mg O₂ l⁻¹, pH 7.2–7.8) or extreme (1–11 mg O₂ l⁻¹, pH 6.8–8.1) diel-cycling hypoxia and pH conditions for 10 d, followed by 10 d of normoxia (7.5 mg O₂ l⁻¹), and pH 7.5 (n = 10). Data analyses assessed whether fish exhibited either growth recovery, demonstrated by increased growth rate during the second 10 d period relative to that during the initial 10 d of diel-cycling hypoxia and pH, or residual effects that would be evident by continued growth detriment. Growth rates during both 10 d periods were compared with the concurrently run control group.

Furthermore, the potential for growth rate acclimation during prolonged exposure to diel-cycling hypoxia and pH was investigated. Fish were subjected to moderate and extreme diel-cycling DO and pH treatments for 30 d (n = 10 per treatment) to determine (1) if prolonged exposure to these conditions resulted

in increased growth rate during the second and third 10 d periods, relative to the control, and (2) whether prolonged exposure resulted in apparent behavioral changes or mortality.

Fish (50–94 mm standard length, SL) were placed individually into experimental chambers in the computer-controlled aquarium systems and given 3 d to acclimate under static DO and pH conditions

Table 1. Diel-cycling DO and pH treatments. Ten fish per cell. Standard, underlined, and *italicized* text indicate treatments run in Trial 1, 2, and 3, respectively.

A control treatment (upper left) was included in all 3 trials

pH treatment	DO treatment		
	Normoxia (7.5 mg O ₂ l ⁻¹)	Moderate cycling (3–9 mg O ₂ l ⁻¹)	Extreme cycling (1–11 mg O ₂ l ⁻¹)
Static (7.5)	Control	<i>Trial 3</i>	Trial 1
Moderate cycling (7.2–7.8)	Trial 1	<u>Trial 2</u>	Trial 1
Extreme cycling (6.8–8.1)	Trial 1	<i>Trial 3</i>	<u>Trial 2</u>

that duplicated the experimental control (Table 1). Fish varied in size among the 3 trials, however, care was taken to ensure that individuals within each given trial had similar body masses at the beginning of the trial (2-way ANOVA: $p = 0.92\text{--}0.99$). At the end of acclimation, food was withheld for 1 d (to minimize the effect of stomach contents on body mass) and fish SL was measured using a digital caliper (± 0.1 mm) and weighed using a DeltaRange Mettler PE 360 balance (± 0.1 mg) and methods described in Stierhoff et al. (2006) whereby fish were placed on a damp sponge and their weight determined by subtracting the weight of the sponge. Measuring and weighing occurred on Days 0, 10, and 20 of each trial, prior to the 09:00 h feeding.

Response variables

Change in mass of each fish was calculated over each 10 d interval. Masses were also used to calculate daily specific growth rate (SGR; % body mass d^{-1}); $SGR = (e^G - 1) \times 100\%$, where G (instantaneous growth rate) = $[(\ln W_f - \ln W_i) / d]$, W_f = final mass, W_i = initial mass and d = number of days between weight measurements. Standard lengths were used to calculate linear growth rate (LGR; mm d^{-1}); $LGR = [(L_f - L_i) / d]$, where L_f = final SL, L_i = initial SL and d = number of days between length measurements.

Effects of experimental treatments on 2 possible growth metrics, SGR and change in mass, were tested using a linear model with initial mass as a covariate to determine the most appropriate growth metric for further analysis. Using SGR as the primary growth metric for analysis was problematic as there was significant treatment \times initial mass interaction. In contrast, there was no such interaction with change in mass; thus, change in mass was chosen as the primary response variable in this study. However, mean SGR of each treatment in the 3×3 matrix was presented, to allow comparison of the results of this study to others that have relied on SGR as a primary growth metric.

Statistical analyses

Analyses of treatments in 3×3 matrix

A series of Dunnett's tests, a multiple comparison procedure that treats all individuals as independent in their response to treatments, was performed to compare the mean change in mass of fish in each

treatment to the mean change in mass in the concurrent control in the 3 partial factorial trials to compare growth over Days 0–10 and 10–20. Another Dunnett's test was performed comparing mean LGR of each treatment to the mean LGR of the concurrent control. Significance was tested using $\alpha = 0.1$, as tests were 1-tailed because they tested for growth reduction due to treatment effect, relative to the control, not overall growth differences. To facilitate more conservative interpretations, the results of the Dunnett's tests were analyzed before and after applying a Bonferroni correction of 3, resulting in a corrected $\alpha = 0.033$, because we ran 3 partial factorial experiments or trials.

Ad hoc procedures were used to further investigate the interactive effect that pH had on DO on summer flounder growth. Growth Curve Analysis (GCA; Mirman 2014), a repeated measures multilevel regression technique, and ANCOVAs, using initial mass as a covariate, were performed to determine whether summer flounder growth rate in the extreme diel-cycling DO and static pH treatment was significantly different from that in the concurrently run extreme diel-cycling DO and moderate diel-cycling pH treatment. The GCA compared the growth curves, based on change in mass from time-repeated weight measurements, of the 2 treatments over Days 0–20 and the ANCOVAs compared the change in mass of the 2 treatments over Days 0–10, 10–20, and 0–20.

Growth rate recovery and acclimation analyses

To investigate presence of growth rate recovery or residual negative growth impacts, a Dunnett's test was performed comparing the mean change in mass of the group subjected to extreme diel-cycling DO and pH treatment for Days 0–10 and then returned to static control levels for Days 10–20, to the concurrent control group. The same comparison was made for the group subjected to moderate diel-cycling DO and pH treatment for Days 0–10 and then returned to static control levels for Days 10–20. In addition to the Dunnett's tests, GCAs compared growth trajectories of the group subjected to the most extreme treatment for 20 d with the concurrently run group subjected to the extreme treatment (Days 0–10) and then returned to static (control) levels (Days 10–20); as well as the group in the most moderate treatment for 20 d with the concurrent group in the moderate treatment (Days 0–10) and returned to static levels (Days 10–20). The GCAs allowed a further investigation of the presence of growth rate recovery or residual

effects through analyzing changes in growth trajectories in groups exposed to static (control) conditions subsequent to diel-cycling DO and pH relative to the groups continuously subjected to same level diel-cycling DO and pH.

The presence of growth rate acclimation was investigated using the same methods to detect growth rate detriment in the 3 × 3 matrix. Dunnett's tests were performed to compare the mean change in mass of fish in the extended most moderate and most extreme diel-cycling DO and pH treatments, maximum duration of 30 d, to the mean change in mass in the concurrent control to determine if initial growth detriment is mitigated over time. The treatments conducted to detect both growth rate recovery or residual growth impact and growth rate acclimation occurred at the same time as treatments to investigate growth impacts of the 3 × 3 matrix.

Measurement of $p\text{CO}_2$

The partial pressure of CO_2 ($p\text{CO}_2$) is a unifying measure in ocean acidification studies and $p\text{CO}_2$ is the standard metric for aquatic carbon species (Riebesell et al. 2010). Water samples for $p\text{CO}_2$ analysis were taken from each treatment system at 09:00, 12:00, and 17:00 h on experimental Days 0, 10, and 20 to capture the range of pH and $p\text{CO}_2$ values during experiments. Water samples were also collected from Pepper Creek, Delaware Coastal Bays, a summer flounder nursery area, on September 5, 2014 at 08:09, 12:03, and 16:41 h. Water collected from the field was ~25°C and ~22.5 ppt, similar to laboratory conditions.

Samples were collected in 20 ml scintillation vials and sterilized with 0.2 ml of 5% HgCl_2 . In preparation for total alkalinity (TA) analysis, samples were filtered through a 0.2 μm Sartorius, 16532-Q™ syringe filter. Total alkalinity was analyzed at Union College (Schenectady, NY) based on methods described in Bouillon et al. (2012, 2014) using a Metrohm 888 Titrando™ autotitrator and 869 compact sample changer with 0.1 mol l^{-1} HCl as titrant; reproducibility was typically better than $\pm 6 \mu\text{mol kg}^{-1}$ based on 22 replicate analyses of an in-house standard. CO2SYS software (Lewis et al. 1998) was used to calculate $p\text{CO}_2$ values using TA, pH, temperature, and salinity in conjunction with the thermodynamic constants outlined by Millero (1979).

Other observations

During the morning feeding (09:00 h), several behavioral observations were made including erratic swimming behavior indicative of an escape response, sluggish activity when fed, and a qualitative assessment of gill ventilation rate and stroke volume throughout all extreme diel-cycling DO treatments. During both the morning and evening (17:00 h) feedings, reduced feeding activity was observed. At these times mortalities were noted and promptly removed to maintain water quality. When multiple mortalities occurred, the aquarium system was thoroughly inspected; water parameters including DO, pH, and salinity were checked using a Yellow Springs Instrument 556MPS hand-held probe; and ammonia, nitrite, and nitrate levels were tested using API test kits. Mortalities during the most extreme diel-cycling DO and pH treatments prevented an extended temporal investigation, beyond 20 d, of growth rate acclimation in this treatment.

RESULTS

Experimental conditions

Prescribed DO and pH conditions (Table 1) were closely achieved during experiments (Table 2). Maximum DO values were not greatly impacted by limitations associated with DO regulation while pH increased.

Response variable

The effect of experimental treatments on growth rates, for both SGR and change in mass, was tested by a linear model using initial mass as a covariate. There was a significant or marginally significant interaction between initial mass and SGR at Day 0 and Day 10 across all trials, with p-values ranging

Table 2. Mean minimum and maximum dissolved oxygen (DO) and pH values during juvenile summer flounder growth experiments (see Table 1). Parentheses enclose the coefficient of variation

Treatment	DO (mg l^{-1})		pH	
	Min.	Max.	Min.	Max.
Static control	7.43 (0.019)	–	7.49 (0.002)	–
Moderate cycling	3.13 (0.094)	8.28 (0.093)	7.22 (0.001)	7.78 (0.001)
Extreme cycling	1.16 (0.081)	10.01 (0.096)	6.87 (0.007)	7.99 (0.005)

from <0.01 to 0.09 . However, no significant interaction was detected between initial mass and change in mass at Day 0 and Day 10 across all trials, with p -values ranging from 0.28 to 0.99 . Change in body mass was therefore the primary response variable utilized to examine treatment effects.

Statistical analyses

Analyses of treatments in 3×3 matrix

The changes in mass of individual fish across all treatments are grouped by trials (Fig. 2) in which the fish were run concurrently in partial factorial experiments. All treatment groups that experienced extreme diel-cycling hypoxia, across all pH treatments, exhibited significant growth rate reduction, compared to concurrent controls, during Days 0–10 and 10–20 (Fig. 2, Table 3). Note that the extreme diel-cycling hypoxia and static pH group exhibited signif-

icant growth detriment during Days 0–10 only prior to the Bonferroni adjustment (Fig. 2, Table 3). The moderate diel-cycling hypoxia and static pH, moderate diel-cycling hypoxia and extreme diel-cycling pH, and static DO and moderate diel-cycling pH treatments exhibited growth reduction only during the first 10 d of their respective trials. No consistent growth reduction was observed outside of the extreme diel-cycling hypoxia treatments (Fig. 2, Table 3). Dunnett's tests using LGR as the growth metric showed the same results.

To further investigate an interactive effect of diel-cycling pH on the impact of diel-cycling DO on growth, tests were conducted to compare the extreme diel-cycling hypoxia and static pH group with the concurrently run extreme diel-cycling hypoxia and moderate cycling pH group (see Fig. 2). First a GCA comparing the 2 groups' growth trajectories over the 20 d experiment yielded $p = 0.055$, suggesting that growth trajectories from the 2 group were not significantly different. Note that a number of

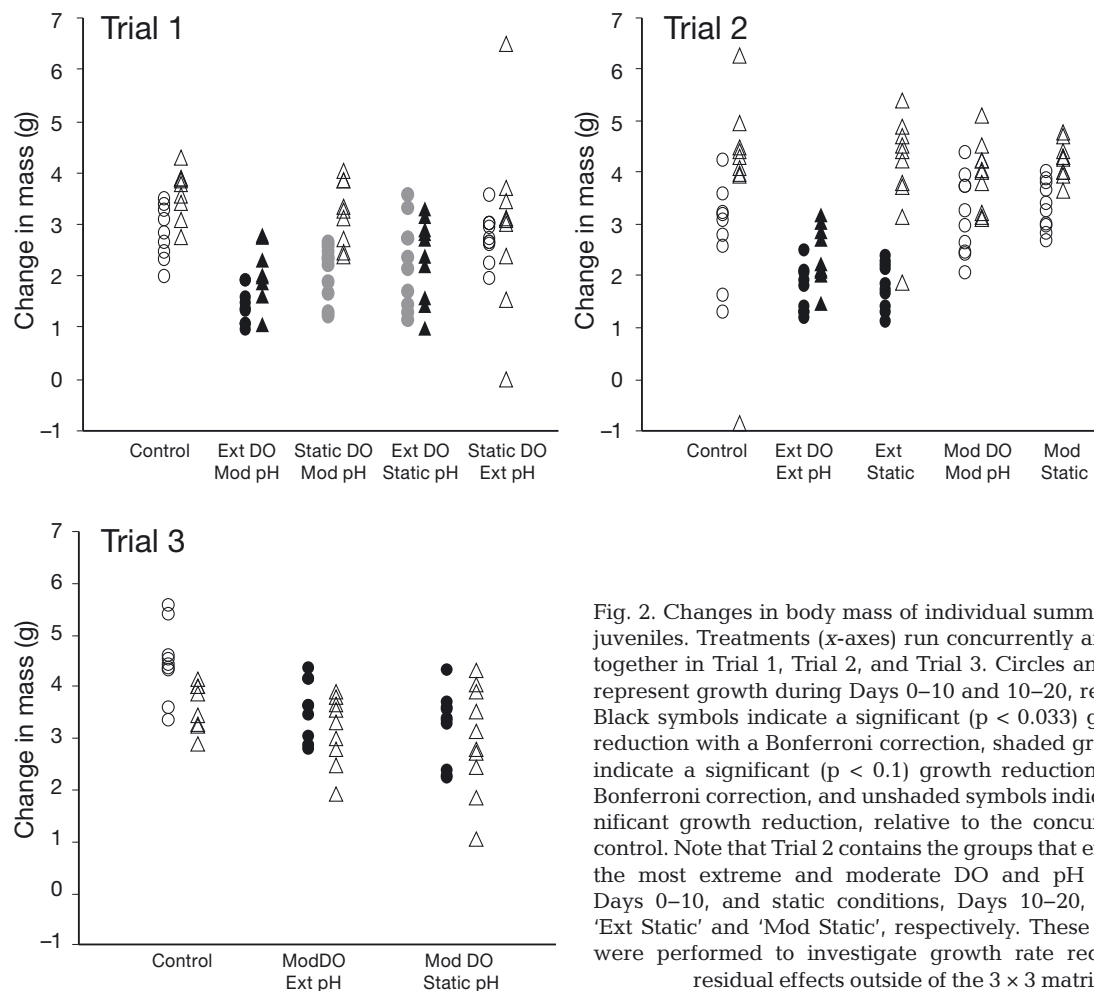


Fig. 2. Changes in body mass of individual summer flounder juveniles. Treatments (x-axes) run concurrently are grouped together in Trial 1, Trial 2, and Trial 3. Circles and triangles represent growth during Days 0–10 and 10–20, respectively. Black symbols indicate a significant ($p < 0.033$) growth rate reduction with a Bonferroni correction, shaded grey symbols indicate a significant ($p < 0.1$) growth reduction without a Bonferroni correction, and unshaded symbols indicate no significant growth reduction, relative to the concurrently run control. Note that Trial 2 contains the groups that experienced the most extreme and moderate DO and pH conditions, Days 0–10, and static conditions, Days 10–20, labeled as 'Ext Static' and 'Mod Static', respectively. These treatments were performed to investigate growth rate recovery and residual effects outside of the 3×3 matrix

Table 3. Dunnett's test results (p-values) comparing mean change in mass of juvenile summer flounder in each treatment (see Table 1 for details) with the concurrently run control (na: not applicable for control). Significant values, $p = 0.1$ in **bold** (^{corr} indicates significance with correction)

	Normoxic		Moderate DO		Extreme DO	
	Day 0–10	Day 10–20	Day 0–10	Day 10–20	Day 0–10	Day 10–20
Static pH	na	na	0.003^{corr}	0.19	0.07	0.02^{corr}
Moderate pH	0.09	0.91	0.83	0.99	<0.001^{corr}	<0.007^{corr}
Extreme pH	0.98	0.40	0.004^{corr}	0.48	<0.001^{corr}	0.008^{corr}

comparisons are possible when performing a GCA and a significance level more stringent than $\alpha = 0.05$, usually $\alpha = 0.01$, is suggested (Mirman 2014). Several ANCOVAs were also conducted analyzing the same groups across Days 0–10, 10–20, and, 0–20 yielding $p = 0.015$, $p = 0.50$, and $p = 0.12$, respectively. These analyses therefore suggest that the extreme diel-cycling DO treatments with moderate diel-cycling pH and static pH yielded similar overall results. Thus there is no evidence of an interactive impact of diel-cycling pH, in addition to DO effects, on growth in juvenile summer flounder.

Growth rate recovery and acclimation

Fish exposed to the extreme diel-cycling DO and pH treatment (Days 0–10) exhibited significant growth differences relative to the concurrently run control group ($p < 0.01$, based on Dunnett's test); however, when returned to static (control) levels (Days 10–20) growth rates were parallel to that of the control ($p = 0.99$), demonstrating growth rate recovery and no significant residual growth effect of the extreme diel-cycling DO and pH treatment (Figs. 2 & 3). The GCA comparing the group subjected to the extreme diel-cycling DO and pH treatment for 20 d to the concurrent group subjected to the extreme treatment (Days 0–10) and returned to static (control) levels during (Days 10–20) yielded $p < 0.01$. These results illustrate a significant difference in growth trajectories between the 2 groups, further demonstrating that these fish exhibit growth rate recovery after a period of growth detriment and do not exhibit residual negative effects of the extreme diel-cycling DO and pH treatment (Fig. 3).

The group subjected to the moderate diel-cycling DO and pH treatment showed no significant growth difference relative to the concurrently run control group during either the first 10 treatment days ($p = 0.40$, based on Dunnett's test) or following the return to static levels over the second 10 d ($p = 0.97$), there-

fore there can be no growth rate recovery or residual negative impacts of moderate diel-cycling DO and pH due to lack of an initial impact. The GCA comparing the group subjected to moderate diel-cycling DO and pH for 20 d to the concurrent group subjected to the moderate treatment (Days 0–10) and returned to static levels (Days 10–20) yielded $p = 0.23$. These results illustrate no significant difference in growth trajectories, further demonstrating that moderate diel-cycling DO and pH does not have a significant impact (Fig. 3).

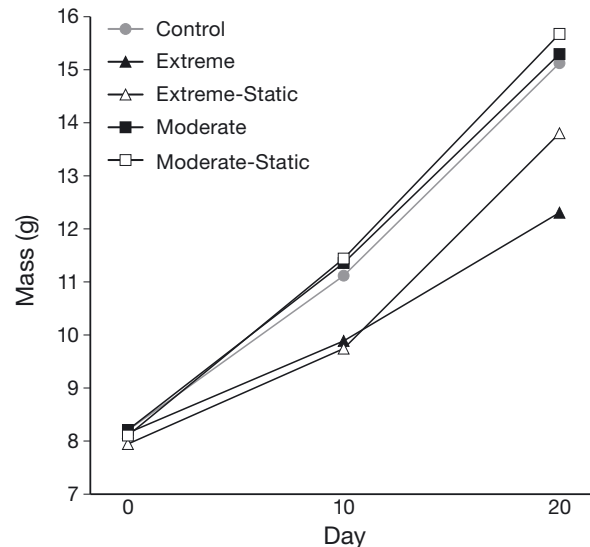


Fig. 3. Growth curves for juvenile summer flounder from the growth rate recovery and residual growth effect treatments. Control refers to the group held under static DO and pH for 20 d, Extreme refers to the group held under extreme diel-cycling DO and pH for 20 d, Extreme-Static refers to the group held under extreme diel-cycling DO and pH for 10 d and subsequently held under static DO and pH conditions for 10 d, Moderate refers to the group held under moderate diel-cycling for 20 d, and Moderate-Static refers to the group held under extreme moderate diel-cycling DO and pH for 10 d and subsequently held under static DO and pH conditions for 10 d. Mean mass of fish per treatment is shown at Days 0, 10, 20 of the trial. The curves show growth rate recovery (open triangle) when fish are subjected to the most extreme diel-cycling DO and pH treatment (Days 0–10) and then returned to static levels (Days 10–20)

$p\text{CO}_2$ values

During diel-cycling and static DO and pH treatments, pH range was 6.85–8.03 and $p\text{CO}_2$ range was 13 706–805 μatm (Fig. 4). Water samples from Pepper Creek, a summer flounder nursery area, showed similar, although slightly lower, $p\text{CO}_2$ values for a given pH. The pH and $p\text{CO}_2$ values from the field were 7.35 and ~2080 μatm at 08:09 h, 7.60 and 1080 μatm at 12:03 h, and 7.79 and ~683 μatm at 16:41 h, respectively.

Specific growth rate

Specific growth rate was calculated for Days 0–10 and Days 10–20 for each treatment group (Table 4). Specific growth rate varied across treatments, in a

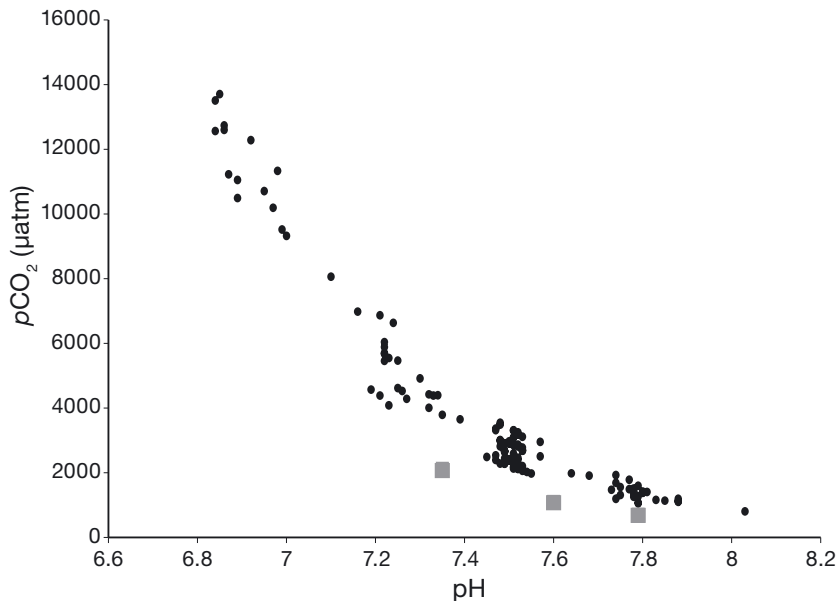


Fig. 4. $p\text{CO}_2$ and pH values during juvenile summer flounder growth experiments. Black circles are $p\text{CO}_2$ values from the aquarium systems during experiments and grey squares are values of water samples taken from Pepper Creek, Delaware

similar manner to change in body mass, and are presented to provide a growth metric for comparison with other studies and for better translation to the field. Decreasing SGR over the course of the 3 trials coincided with an increase in initial body size.

DISCUSSION

Diel-cycling hypoxia and pH impacts on summer flounder growth

Juvenile summer flounder exposed to extreme diel-cycle hypoxia (1–11 $\text{mg O}_2 \text{ l}^{-1}$) exhibited significantly reduced growth, across all pH treatments. No consistent growth reduction was observed at moderate levels of diel-cycling hypoxia (3–9 $\text{mg O}_2 \text{ l}^{-1}$), across pH treatments. Statistical analyses showed no

overall independent or interactive effect of diel-cycling pH on juvenile summer flounder growth rate, therefore these data do not suggest that the pH ranges tested, and commonly observed in summer flounder nursery habitat (MD DNR 2012, Baumann et al. 2015) were stressful to juvenile summer flounder. The only previous investigation of diel-cycling hypoxia and pH impacts on fish growth reported similar results of no independent or interactive effects on growth rate of the mummichog *Fundulus heteroclitus*, a hypoxia-tolerant estuarine resident species (Bogue 2013). This study provides another example of a juvenile estuarine fish showing no impact of acidification (means during the low part of the diel cycle as low as pH 6.87 and $p\text{CO}_2$ as high as ~10 000 μatm) on growth rate (Hendrey 1987, Hudd 2000).

Table 4. Mean specific growth rate (SGR; % body mass d^{-1}) of juvenile summer flounder for each treatment (Trial [T] 1, 2 and 3; see Table 1 for details)

	Normoxic		Moderate DO		Extreme DO	
	Day 0–10	Day 10–20	Day 0–10	Day 10–20	Day 0–10	Day 10–20
Static pH	4.12 (T1) 3.15 (T2) 3.79 (T3)	3.57 (T1) 3.11 (T2) 3.67 (T3)	2.87 (T3)	2.07 (T3)	3.27 (T1)	2.59 (T1)
Moderate pH	3.72 (T1)	3.67 (T1)	3.48 (T2)	3.12 (T2)	2.25 (T1)	2.42 (T1)
Extreme pH	4.04 (T1)	2.97 (T1)	3.11 (T3)	2.09 (T3)	2.04 (T2)	2.26 (T2)

Growth rate recovery

Juvenile summer flounder exhibited growth rate recovery after exposure to extreme diel-cycling hypoxia and pH for 10 d. After cessation of the extreme diel-cycling hypoxia and pH treatments, growth rate increased to control levels. No compensatory growth (growth greater than the control that could compensate for the initial growth reduction) was observed (Fig. 3). In the field, when extreme diel-cycling hypoxia and pH conditions improve (Tyler et al. 2009, MD DNR 2012, Baumann et al. 2015), we would expect growth of juvenile summer flounder to recover. The lack of negative residual growth effects following a 10 d exposure to growth limiting diel-cycling hypoxia and pH demonstrates a degree of resilience of this species to current and future diel-cycling hypoxia conditions.

Bejda et al. (1992) showed that juvenile winter flounder *Pseudopleuronectes americanus* could fully compensate for growth limitation experienced during 10–11 wk of exposure to chronic ($2.2 \text{ mg O}_2 \text{ l}^{-1}$) and diel-cycling ($2.5\text{--}6.4 \text{ mg O}_2 \text{ l}^{-1}$) hypoxia. When returned to optimal DO conditions ($7.2 \text{ mg O}_2 \text{ l}^{-1}$) for 5 wk, these fish exhibited growth rates significantly greater than fish held under optimal conditions throughout the entire experiment. Paul et al. (1995) showed that another flatfish, juvenile yellowfin sole *Pleuronectes asper* were unable to demonstrate growth rate compensation after being withheld food for several weeks while Ali et al. (2003) reported compensatory growth across several families of fishes subsequent to food deprivation. Given the duration of the present study it was not possible to determine whether juvenile summer flounder are capable of increasing growth rate sufficiently, over the longer term, to completely compensate for the detrimental growth effects of diel-cycling hypoxia.

Behavioral observations

Qualitative observations indicated that juvenile summer flounder increased gill ventilation rate and stroke volume during DO minima under moderate and extreme diel-cycling hypoxia treatments. Reduced feeding was observed under all extreme diel-cycling hypoxia treatments, during both the morning and evening feedings. During the morning feeding, which coincided with the DO minimum, fish in the extreme diel-cycling hypoxia treatments exhibited either sluggish behavior, reduced activity when fed, or erratic swimming indicative of an escape response.

Behaviors observed in the extreme diel-cycling hypoxia treatments were insufficient to mitigate growth rate reduction. Increased gill ventilation rate and stroke volume and erratic swimming behavior, indicative of an escape response, are energy intensive, but increased gill ventilation, in the absence of other observed behaviors, had insignificant impact on growth rate, as this behavior was observed during DO minima in most fish in the moderate diel-cycling DO treatments which showed no growth rate reduction. Reduced feeding rates observed in the extreme diel-cycling hypoxia treatments likely contributed to the observed growth reduction. A direct relationship between hypoxia-induced growth limitation and reduced feeding rate has been previously established in juvenile summer flounder and winter flounder (Stierhoff et al. 2006) and mummichog (Stierhoff et al. 2003).

The sedentary lifestyle of juvenile summer flounder may result in less scope to decrease routine metabolism under extreme diel-cycling hypoxia, and thus less latitude to mitigate detrimental growth impacts associated with diel-cycling hypoxia and pH, compared to other more active mid-water and benthopelagic fishes (Schurmann & Steffensen 1994, Crocker & Cech 1997, Brady et al. 2009, Stierhoff et al. 2009b). Stierhoff et al. (2006), for example, reported that juvenile weakfish *Cynoscion regalis* reduced swimming activity at low DO in the laboratory (see also Brady et al. 2009); and speculated that this behavioral scope allowed high growth rates to be maintained in the laboratory under *ad libitum* feeding conditions. However, *in situ* growth in the field was reduced under comparably low diel-cycling DO levels where reduced swimming likely reduced feeding.

Mortality and acclimation

Mortalities precluded an extended evaluation (>20 d) of growth rate acclimation, defined as increased growth during prolonged exposure to extreme diel-cycling DO and pH, a phenomenon, that occurs in juvenile summer flounder habitat (MD DNR 2012). Summer flounder in Trial 2, under the most extreme diel-cycling DO and pH treatment, experienced 100% mortality on Day 21. This treatment was repeated and 9 of 10 fish died on Day 15 of Trial 3, although 1 fish survived for 30 d. No mortality occurred during 30 d of moderate diel-cycling hypoxia and pH. Given the results of the Dunnett's test that indicate reduced growth throughout the 20 d trial (Table 3, Fig. 2) and the fact that juvenile sum-

mer flounder exhibited mortality when subjected to prolonged exposure (~2–3 wk) to extreme diel-cycling hypoxia and pH it can be inferred that the fish are incapable of growth rate acclimation under such extreme diel cycles.

During the mortality events under extreme diel-cycling hypoxia and pH (Day 21 of Trial 2 and Day 15 of Trial 3) DO and pH regulation was checked and was functioning normally and ammonia, nitrite, and nitrate levels were ~0 ppm. These results suggest juvenile summer flounder do not exhibit growth rate acclimation to extreme diel-cycling hypoxia and pH, consistent with results on juvenile summer flounder reported by Stierhoff et al. (2006) during a 14 d chronic (2 mg O₂ l⁻¹) and diel-cycling (2–11 mg O₂ l⁻¹) hypoxia trial. These results add to the known risks of summer flounder, specifically an LC₅₀ of 1.1–1.6 mg O₂ l⁻¹ after 3 d of static exposure (Poucher & Coiro 1997, Miller et al. 2002), and will enable a better understanding of the impacts of diel-cycling hypoxia and pH and estuarine eutrophication.

Nursery habitat quality

Results reported here suggest that extreme diel-cycling hypoxia and pH due to increased levels of productivity, caused by eutrophication, will reduce the nursery habitat quality of shallow estuarine tributaries for juvenile summer flounder. Diel-cycling pH and pCO₂ (means during the low part of the diel cycle as low as pH 6.87 and pCO₂ as high as ~10 000 µatm) appear to not exacerbate the growth reduction for young summer flounder over the effects of diel-cycling hypoxia alone. Therefore, it appears that lower growth rates of young summer flounder in the field, calculated from RNA:DNA analysis (Stierhoff et al. 2009a), relative to growth rates observed in experiments using only diel-cycling hypoxia (Stierhoff et al. 2006) are unlikely due to co-occurring pH and pCO₂ cycles in estuarine nursery grounds. Reduced feeding opportunities in the field, foraging activity, and predator avoidance likely reduce growth potential and exacerbate hypoxia impacts on growth rate in nursery habitat, regardless of pH, within the range tested in the present work.

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