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Effects of diel-cycling hypoxia and acidification on juvenile weakfish *Cynoscion regalis* growth, survival, and activity

Danielle M. Lifavi, Timothy E. Targett*, Paul A. Grecay

University of Delaware, College of Earth, Ocean, and Environment, School of Marine Science and Policy, Lewes, DE 19958, USA

ABSTRACT: During summertime, dissolved oxygen (DO) and pH/pCO_2 cycle daily in shallow estuarine waters used by young fishes as nursery habitat. Laboratory experiments on juvenile weakfish *Cynoscion regalis* examined individual and interactive impacts of present-day levels of diel-cycling hypoxia and acidification on growth and activity. Fish were exposed to 3 DO treatments (extreme cycling DO, 1–11 mg O_2 l^{-1}; moderate cycling DO, 3–9 mg O_2 l^{-1}; and constant normoxia, 7.5 mg O_2 l^{-1}) and 3 pH treatments (extreme cycling pH, 6.8–8.1; moderate cycling pH, 7.2–7.8; and constant pH, 7.5) for 20 d in a 3 × 3 factorial design. Growth was not impacted by moderate diel cycles of DO and acidification with mean daily lows of 3.0–3.3 mg O_2 l^{-1} and mean daily highs of 7.8–9.5 mg O_2 l^{-1} (mean daily low pH 7.03–7.47 and high pCO_2 ~16 000–5000 µatm). However, 100% mortality occurred within ~10 d at extreme diel-cycling hypoxia during which DO cycled between mean daily lows of 1.5–2.1 mg O_2 l^{-1}, mean daily low pH 6.99–7.44, and associated high pCO_2 of ~16 500–5500 µatm. We found no clear independent or interactive effect of pH/pCO_2 on growth or survival despite pH being as low as 6.86 and pCO_2 as high as ~20 000 µatm. Furthermore, fish that survived as much as 10 d exposure to extreme and moderate diel cycles of DO and acidification experienced no residual negative impact on growth following return to normoxia and static pH. Our results suggest that juvenile weakfish have substantial tolerance of diel cycles of oxygenation and acidification encountered in shallow estuarine nursery habitat.

KEY WORDS: *Cynoscion regalis* · Weakfish · Growth · Hypoxia · pH · pCO_2 · Ocean acidification · Diel-cycling · Estuary

INTRODUCTION

Estuary-dependent fishes spend their first few months as juveniles in estuaries, often following hatching and larval life in marine waters (Elliott et al. 2007). Estuaries provide conditions favorable for rapid growth and survival before young fishes recruit to adult populations (Weinstein 1979, Lubbers et al. 1990, Peterson & Ross 1991, Able 1999, Minello 1999). However, during summer months, shallow estuarine waters can experience diel-cycling hypoxia, whereby dissolved oxygen (DO) concentrations vary substantially over the day–night cycle (Kemp & Boynton 1980, D'Avanzo & Kremer 1994, Tyler & Targett 2007, Diaz & Rosenberg 2008, Tyler et al. 2009). During the day, photosynthesis produces O_2 and consumes CO_2, and can produce maximum DO concentrations >15 mg O_2 l^{-1} in late afternoon, whereas at night, aerobic respiration can cause DO to fall to a minimum (often <2 mg O_2 l^{-1}) just after dawn (Kemp & Boynton 1980, D'Avanzo & Kremer 1994, Tyler et al. 2009, Baumann et al. 2015). These diel cycles also cause large diel fluctuations in CO_2, with pCO_2 rising to >20 000 µatm and associated pH values dropping from ~8 to <7 (Howarth et al. 2011, Ullman et al. 2013, Baumann et al. 2015) over the course of 24 h.

Anthropogenic influences can exacerbate these biogeochemical cycles in estuaries, and ocean acidifi-

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*Corresponding author: ttargett@udel.edu

cation is an increasing issue worldwide (Duarte et al. 2013, Heuer & Grosell 2014, Wallace et al. 2014, Greene et al. 2015). Eutrophication increases the loads of reactive nitrogen and phosphorus in estuaries, intensifying the naturally occurring diel cycles of DO and pH/pCO_2 (Boynton et al. 1996, Paerl et al. 1998, Cloern 2001, Diaz 2001, Howarth et al. 2011, Duarte et al. 2013). These diel cycles force estuarine organisms to cope with several potential stressors simultaneously (Orr et al. 2005, Pörtner 2008, Denman et al. 2011, Wallace et al. 2014, Gobler & Baumann 2016).

Little is known about the impacts of diel-cycling hypoxia on growth of young fishes, and even less has been published on the potential independent or combined effects of diel-cycling pH/pCO_2 (Duarte et al. 2013, Davidson et al. 2016, Gobler & Baumann 2016). In laboratory experiments manipulating DO only, Stierhoff et al. (2009b) did not find a significant effect of diel-cycling hypoxia (2–11 mg O_2 l^{-1}) on the growth rate of juvenile weakfish *Cynoscion regalis* at 20–30°C. Additionally, growth rate of juvenile spot *Leiostomus xanthurus* was not impacted by diel-cycling hypoxia (2–10 mg O_2 l^{-1}) at 30°C in the laboratory and did not decrease under constant DO until concentrations reached near-lethal limits (1.5 mg O_2 l^{-1}) (McNatt & Rice 2004). In contrast, growth rates of other juvenile estuary-dependent fishes, such as summer flounder *Paralichthys dentatus* and winter flounder *Pseudopleuronectes americanus*, have been shown to be significantly reduced by diel-cycling hypoxia (2–11 mg O_2 l^{-1}) in laboratory experiments (Stierhoff et al. 2006).

In a recent field study examining growth of estuary-dependent juvenile fishes in estuarine nursery habitat, Stierhoff et al. (2009a) found that juvenile weakfish and summer flounder are both negatively impacted by diel-cycling hypoxia (<2 to >15 mg O_2 l^{-1}) throughout the summer. It is possible that co-varying diel pH/pCO_2 cycles in the field also negatively impacted growth rate, particularly in the case of juvenile weakfish. However, no studies have incorporated the potentially significant independent and synergistic effects of co-varying acidification on growth and behavior of estuary-dependent fishes. A recent review by Gobler & Baumann (2016) highlighted the need for such research.

It is important to understand the effects of these biogeochemical cycles, in concert, on growth of young fishes to assess their relative impacts on the quality of shallow estuarine nursery grounds worldwide. The magnitude of these diel cycles varies spatiotemporally (Tyler et al. 2009, Ullman et al. 2013, Baumann et al. 2015), and the goals of this study were to examine the effects of different magnitudes of diel-cycling hypoxia and pH/pCO_2, independently and together, on juvenile weakfish growth rate and activity level. Weakfish were also subjected to 10 d of exposure to diel-cycling DO and pH/pCO_2, and then returned to control conditions (for 20 d) to assess whether short-term exposure caused any negative residual impacts on longer-term growth and/or survival.

MATERIALS AND METHODS

Fish collection and acclimation

Juvenile weakfish were collected using an otter trawl in Pepper Creek, a tributary of Indian River Bay, Delaware, USA. Fish were collected over several days in 2014 and accumulated into a group for the experiments. Weakfish were acclimated for ≥14 d in ~450 l recirculating aquaria at 25°C, salinity 25, and normoxia (7.0 mg O_2 l^{-1}) with a 14:10 h light:dark photoperiod. Fish were fed frozen mysid shrimp *Mysis relicta* ad libitum once per day (Stierhoff et al. 2009b). Laboratory seawater came from Indian River Inlet, Delaware, and salinity was adjusted by adding tap water.

Experimental design

Growth experiments were conducted in 5 recirculating aquarium systems (Grecay & Stierhoff 2002), as modified by Davidson et al. (2016) to study impacts of diel-cycling hypoxia and acidification on growth and behavior of juvenile estuarine fishes. Temperature (25°C) and photoperiod (14:10 h light:dark) were controlled in the laboratory to mimic conditions in the field. Each aquarium system (Fig. 1) contained a total volume of ~415 l, in 10 individual 18 l polyethylene fish-holding tanks, an air-tight overflow tray, and a sump tank. Water flowed into each holding tank through a supply manifold and eventually overflowed into the surrounding tray, where it then entered the sump and was recirculated.

Computer control created diel cycles of both DO and pH in each of the 5 aquarium systems simultaneously and independently (Davidson et al. 2016). A PC running LabVIEW instrumentation software (2010 Version 10.0.1 SP1) was interfaced with a Hach sc200 Universal Controller, a Hach LDO dissolved oxygen probe, and a Hach Differential pH/ORP sensor. The PC monitored DO and pH (NBS scale) in each system and controlled treatment conditions (Table 1) by ad-

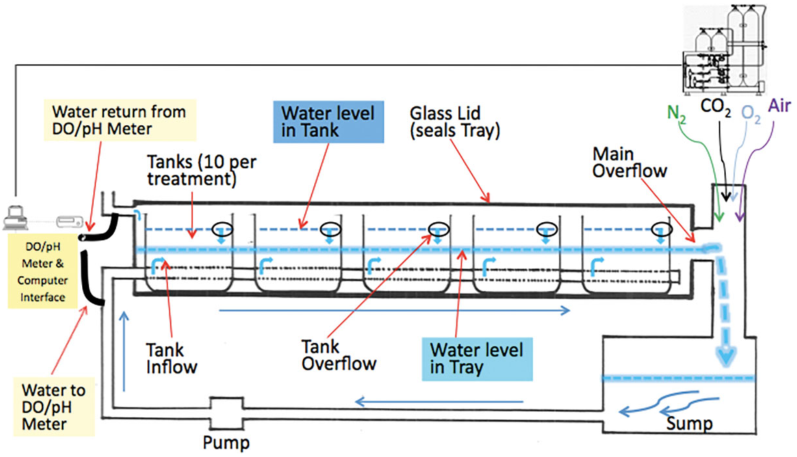

Fig. 1. One of the 5 computer-interfaced aquarium systems used for dissolved oxygen (DO) and pH control. Each system contained 10 holding tanks (18 l each). Only 5 chambers are visible in this side view. A portion of the water flow (described in 'Materials and methods – Experimental design') was directed to the DO and pH probes to be monitored and ensure DO and pH levels were in agreement with treatment levels. This water was then returned to the air-tight overflow tray. A PC running LabVIEW maintained desired treatment levels of DO and pH (see 'Experimental design')

ding gases (air, CO_2, N_2, and O_2) as needed into the sump tank. Solenoid-controlled water flow past the DO and pH probes allowed the LabVIEW program to determine DO and pH levels in each system in sequence. The program activated appropriate solenoids to bubble gases in appropriate amounts to achieve desired levels. DO was controlled by bubbling N_2 and O_2, and pH was controlled by bubbling CO_2 and compressed air. Minimum and maximum DO and/or pH in diel-cycling treatments coincided with the beginning of the light (06:00 h) and dark (20:00 h) periods, respectively.

Although this process was able to control DO and pH simultaneously, there were some limitations. Compressed air was used to raise the pH of each system, and while air was being bubbled it was not possible to maintain supersaturated DO concentration. In diel-cycling treatments where both DO and pH fluctuated, pH reached the desired high levels before DO. Therefore, Kent Marine SuperbufferdKh™ was added to a total alkalinity of 4388 ± 49.5 (SE) µmol kg^{-1} water, preventing pH from changing too quickly and allowing precise and prolonged pH, and therefore DO, control. This alkalinity is within the range of natural levels in the shallow estuarine tributaries, draining marshes, that juvenile weakfish inhabit (Neubauer & Anderson 2003, Tzortziou et al. 2011, Ullman et al. 2013).

Table 1. Matrix of diel-cycling DO and pH treatments (with abbreviations in parentheses). Blue, green and red shading indicate treatments used in Trials 1, 2, and 3, respectively. A control (yellow) was included in all 3 trials

	DO: 7.5 mg O_2 l^{-1}	DO: 3–9 mg O_2 l^{-1}	DO: 1–11 mg O_2 l^{-1}
pH: 7.5	🟨 Control: Normoxia, Static pH (Norm–Stat pH)	🟩 Moderate DO Cycle, Static pHm (Mod DO–Stat pH)	🟩 Extreme DO Cycle, Static pH (Ext DO–Stat pH)
pH: 7.2–7.8	🟥 Normoxia, Moderate pH Cycle (Norm–Mod pH)	🟦 Moderate DO Cycle, Moderate pH Cycle (Mod DO–Mod pH)	🟩 Extreme DO Cycle, Moderate pH Cycle (Ext DO–Mod pH)
pH: 6.8–8.1	🟥 Normoxia, Extreme pH Cycle (Norm–Ext pH)	🟩 Moderate DO Cycle, Extreme pH Cycle (Mod DO–Ext pH)	🟦 Extreme DO Cycle, Extreme pH Cycle (Ext DO–Ext pH)

Growth trials

Treatment conditions mimicked diel cycles of DO and pH/pCO_2 in shallow estuarine tributaries such as Pepper Creek, Delaware (Tyler et al. 2009), and tributaries of the Chesapeake Bay (Maryland Department of Natural Resources 2012). Fish were exposed to a range of diel DO and pH cycles to determine the synergistic and independent impacts of these co-varying cycles on growth rates and activity levels of juvenile weakfish. Treatments were chosen to represent moderate and extreme diel cycles encountered in the field, along with a static control representing more benign field conditions. DO treatments included: extreme diel-cycling hypoxia (1.0–11.0 mg O_2 l^{-1}), moderate diel-cycling hypoxia (3.0–9.0 mg O_2 l^{-1}), and a static normoxic control (7.5 mg O_2 l^{-1}). pH treatments included: extreme diel-cycling pH (6.8–8.1), moderate diel-cycling pH (7.2–7.8), and a static pH control (7.5). Each DO and pH cycle was crossed, yielding a 3 × 3 matrix including 8 different DO/pH treatments plus a static control (Table 1). Experiments were run in 3 trials, each consisting of several treatments and a concurrently run control (Table 2). Fish were not reused. Daily DO and pH minima and maxima values were recorded throughout the trials to ensure accuracy of the DO and pH treatments (Tables 2 & 3; Lifavi 2015).

After fish were acclimated to laboratory conditions, fish of similar size were selected and placed in the 18 l holding tanks (1 fish tank^{-1}, 10 tanks treatment^{-1}, different treatments in separate systems). Most treatments started with 6–10 fish (Table 2); numbers differed based on availability of experimental fish. Weakfish in a given trial had similar body weights prior to the start of each trial (confirmed by 1-way ANOVA; $p > 0.05$).

Fish were acclimated to the tanks for 3 d under control DO (7.5 mg O_2 l^{-1}) and pH (7.5) conditions. Fish were fed mysid shrimp ad libitum twice daily, at 09:00 and 17:00 h. Twenty-four hours prior to the end of acclimation, food was withheld to minimize the effect of stomach content on body mass. Standard length (SL) and mass of each fish were measured on Days 0, 10, and 20 immediately prior to the 09:00 h feeding. Mass was measured (0.01 g) by placing a fish on a damp sponge, determining the total mass (fish + sponge), then re-weighing the sponge and subtracting its weight. Standard length was measured (0.1 mm) using digital calipers. Mass and SL were used to calculate growth rate during trials. Feces and uneaten food were removed each morning before the 09:00 h feeding to maintain water quality. Water was tested approximately twice a week for ammonia, nitrite, and nitrate using API test kits, and levels remained at <0.25, <0.25, and <5 ppm, respectively.

A final 30 d trial was run to determine whether short-term exposure to diel DO and pH cycles had residual effects and thus negatively impacted subsequent longer-term growth rate and/or survival under control conditions (Table 3). Fish were exposed to extreme diel-cycling DO and pH (1–11 mg O_2 l^{-1}, pH 6.8–8.1) and moderate diel-cycling DO and pH (3–9 mg O_2 l^{-1}, pH 7.2–7.8) for 10 d, after which normoxia and static pH were reinstated for 20 d. Residual effects of short-term exposure were shown if fish had a statistically lower growth rate and/or an increase in mortality during the final 20 d when compared to the control. We used 10 fish treatment^{-1}

Table 2. Three trials run to examine the full set of treatment conditions in Table 1. Trials consisted of the treatments listed and each contained a concurrently run control. Individual weakfish *Cynoscion regalis* were used for all 20 d. For each time interval (days), the number of fish (N) at the beginning of the interval is shown, along with mortalities (no., day occurred), and the daily maximum and minimum dissolved oxygen (DO, mg O_2 l^{-1}) and pH. Values are means ± SE. For treatment intervals during which no fish survived until the end, mean daily maximum and minimum DO and pH values are given only for days through the last mortality

Trial	Treatment	Interval	N	Mortalities	DO Maximum	DO Minimum	pH Maximum	pH Minimum
1	Control	d0–10	10	0	7.95 ± 0.04	6.58 ± 0.10	7.57 ± 0.00	7.45 ± 0.00
1	Mod DO–Mod pH	d0–10	10	0	9.38 ± 0.03	3.30 ± 0.33	7.79 ± 0.00	7.24 ± 0.02
1	Ext DO–Ext pH	d0–10	10	3, d2; 7, d10	9.09 ± 0.31	1.75 ± 0.47	8.01 ± 0.00	6.99 ± 0.04
1	Control	d11–20	10	0	7.64 ± 0.01	7.26 ± 0.02	7.51 ± 0.00	7.46 ± 0.00
1	Mod DO–Mod pH	d11–20	10	0	9.25 ± 0.05	3.06 ± 0.01	7.78 ± 0.00	7.22 ± 0.00
1	Ext DO–Ext pH	d11–20	0	–	–	–	–	–
2	Control	d0–10	9	0	7.88 ± 0.02	5.94 ± 0.06	7.62 ± 0.01	7.24 ± 0.02
2	Mod DO–Stat pH	d0–10	6	0	9.49 ± 0.04	3.08 ± 0.04	7.54 ± 0.00	7.47 ± 0.00
2	Mod DO–Ext pH	d0–10	6	1, d7; 1, d9; 1, d10	7.76 ± 0.03	3.00 ± 0.07	7.73 ± 0.01	7.03 ± 0.04
2	Ext DO–Stat pH	d0–10	6	6, d3	11.16 ± 0.08	2.14 ± 0.23	7.58 ± 0.01	7.44 ± 0.01
2	Ext DO–Mod pH	d0–10	6	6, d1	11.49	1.49	7.78	7.22
2	Control	d11–20	9	0	7.68 ± 0.23	7.02 ± 0.17	7.50 ± 0.03	7.36 ± 0.03
2	Mod DO–Stat pH	d11–20	6	0	9.51 ± 0.07	3.16 ± 0.03	7.53 ± 0.00	7.48 ± 0.00
2	Mod DO–Ext pH	d11–20	3	2, d11; 1, d12	8.12 ± 0.24	2.82 ± 0.02	7.67 ± 0.00	7.02 ± 0.00
2	Ext DO–Stat pH	d11–20	0	–	–	–	–	–
2	Ext DO–Mod pH	d11–20	0	–	–	–	–	–
3	Control	d0–10	5	0	7.31 ± 0.03	7.02 ± 0.05	7.60 ± 0.01	7.23 ± 0.04
3	Norm–Mod pH	d0–10	5	0	7.39 ± 0.01	7.13 ± 0.07	7.76 ± 0.00	7.17 ± 0.00
3	Norm–Ext pH	d0–10	5	0	7.37 ± 0.01	7.08 ± 0.05	7.83 ± 0.00	6.87 ± 0.01
3	Control	d11–20	5	0	7.26 ± 0.03	6.83 ± 0.19	7.58 ± 0.00	7.21 ± 0.01
3	Norm–Mod pH	d11–20	5	0	7.38 ± 0.03	7.19 ± 0.03	7.71 ± 0.01	7.17 ± 0.00
3	Norm–Ext pH	d11–20	5	0	7.32 ± 0.03	7.08 ± 0.03	7.80 ± 0.00	6.86 ± 0.01

Table 3. Residual effects trial examining whether short-term (10 d) exposure to diel dissolved oxygen (DO) and pH cycles negatively impacted longer term (subsequent 20 d) growth rate and/or survival of weakfish *Cynoscion regalis*. Individual fish were used for all 30 d. For each time interval (days), the number of fish (N) at the beginning of the interval is shown, along with mortalities (no., day occurred), and the daily maximum and minimum DO (mg O_2 l^{-1}) and pH. Values are means ± SE, treatment abbreviations as in Table 1

Treatment	Interval	N	Mortalities	DO Maximum	DO Minimum	pH Maximum	pH Minimum
Control	d0–10	10	0	7.95 ± 0.04	6.58 ± 0.10	7.57 ± 0.00	7.45 ± 0.00
Mod DO–Mod pH	d0–10	10	0	9.58 ± 0.06	3.31 ± 0.33	7.79 ± 0.00	7.22 ± 0.02
Ext DO–Ext pH	d0–10	9	1, d2	10.60 ± 0.37	1.68 ± 0.48	8.02 ± 0.00	6.95 ± 0.04
Control	d11–20	10	0	7.64 ± 0.01	7.26 ± 0.02	7.51 ± 0.00	7.46 ± 0.00
Control following 10 d of Mod DO–Mod pH	d11–20	10	0	7.86 ± 0.02	6.72 ± 0.06	7.57 ± 0.00	7.44 ± 0.00
Control following 10 d of Ext DO–Ext pH	d11–20	9	0	7.69 ± 0.03	7.15 ± 0.11	7.52 ± 0.01	7.46 ± 0.00
Control	d21–30	10	0	7.73 ± 0.05	7.16 ± 0.07	7.50 ± 0.00	7.46 ± 0.00
Control following 10 d of Mod DO–Mod pH	d21–30	9	1, d27	7.84 ± 0.09	6.73 ± 0.26	7.52 ± 0.01	7.45 ± 0.00
Control following 10 d of Ext DO–Ext pH	d21–30	9	0	7.70 ± 0.04	7.24 ± 0.04	7.51 ± 0.01	7.46 ± 0.00

(Table 3), and acclimation, feeding, and measuring routines were identical to the methods described previously.

Activity observation and mortality assessment

Weakfish activity levels were monitored every other day just before and during morning and evening feedings, throughout Trials 1 to 3. Five fish were randomly selected from each treatment and their activity level was recorded on a scale from 1 to 10, with 1 being no swimming and 10 being hyperactive swimming (treatments were known to the observer). Mortality was also documented during morning and evening feedings. In the event of multiple mortalities in a given aquarium system, the system was thoroughly inspected and water parameters (DO, pH, and salinity, ammonia, nitrite, and nitrate) were checked to ensure that these parameters were within treatment and water quality limits.

Data analyses

Changes in fish mass were calculated over 2 time intervals during Trials 1 to 3: Days 0 to 10 and Days 11 to 20. Daily specific growth rate (SGR; % mass growth d^{-1}) was calculated using the formula SGR = $(e^G - 1) \times 100\%$, where G (instantaneous growth rate) = $[(\ln W_f - \ln W_i)/d]$; W_f = final weight, W_i = initial weight and d = number of days between measurements. Linear growth rate (LGR; mm d^{-1}) was calculated using the formula LGR = $[(L_f - L_i)/d]$, where L_f = final SL, L_i = initial SL and d = number of days between measurements. Normal distribution of the data was confirmed with a Shapiro-Wilk test.

Statistical routines in R were used to analyze fish growth and swimming activity during trials. For each time interval, the effect of treatment (Table 1) on growth (as change in mass, SGR, and LGR) was determined using linear models with initial mass as a covariate. For SGR and LGR, interaction between initial mass and treatment complicated a clear interpretation of treatment effect. However, for absolute growth (change in mass), no interaction between treatment and initial mass was observed, favoring absolute growth as the primary growth metric for statistical analyses.

Growth among treatments within a trial was compared using the R package 'lme4' to conduct growth curve analyses (GCA) with linear mixed-effect models evaluating differences in growth curves associated with treatments using likelihood ratio tests for differences among treatment groups in slopes and intercepts (Mirman 2014). When significant heterogeneity in growth curves among treatments was observed within a trial, pairwise GCA (control vs. selected treatment) was conducted to compare treatments, with adjustment for multiple testing using the Hochberg sequential Bonferroni method. In the final trial, a 30 d experiment to determine whether short-term (10 d) exposure to diel DO and pH cycles negatively impacted longer-term (20 d) growth following return to control conditions, changes in mass during the 20 d period of normoxia and static pH were compared to the control group by a 1-way ANCOVA.

Weakfish swimming activity during Trials 1 to 3 was analyzed for differences among treatments using R × C chi-squared tests of independence. Correlation between mean activity and SGR across treatments was also calculated for each time interval (Days 0–10 and Days 11–20). The effect of DO on mean activity for each interval was evaluated by a 2-way mixed model with DO level and individual fish as predictor variables. Difference in mean activity level between intervals was also examined using a 2-way mixed model with fish and activity as predictor variables. Finally, activity level of weakfish across all moderate diel-cycling DO treatments was compared with activity level across all normoxic treatments using a *t*-test.

Measurement of pCO_2 during experiments

Two water samples for pCO_2 determination were taken from each treatment tray 3 times a day (during pH minima, mid-range, and pH maxima for each diel-cycling treatment) every 10 d, when fish were measured and weighed. pH during those times was recorded. Water was collected in 25 ml scintillation vials and samples were sterilized with 0.2 ml of 5% $HgCl_2$ and refrigerated to prevent further production of CO_2. During analysis, 0.5 to 1 ml samples were acidified, and the obtained CO_2 gas was measured using an infrared CO_2 gas detector (Li-Cor 7000). The dissolved inorganic carbon (DIC) values were then checked against certified reference material from A. Dickson (Scripps Institution of Oceanography). CO2SYS software (Pierrot et al. 2006) was used to calculate pCO_2 and total alkalinity using known values of salinity, temperature, pH, and DIC.

RESULTS

Change in mass and SGR

Growth curve analyses (Fig. 2), comparing change in body weight during each time period, did not detect treatment effects, with the exception of a significant difference between the control and the Moderate DO Cycle & Extreme pH Cycle treatment (Mod DO–Ext pH; see Table 1 for treatment abbreviations) during Days 0 to 10 ($p < 0.001$; Fig. 2B). Note that all fish in the Ext DO–Stat pH and the Ext DO–Mod pH treatments died before Day 10 (see below; Table 2). Treatment effects using SGR as the growth metric were the same as those using change in body weight, across all trials. Only the Mod DO–Ext pH treatment in Trial 2 from Days 0 to 10 differed significantly from the control (Dunnett's test, $p = 0.0154$). LGR showed no significant treatment effects.

In the 30 d residual effects trial to determine whether short-term (10 d) exposure to diel DO and pH cycles negatively impacted subsequent longer-term (20 d) growth under control conditions (Table 3, Fig. 3), no differences in growth rate (slope) were observed during Days 0 to 10 (Fig. 3). Following return to control conditions (Days 11–30), no negative residual effects on growth were observed in either the Mod DO–Mod pH or the Ext DO–Ext pH treatments. There were also few mortalities in these treatments (Table 3); 1 fish died on Day 27 in the Mod DO–Mod pH treatment, and 1 fish died on Day 1 in the Ext DO–Ext pH treatment.

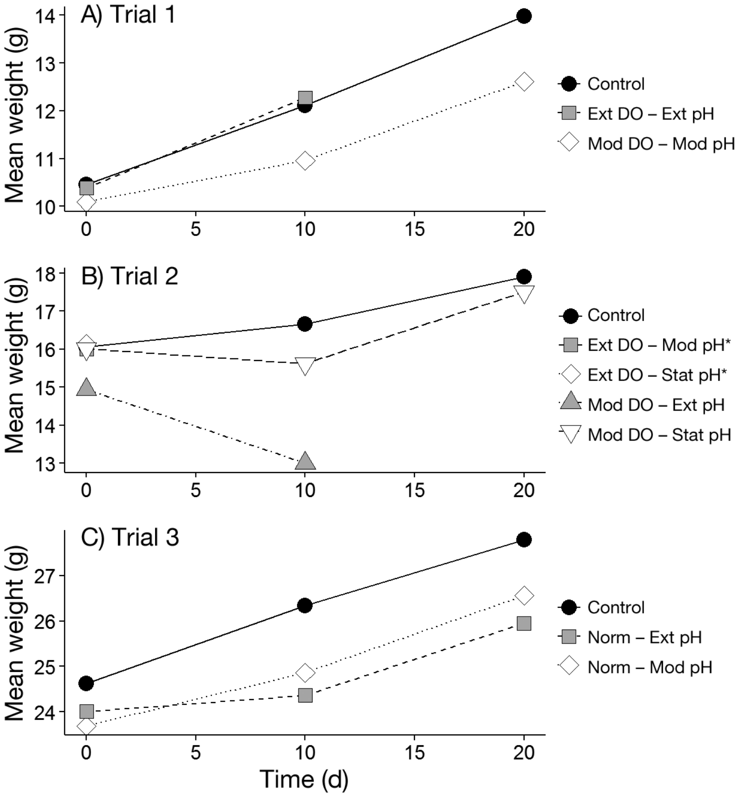

Fig. 2. Mean body weight of juvenile weakfish *Cynoscion regalis* over time in each trial (see Table 1). *In Trial 2, all fish in the Ext DO–Stat pH and the Ext DO–Mod pH treatments (see Table 1) died before Day 10

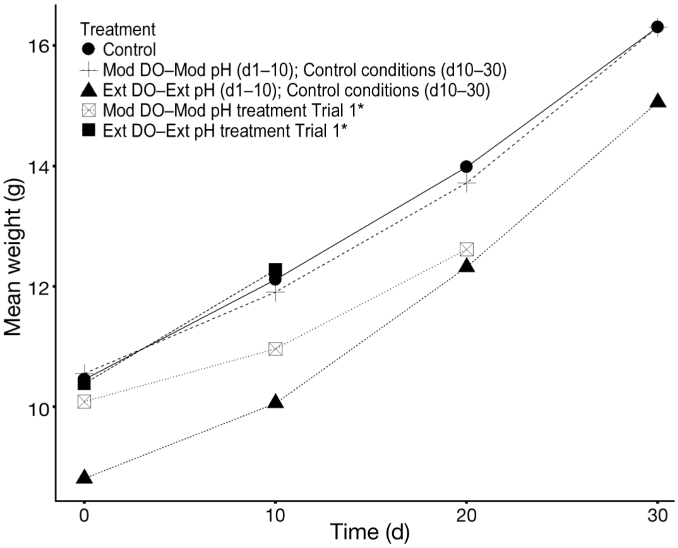

Fig. 3. Mean body weight of juvenile weakfish *Cynoscion regalis* over 30 d in the trial assessing whether short-term exposure to diel DO and pH cycles had a negative residual impact on longer-term growth and/or survival following return to control conditions on Day 10. Mean weight (g) of fish is shown at Days 0, 10, 20, and 30 for 3 treatments (see Table 1): Mod DO–Mod pH, Ext DO–Ext pH, and a concurrently run Control. Fish in the diel-cycling DO and pH treatments were exposed to diel cycles from Days 0 to 10 and returned to control conditions for Days 11 to 30. *Growth curves from the 20 d Mod DO–Mod pH and the Ext DO–Ext pH treatments from Trial 1 (see Table 1) are included for comparison

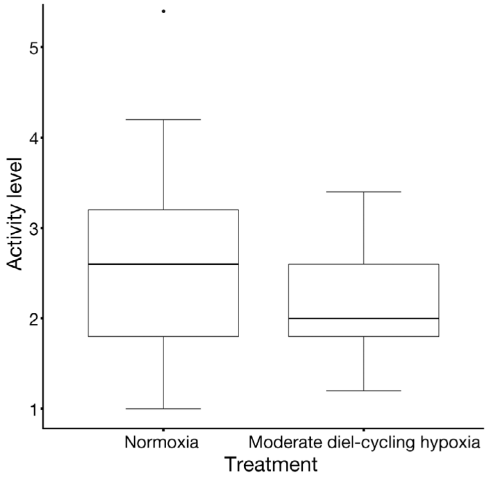

Fig. 4. Activity level of juvenile weakfish *Cynoscion regalis* from all normoxia treatments and all moderate diel-cycling hypoxia treatments, combined across Trials 1, 2, and 3 (see Table 1). Activity level was scaled from 1 to 10, with 1 being no swimming and 10 being hyperactive swimming. Medians are shown as horizontal lines inside the boxes, boxes enclose the middle 50% of the data, and vertical lines represent the data range, excluding any outliers. The single outlier in the normoxia treatment is shown. Activity level in the moderate diel-cycling hypoxia treatments is significantly lower than in normoxic conditions ($p = 0.007$)

Activity observation and mortality assessment

Juvenile weakfish exhibited atypical behavior in the moderate diel-cycling hypoxia treatments and experienced 100% mortality within ~10 d in the extreme diel-cycling hypoxia treatments in Trials 1 and 2 (Table 2). The aquarium systems in which mortality occurred did not exhibit pH or DO levels outside of treatment values, and the ammonia, nitrite, and nitrate levels remained <0.25, <0.5, and <20 ppm, respectively. In the moderate diel-cycling hypoxia treatments, some weakfish displayed sluggish swimming and/or reduced activity when fed. Average activity level of the fish in moderate diel-cycling hypoxia treatments was significantly lower ($p = 0.007$) than for fish under normoxic conditions (Fig. 4). However, no significant correlation was detected between SGR and mean activity level during any time interval (Days 0–10 and Days 11–20). Mean activity level within and among treatments did not differ between high and low DO periods, or between time intervals (Days 0–10 and Days 11–20). In addition, activity level was not affected by diel-cycling pH, nor was there an interactive effect of pH with diel-cycling hypoxia.

All weakfish in the extreme diel-cycling hypoxia conditions in Trials 1 and 2 died within ~10 d, regardless of pH conditions (Table 2): Ext DO–Ext pH (3 fish died on Day 2 [DO minimum that day = 1.3 mg O_2 l^{-1}] and 7 died on Day 10 after being measured and weighed [DO minimum = 1.11 mg O_2 l^{-1}]), Ext DO–Mod pH (all 6 fish died on Day 1 [DO minimum = 1.49 mg O_2 l^{-1}]), and Ext DO–Stat pH (all 6 fish died on Day 3 [minimum DO = 1.8 mg O_2 l^{-1}]). There was also 100% mortality in the Mod DO–Ext pH treatment (Table 2). One fish died on Day 7, 1 on Day 9, 1 on Day 10 after being measured and weighed, 2 on Day 11, and 1 on Day 12. Minimum DO levels on those days were 2.92, 2.94, 2.85, 2.81, and 2.84 mg O_2 l^{-1}, respectively. Mass mortalities were not observed in any other treatments in Trials 1, 2, or 3.

pCO_2 and pH conditions during experiments

There was a consistent relationship between pCO_2 and pH in the aquarium systems (Fig. 5). pH and pCO_2 values ranged from 6.86 to 7.79 and 20 665 to 2586 µatm, respectively, during the diel cycles tested.

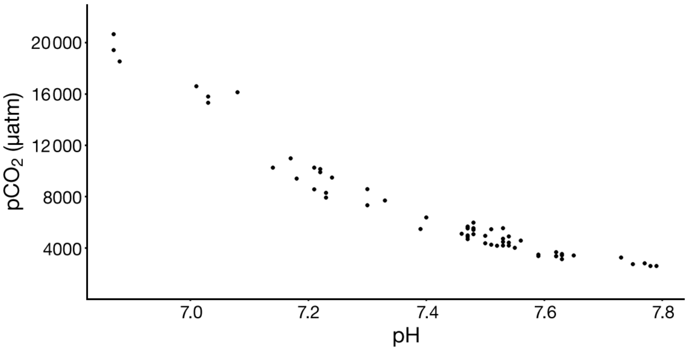

Fig. 5. pH and pCO_2 values of water samples taken from aquarium systems during experiments

DISCUSSION

Effects of diel-cycling hypoxia and pH/pCO_2 on weakfish growth and survival

All juvenile weakfish exposed to extreme diel-cycling hypoxia in Trials 1 and 2, regardless of pH, died within ≤10 d. During these extreme conditions, DO cycled between mean daily lows of 1.5–2.1 mg O_2 l^{-1} and mean daily highs of 9.1–11.5 mg O_2 l^{-1}; mean daily low pH ranged from 6.99 to 7.44 and associated high pCO_2 ranged from ~16 500 to ~5500 µatm. However, there was little evidence that growth of juvenile weakfish was impacted by sublethal levels of diel-cycling hypoxia and pH/pCO_2 over the 20 d exposure. The 7 fish exposed to extreme diel-cycling hypoxia and extreme diel-cycling acidification that survived through measuring and weighing on Day 10 in Trial 1 exhibited no growth detriment, despite not surviving another day. Also, fish in the Ext DO–Ext pH and Mod DO–Mod pH treatments for the first 10 d in the residual effects trial did not exhibit reduced growth either. Thus, it appears that individuals which survive 10 d of extreme diel-cycling hypoxia do so without growth consequences, even with co-varying extreme pH/pCO_2 cycles. Furthermore, moderate diel cycles of DO with mean daily lows of 3.0–3.3 mg O_2 l^{-1} and mean daily highs of 7.8–9.5 mg O_2 l^{-1} (mean daily low pH 7.03–7.47; pCO_2 ~16 000–5000 µatm) generally did not negatively impact juvenile weakfish growth.

The only treatment in which growth was negatively impacted was the Mod DO–Ext pH treatment in Trial 2. These fish lost an average of 13% body weight, from Days 0 to 10, based on only 4 fish that survived until Day 10. It is also evident from the residual effects trial that juvenile weakfish which withstand 10 d of exposure to either moderate or extreme diel cycles of DO and pH/pCO_2 experience minimal mortality and no negative impact on growth over the subsequent longer (20 d) term, if conditions are returned on Day 10 to normoxia and static pH conditions. Note that most fish held at extreme diel-cycling DO and pH/pCO_2 in Trial 1 died on Day 10 after being measured, weighed, and returned to the same extreme diel-cycling conditions, suggesting that relief from these conditions within this time frame is important for survival.

Stierhoff et al. (2009b) reported a lack of growth detriment in juvenile weakfish exposed in the laboratory to chronic (2, 3.5, 5 mg O_2 l^{-1}) or diel-cycling hypoxia with mean daily low concentrations as low as 2.4 mg O_2 l^{-1} for 7 d, at 20, 25, and 30°C. It is important to emphasize that, as is the case in most previous studies on impacts of diel-cycling DO on macrofauna, only DO was manipulated in these experiments. Thus the present study provides a valuable assessment of the potential confounding impact of acidification, because DO and pH/pCO_2 co-vary on a diel cycle in the field (Baumann et al. 2015). Overall there was no clear independent or interactive pH/pCO_2 effect on either growth or survival of juvenile weakfish, within or across trials, despite instances when pH was as low as 6.86 and pCO_2 as high as ~20 000 µatm. However, there is a suggestion of a synergistic negative impact (sensu DePasquale et al. 2015, Gobler & Baumann 2016) of low pH on survival in moderate diel DO cycles.

Juvenile spot, a closely related sciaenid fish with similar occurrence in shallow estuarine nursery habitats, exhibited a similar lack of impaired growth at chronic DO concentrations down to near-lethal levels (1.5 mg O_2 l^{-1}) at 25°C in 14 d laboratory experiments (McNatt & Rice 2004). Growth was also not impacted by diel-cycling hypoxia (2–10 mg O_2 l^{-1}) nor by chronic exposure to 2.0 mg O_2 l^{-1} at 30°C, although growth was reduced by chronic exposure to 1.5 mg O_2 l^{-1} at this temperature. It is possible that juvenile weakfish would show greater hypoxia effects at temperatures higher than tested in the present study. Regardless, together these results suggest that juvenile sciaenid fishes that inhabit shallow estuarine nursery grounds have considerable tolerance of hypoxia. In contrast, other juvenile estuary-dependent fishes, such as summer flounder and winter flounder, exhibited reduced growth at only moderate levels of hypoxia (between 3.5 and 5.0 mg O_2 l^{-1}; Stierhoff et al. 2006).

However, juvenile weakfish appear to be more negatively impacted by hypoxia in the field than would be predicted based on previous published laboratory results on DO effects (Stierhoff et al.

2009a,b). The present research provides the opportunity to assess whether diel pCO_2 and pH cycles which co-vary with DO in the field, and were not included in the experiments of Stierhoff et al. (2009b), negatively impact growth rate. Interestingly, we found no evidence that the low pH and high pCO_2 levels tested impact either growth or survival of this species. Growth of juvenile summer flounder has also been recently found to be unaffected by diel cycles of low pH and high pCO_2 (means during the low part of the diel cycle as low as pH = 6.87 and pCO_2 as high as ~10 000 µatm), either independently or interactively with hypoxia (Davidson et al. 2016). Further research is needed to establish whether growth tolerance of such diel-cycling pH and pCO_2 conditions also extends to other estuary-dependent fishes.

Ocean acidification-relevant CO_2 levels are generally considered to be <~2000 µatm CO_2 (Heuer & Grosell 2014), a value predicted for mean oceanic CO_2 by the year 2300 (Caldeira & Wickett 2003). Studies of the potential impacts of chronic ocean acidification levels in this range, and much higher, have reported variable impacts on juvenile and adult fish growth (see online supplemental Table 1 in the review by Heuer & Grosell 2014). Juveniles and adults have, in general, been found to be resistant to decreased growth effects of prolonged (up to 28 wk) exposure to pCO_2 <~2000 µatm (Heuer & Grosell 2014). Interestingly, sub-yearling walleye pollock *Theragra chalcogramma* showed increased growth at pCO_2 levels of ~1300 and 2900 µatm compared to levels <~850 µatm (Hurst et al. 2012). Several, but not all, species tested have been found to be resistant to decreased growth impacts of up to 10 wk exposure to pCO_2 as high as ~15 000 µatm and pH ~6.6, but not to more extreme levels (Fivelstad et al. 1998, Foss et al. 2003, Ishimatsu et al. 2008). Juvenile turbot *Scophthalmus maximus*, for example, exhibited reduced growth over 8 wk exposure to 15 000 and 25 000 µatm pCO_2 (pH = 6.7 and 6.4, respectively) compared to 3000 µatm pCO_2 (pH = 7.4) (Stiller et al. 2015). At very high pCO_2 levels, European sea bass *Dicentrarchus labrax* showed no growth detriment from 45 d exposure to pCO_2 of ~34 000 µatm and pH = 6.4 (Petochi et al. 2011).

Thresholds for negative pCO_2 effects are clearly species-specific. DePasquale et al. (2015) examined the impacts of chronic low pH (pCO_2 levels of ~2000 µatm, pH = 7.4) and low DO (~1.6–2.7 mg l^{-1}) on survival and growth of larval inland silverside *Menidia beryllina*, Atlantic silverside *M. menidia*, and sheepshead minnow *Cyprinodon variegatus* from hatching to ~10 d post-hatch in the laboratory. These species inhabit shallow estuarine habitats along the Atlantic coastline of the USA and can experience diel cycles of oxygenation and acidification as larvae. The 3 species exhibited different sensitivities to the static levels of acidification and hypoxia examined, with only inland silverside larvae showing a growth detriment from chronic exposure to ~2000 µatm pCO_2.

The present research has shown that juvenile weakfish growth is generally tolerant of diel cycles of low pH and high pCO_2 lasting at least 10 d, despite means during the low part of the diel cycle as low as pH = 7.03 and pCO_2 as high as ~16 000 µatm. These cycles are present-day occurrences in estuarine nursery habitat (Baumann et al. 2015). Longer-term experiments on this species and other juvenile estuary-dependent fishes are needed to more fully assess these fishes' overall tolerance to cyclical pCO_2 and pH conditions.

Activity observation and mortality assessment

Juvenile weakfish exposed to moderate diel-cycling hypoxia displayed sluggish swimming and/or reduced activity when fed. Although the observer was not blind to the treatments, which could have influenced the results, similar findings reported by Brady et al. (2009) and Stierhoff et al. (2009b) suggest that these fish reduce swimming speeds when exposed to moderately low DO cycles. Reduced activity could permit juvenile weakfish to allocate proportionally more energy toward growth, despite reduced feeding, as has been speculated by Stierhoff et al. (2009b). This is consistent with the similar growth rates found across treatments in this study. In fact, because fish were fed *ad libitum* twice a day in the laboratory, any reduced feeding consequences of decreased swimming activity were likely less than would be the case in the field, and may account for the greater impact of diel-cycling hypoxia on growth in the field than in the laboratory reported by Stierhoff et al. (2009a).

Weakfish swimming activity did not change significantly when exposed to diel-cycling pH/pCO_2, nor was there an interaction with diel-cycling hypoxia during these 2 wk trials. Most behavioral changes found during experiments testing for chronic ocean acidification impacts are due to a variety of internal disturbances (Duarte et al. 2013, Heuer & Grosell 2014). Sensory systems including olfaction, hearing, and vision and other processes related to cognitive function including lateralization, activity, boldness, and learning have been found to be impaired in numerous species when exposed to prolonged

(4–20 d) decreased pH conditions (see online supplemental Table 1 in Heuer & Grosell 2014). Further experiments on juvenile estuary-dependent fishes, examining a wider variety of behavioral responses to diel-cycling pH and pCO_2, will be necessary to more fully assess these fishes' overall tolerance to these cyclical conditions. In general, fishes may be more tolerant of short-term (diel-cycling in this case) exposure to low pH and high pCO_2 compared with chronic ocean acidification (Duarte et al. 2013, Heuer & Grosell 2014). More research on this topic, particularly with early life stages, including larvae, is warranted.

The lower lethal DO for juvenile weakfish is unknown (Brady & Targett 2013). However, this experiment suggests that weakfish cannot withstand the accumulated stress of daily DO levels in the 1.5–2.0 mg O_2 l^{-1} range. All fish exposed to these levels in the extreme diel-cycling hypoxia treatments died within ~10 d. Juvenile weakfish, however, generally avoid hypoxic areas with daily lows of 1.4–2.8 mg O_2 l^{-1} by increasing swimming speed during extremely low DO, and using tidal flow to facilitate escape (Brady & Targett 2013). Tyler & Targett (2007) found that juvenile weakfish have an avoidance threshold of ~2.0 mg O_2 l^{-1} and demonstrate dynamic temporal movement related to DO concentrations in the field.

Effects of diel-cycling hypoxia and pH/pCO_2 on nursery habitat quality

Many fish species rely on estuaries as nursery habitat during their juvenile stages. Diel cycles of hypoxia and pH/pCO_2, exacerbated by eutrophication, have the potential to negatively impact fitness of fishes residing in shallow nursery areas, and thus degrade nursery habitat quality and quantity. Results of the present study, along with previous field investigations (Tyler & Targett 2007, Brady & Targett 2013) and a laboratory mesocosm experiment (Brady et al. 2009) suggest that juvenile weakfish will avoid extreme diel cycles of hypoxia and pH before they become lethal, and that this behavior is driven by low DO. These fish are physiologically capable of growing well under moderate diel cycles of DO and acidification with mean daily lows of 3.0–3.3 mg O_2 l^{-1}, low pH 7.03–7.47, and high pCO_2 ~16000–5000 µatm. In addition, juvenile weakfish that survive as much as 10 d of exposure to extreme and moderate diel cycles of DO and acidification (with mean daily lows ranging from 1.7–3.3 mg O_2 l^{-1} and mean daily highs from 9.6–10.6 mg O_2 l^{-1}, mean daily low pH 6.95–7.22, and associated high pCO_2 ~18000–9000 µatm) experience no residual negative impact on growth following return to normoxia and static pH conditions.

Although decreased swimming in moderate diel-cycling hypoxia (Brady et al. 2009, Stierhoff et al. 2009b, this study), and potential associated decline in foraging success in the field, may account for the greater impact of diel-cycling hypoxia on juvenile weakfish growth in the field than in the laboratory (Stierhoff et al. 2009a), this study shows that substantial tolerance to cycling hypoxia and acidification facilitates taking advantage of hypoxic habitats for feeding and predator avoidance. Additional laboratory and field studies investigating impacts of diel-cycling DO and pH/pCO_2 on juveniles of other species in the family Sciaenidae, and other estuary-dependent fishes, will help establish response patterns to dynamic conditions of dissolved oxygen and acidification in shallow estuarine nursery habitats.

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