

**DIEL-CYCLING HYPOXIA AND pH IMPACTS ON JUVENILE SUMMER
FLOUNDER GROWTH AND SURVIVAL**

by

Max I. Davidson

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of
the requirements for the degree of Master of Science in Marine Studies

Spring 2015

© 2015 Max I. Davidson
All Rights Reserved

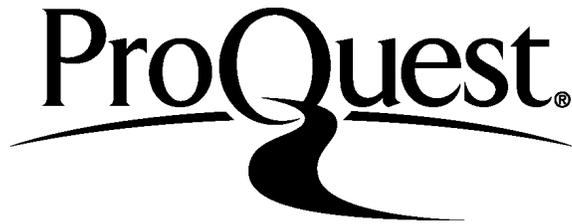
ProQuest Number: 1596843

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 1596843

Published by ProQuest LLC (2015). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

**DIEL-CYCLING HYPOXIA AND pH IMPACTS ON JUVENILE SUMMER
FLOUNDER GROWTH AND SURVIVAL**

by

Max I. Davidson

Approved:

Timothy E. Targett, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved:

Mark A. Moline, Ph.D.
Director of the School of Marine Science and Policy

Approved:

Nancy M. Targett, Ph.D.
Dean of the College of Earth, Ocean, and Environment

Approved:

James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education

ACKNOWLEDGEMENTS

I would like to thank my committee members, Dr. Timothy Targett, Dr. Paul Greco, and Dr. Patrick Gaffney for their support and guidance throughout the entirety of the project. Each committee member played an extremely active and integral role in the success of this project and my personal development. I was blessed to have had the support of my lab mates, Danielle Lifavi, Rachel Dixon, and Mike Torre. All of your help throughout the project is much appreciated.

Lastly, I want to thank my family and friends who have been extremely supportive of my endeavors at UD and beyond. Special thanks to Sam Arvanitis, whose guidance at a young age and introduction to the sport of fishing, and subsequent love for the ocean, has shaped my life tremendously.

This research was supported by an award from NOAA, National Centers for Coastal Ocean Science, Center for Sponsored Coastal Ocean Research, through the Coastal Hypoxia Research Program (Grant Number: NA10NOS4780156 to Dr. Timothy Targett).

TABLE OF CONTENTS

LIST OF TABLES	vi
LIST OF FIGURES	vii
ABSTRACT	viii

Chapter

1	DIEL-CYCLING HYPOXIA AND pH IMPACTS ON JUVENILE SUMMER FLOUNDER GROWTH AND SURVIVAL	1
	Introduction	1
	Materials and Methods	4
	Summer Flounder Collection & Acclimation	4
	Laboratory Design	5
	Growth Trials	6
	Data Analysis	8
	Behavioral Observations & Mortality	9
	Measuring $p\text{CO}_2$	10
	Results	11
	Experimental Conditions	11
	Change in Mass as Primary Growth Metric	11
	Treatment Effects	11
	Specific Growth Rate	13
	Behavioral Observations & Mortality	14
	$p\text{CO}_2$ Values	15
	Discussion	16
	Extreme Diel-Cycling Hypoxia Negatively Impacted Summer Flounder Growth	16
	Extreme Diel-Cycling Hypoxia Negatively Impacted Summer Flounder Growth	16
	Growth Rate Acclimation & Recovery Growth	17

Behavioral Observations & Mortality.....	18
Nursery Habitat Quality	20
Most Significant Findings.....	20
REFERENCES	30
Appendix	
IACUC LETTER	38

LIST OF TABLES

Table 1	Diel-cycling DO and pH treatments.	22
Table 2	Treatment information	23
Table 3	Results of Dunnett’s test comparing mean change in mass of each treatment to concurrent control.....	24
Table 4	SGR values.....	25

LIST OF FIGURES

Figure 1	DO and pH treatment aquarium.....	26
Figure 2	Change in flounder mass.....	27
Figure 3	Growth curves from recovery/compensatory growth treatments	28
Figure 4	$p\text{CO}_2$ and pH values.....	29

ABSTRACT

A series of laboratory experiments was conducted to determine the impact of diel-cycling dissolved oxygen (DO) and pH on young-of-the-year summer flounder (*Paralichthys dentatus*). Flounder were exposed to two cycling DO levels (extreme = 1-11 mg O₂ l⁻¹; moderate = 3-9 mg O₂ l⁻¹), two cycling pH levels (extreme = 6.8-8.1; moderate = 7.2-7.8), and a constant normoxia (7.5 mg O₂ l⁻¹) & pH (7.5) control treatment in a fully crossed 3X3 experimental design at 25°C. DO and pH levels were chosen to reflect summertime conditions in shallow estuarine nursery habitat. Growth rate, overall change in mass, was significantly reduced in fish exposed to the most extreme diel-cycling hypoxia, across all pH treatments, throughout the course of 20 d experiments. No consistent growth detriment was observed in the other treatments, indicating a) that a moderate DO cycle has little or no impact on growth and b) that pH has neither an independent nor interactive effect with hypoxia on growth rate. In a series of experiments to examine growth rate recovery and acclimation, flounder experienced an initial growth rate detriment when subjected to extreme diel-cycling hypoxia and pH for 10 days, but then exhibited growth rate recovery (growth returned to control levels) when exposed to static normoxia and normal pH conditions the following 10 days. Flounder did not exhibit growth rate acclimation when subjected to extreme diel-cycling hypoxia and pH for an extended period, as growth rate detriment persisted for 20 d of exposure. Flounder exhibited behavioral observations indicative of stress when exposed to diel-cycling hypoxia and eventually exhibited mortality after two to three weeks of exposure to extreme diel-cycling hypoxia and pH. These results demonstrate that prolonged and

severe diel-cycling hypoxia and pH degrades summer flounder nursery habitat as flounder exhibited growth rate detriment, and ultimately mortality, under laboratory conditions exhibited in shallow, highly eutrophied estuaries.

Chapter 1

DIEL-CYCLING HYPOXIA AND pH IMPACTS ON JUVENILE SUMMER FLOUNDER GROWTH AND SURVIVAL

Introduction

A variety of fishes utilize shallow productive estuaries as nursery habitat (Beck et al., 2001; Able & Fahay, 2010). Such fishes benefit from the physicochemical conditions that increase growth and survival rates in early ontogenetic stages, including: shelter, increased food availability, and optimal water temperatures (Weinstein, 1979; Lubbers et al., 1990; Able, 1999; Minello, 1999). The suitability of estuarine habitats has been shown to have a significant positive correlation with adult population recruitment rates (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).

Estuarine physicochemical conditions; including dissolved oxygen (DO), pH, temperature, and salinity; exhibit daily, seasonal, and annually cycles (Boynton et al., 1996; Wong, 1998; Stierhoff et al., 2009a; Tyler et al., 2009; Howarth et al., 2011). For example, algal photosynthesis during the day, particularly during the summer, and subsequent respiration during the night may cause shallow estuarine waters to exhibit diel-cycling hypoxia and pH (Tyler & Targett, 2007; MD DNR, 2012). During the diel cycle, in summer, summer flounder nursery habitat may experience DO values ranging from 0 mg O₂ l⁻¹, anoxia, just after dawn to >15 mg O₂ l⁻¹, hyperoxia, 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 force estuarine organisms to cope with multiple potential stressors simultaneously (Orr et al., 2005; Denman et al., 2011). Such diel cycles are exacerbated 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 has been shown to cause various sub-lethal impacts on fishes including reduction in growth, decreased feeding, and decreased fecundity (McNatt & Rice, 2004; Bell & Eggleston, 2005; Stierhoff et al., 2006; Thomas et al., 2006; Stierhoff et al., 2009a; Rees et al., 2012). Exposure to diel-cycling hypoxia may lead to reductions in food intake as foraging efficiency decreases due to hindered locomotion, manifesting in long-term reductions in growth (Stierhoff et al., 2003; Stierhoff et al., 2006; Richards et al., 2009; Brandt et al., 2009). Juvenile fishes subjected to extreme diel-cycling hypoxia, when DO approaches $\sim 1 \text{ mg l}^{-1}$, may exhibit avoidance behaviors, usually swimming in a panicked manner in search of sufficiently oxygenated water (Howell & Simpson, 1994; Eby & Crowder, 2002; Tyler & Targett, 2007; Brady et al., 2009; Brady & Targett, 2010; Brady & Targett, 2013). Although avoidance behavior increases survival of fishes subjected to severe hypoxia or anoxia, it is likely that a detriment is experienced before fish actively avoid hypoxic environments (Stierhoff et al., 2009b). Several juvenile estuarine species do not exhibit avoidance behavior until DO decreases to a static level $\sim 1 \text{ mg O}_2 \text{ l}^{-1}$, well below the EPA growth protective criterion of $>4.8 \text{ mg O}_2 \text{ l}^{-1}$ (Wannamaker & Rice, 2000; Stierhoff et al., 2009b). Other behavioral

responses to hypoxia, such as increased gill ventilation rate and stroke volume (Breitburg, 1994; Wannamaker and Rice, 2000; Taylor & Miller, 2001; Richards et al., 2009), are energy intensive, forcing fish to allocate less energy towards growth, reproduction, and survival (Steffensen et al., 1982; Herbert & Steffensen, 2006). Furthermore, if fish are able find normoxic waters they may become overcrowded, increasing competition for prey and exposure to density dependent growth impacts (Eby & Crowder, 2005; Campbell & Rice, 2014).

In laboratory experiments, summer flounder have been shown to exhibit significant growth rate reductions of ~25% at static levels of 3.5 mg O₂ l⁻¹, 50-60% at 2.0 mg O₂ l⁻¹ at 20°C and 25°C, and ~90% at 2.0 mg O₂ L⁻¹ at 30°C (Stierhoff et al., 2006). These experiments also suggest increased detriment occurred at 30°C compared to 25°C across all static and diel-cycling DO treatments. During summer months in summer flounder nursery habitats, diel-cycling hypoxia can cause DO levels to decrease below the static DO LC₅₀, 1.1 – 1.6 mg O₂ l⁻¹, for summer flounder (Poucher & Coiro, 1997; Miller et al., 2002, Tyler et al., 2009). Juvenile summer flounder exhibit active avoidance responses to diel-cycling hypoxia, at ~2.8 mg O₂ l⁻¹, and static hypoxia, at ~1.4 mg O₂ l⁻¹. (Stierhoff et al., 2009a ; Brady & Targett, 2010; Brady & Targett, 2013).

The literature lacks sufficient studies investigating the independent and interactive impacts of diel-cycling hypoxia and pH on young fishes that utilize hypoxia impacted estuarine habitats as nursery grounds. No studies to date have investigated the synergistic impacts of diel-cycling hypoxia and co-varying pH on growth and mortality of juvenile

summer flounder. The combined impacts of hypoxia and decreased pH have been shown to increase both mortality and decrease cellular metabolic efficiency compared to hypoxia alone in tilapia, *Oreochromis mossambicus* (van Ginnekan et al., 1996).

The objectives of this study were: (1) to investigate the individual and synergistic effects of diel-cycling hypoxia and pH on growth and mortality of juvenile summer flounder, (2) determine whether summer flounder have the ability to exhibit growth rate acclimation to diel-cycling hypoxia and pH, (3) investigate the ability of summer flounder to exhibit growth rate recovery when exposed to optimal DO and pH levels subsequent to exposure to diel-cycling hypoxia and pH.

Materials and Methods

Summer Flounder Collection & Acclimation

Juvenile summer flounder were collected using seine and otter trawl from Pepper Creek and Indian River, tributaries of the Delaware Coastal Bays. Fish were held in ~450 l recirculating aquaria at 25°C, a salinity of 25, and normoxia ($7.0 \text{ mg O}_2 \text{ L}^{-1}$) for \geq 14 days, to ensure acclimation to laboratory conditions. Photoperiod was 14 h light: 10 h dark and fish were be fed mysid shrimp (*Mysis relicta*) twice-daily *ad libitum* at 09:00 and 17:00. After an acclimation period fish were transferred to recirculating computer-controlled DO/pH aquarium systems.

Laboratory Design

Growth experiments took place in five air-tight recirculating aquaria systems modified by Bogue et al., (*In prep*) to study impacts of diel-cycling hypoxia and pH on mummichogs (*Fundulus heteroclitus*) from the DO controlling systems previously designed by Gre cay & Stierhoff (2002) to study impacts of diel-cycling and chronic hypoxia on growth and behavior of juvenile estuarine fishes (Stierhoff et al., 2006; Stierhoff et al., 2009b). Each aquarium system (Figure 1) was capable of regulating DO and pH independently, and all were maintained at 25°C, a salinity of 25, and photoperiod controlled, 14 h light: 10 h dark, to mimic conditions in the field. Each aquarium system has a volume ~415 l and held 10 juvenile summer flounder in 10 individual 18 l polyethylene holding tanks. Water constantly flowed into each holding tank and overflowed into the surrounding tray, this ensured the metabolism of each flounder did not alter the DO and pH of their ambient environment. A PC running LabVIEW instrumentation software, interfaced with a HACH sc200 Universal Controller, Hach LDO DO probe, and Hach Differential pH/ORP sensor, regulated the DO and pH of each system by adding necessary amounts of compressed gasses: N₂, CO₂, O₂, and air via solenoid-controlled gas manifolds into water in the sump prior to recirculation. DO levels are controlled by injecting N₂ and O₂ while pH levels are controlled by injecting CO₂ and air into each system (Figure 1). Each aquarium system is capable of manipulating both DO and pH independently, allowing five trials to be run concurrently.

Maximum pH levels superseded maximum DO levels in all treatments where DO and pH cycled concurrently as air was bubbled to increase pH, and therefore DO was maintained at a saturated level ($\sim 7.5 \text{ mg l}^{-1}$). DO would supersaturate in all moderate and extreme diel-cycling DO treatments once the desired pH was achieved. The water was buffered and alkalinity was raised using Kent Marine Superbuffer-dkH™ allowing precise and prolonged pH, and therefore DO, control.

Growth Trials

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). Flounder were exposed to two cycling DO levels (extreme = $1\text{-}11 \text{ mg O}_2 \text{ l}^{-1}$; moderate = $3\text{-}9 \text{ mg O}_2 \text{ l}^{-1}$), two cycling pH levels (extreme = $6.8\text{-}8.1$; moderate = $7.2\text{-}7.8$), and a constant normoxia ($7.5 \text{ mg O}_2 \text{ l}^{-1}$) and pH (7.5) control treatment in a fully crossed 3×3 factorial design yielding 8 different 20 d diel-cycling hypoxia and pH treatments and one control treatment (Table 1). Treatments were conducted over three separate time intervals in the summer of 2014, each with a concurrent control. Daily DO and pH minima and maxima values were recorded during experimentation to ensure accurate reporting of the DO and pH treatments. Prior to each acclimation period for a given trial the experimental aquaria were tested for 2-4 d to ensure consistent and accurate regulation of prescribed DO and pH conditions

Fish (50 to 94 mm standard length, SL) were subjected to a three-day acclimation under static optimal conditions when placed in the computer-controlled aquarium

systems. All flounder in a given trial had similar body masses prior to the commencement of each trial confirmed by two-way ANOVA analysis: trial 1, $p = 0.99$, trial 2, $p = 0.99$, trial 3, $p = 0.92$. Prior to the end of acclimation fish were withheld food for one day (in order to minimize the effect of stomach contents on body mass) and were measured for standard length (SL) using a digital caliper to the nearest 0.1 mm. Fish were massed using a DeltaRange Mettler PE 360 balance using similar to methods described in Stierhoff et al., (2006). Measuring and weighing occurred on days 0, 10, and 20 of each trial, prior to the 09:00 feeding. Treatments were run at 25°C, a salinity of 25 ppt, and a photoperiod of 14 h light: 10 h dark. Summer flounder were fed mysid shrimp (*Mysis relicta*) *ad libitum* twice daily at 09:00 and 17:00. Experimental aquaria water was seeded with sufficient bacterial communities to ensure control over ammonia and nitrite levels.

In addition to the 3X3 factorial design a recovery and compensatory growth experiment was conducted. Fish were subjected to moderate (3-9 mg O₂ l⁻¹, pH 7.2-7.8) and extreme (1-11 mg O₂ l⁻¹, pH 6.8-8.1) diel-cycling hypoxia and pH treatments for 10 d. Subsequent to 10 d of exposure to the most moderate and most extreme diel-cycling hypoxia and pH treatments, water was returned to normoxia, 7.5 mg O₂ l⁻¹, and a pH of 7.5 for 10 d. This provided data to investigate whether juvenile summer flounder exhibit compensatory growth, an increase in growth relative to the concurrent control when water is returned to optimal conditions that compensates for growth detriment during the diel-cycling hypoxia and pH treatments, or recovery growth, an increase in growth equal to that of the concurrent control group when water is returned to optimal conditions (Bejda et al., 1992).

Juvenile summer flounder also were subjected to the most moderate and most extreme diel-cycling DO and pH treatment for 30 d to determine if prolonged exposure to these conditions results in mortality or any apparent behavioral changes.

Data Analysis

Overall changes in mass of each fish were calculated over two time intervals, d 0-10 and d 10-20, during each trial. Measured masses were used to calculate daily specific growth rate (SGR; % body mass growth d⁻¹). $SGR = (e^G - 1) * 100\%$, where G (instantaneous growth rate) = $[(\ln W_{t_f} - \ln W_{t_i})/d]$; W_{t_f} = final weight, W_{t_i} = initial weight and d = number of days between measurements. Standard length (SL) measurements were used to calculate linear growth rate (LGR; mm d⁻¹). LGR will be calculated as $LGR = [(L_f - L_i)/d]$; L_f = final SL, L_i = initial SL and d = number of days between measurements.

A one-way ANOVA was conducted, with a covariate analyzing the effects of treatment and initial mass on growth using linear models, growth was measured either as change in mass or SGR. Using SGR as the primary growth metric for analysis was problematic as this showed a significant treatment x initial mass interaction. By contrast, there was no significant overall interaction effect of treatment x initial mass with change in mass, thus change in mass was chosen as the primary growth metric. A Dunnett's test was performed to compare the mean change in mass of each treatment, our main metric for growth, to the mean change in mass of the concurrent control. Another Dunnett's test

was performed comparing mean LGR of each treatment to the mean LGR of the concurrent control.

To further investigate the presence of recovery in appropriate trials over the multiple time intervals (d 0-10 and d 10-20) a repeated measures approach was needed, as individual fish were repeatedly measured at given intervals. The multilevel regression procedure, the Growth Curve Analysis (GCA) provided a useful method for time course data of growth trajectories (Mirman, 2014). The GCA compared growth curves of the group subjected to the most extreme treatment for 20 d and the concurrent group that was subjected to the most extreme treatment (d 0-10) and returned to static levels (d 10-20); as well the group subjected to the most moderate treatment for 20 d and the concurrent group that was subjected to the most moderate treatment (d 0-10) and returned to static levels (d 10-20). The GCA could not be used to examine compensatory growth due to temporal constraints of the experiments.

To further investigate the interactive effect of pH on growth a GCA and several ANCOVAs, using initial mass as a covariate, were performed to determine whether the extreme diel-cycling hypoxia and static pH treatment was significantly different than the concurrent extreme diel-cycling hypoxia and moderate diel-cycling pH treatment. The ANCOVAs compared growth from the two treatments across three temporal intervals, days 0-10, 10-20, and 0-20.

Behavioral and Mortality Observations

Qualitative behavioral observations were made during morning and evening feedings, 09:00 and 17:00. Behavioral observations examined included panicked

swimming behavior that was indicative of an escape response, sluggish activity when fed, reduced feeding, and gill ventilation rate and stroke volume. All mortalities were noted and dead fish were removed promptly to maintain water quality. During the event of multiple mortalities the laboratory system was thoroughly inspected, water parameters were monitored including DO, pH, and salinity using a Yellow Springs Instrument 556MPS probe, and ammonia, nitrite, and nitrate levels were tested using API test kits.

Measuring pCO_2

To optimize the usefulness of the experiment for current and future assessments regarding pH effects, pCO_2 values were calculated for each treatment. Furthermore, water samples were also taken on September 5, 2014 at 08:09, 12:03, and 16:41 from Pepper Creek, Delaware Coastal Bays. Water collected from the field was $\sim 25^\circ C$ and ~ 22.5 ppt, similar to our laboratory conditions, and was measured to determine similarity of the carbonate chemistry in the field and laboratory. The partial pressure of CO_2 values are a unifying measure used in ocean acidification studies and pCO_2 is the standard metric for aquatic carbon species (Riebesell et al., 2010).

Water samples were collected in 25 mL scintillation vials from each treatment at three times: 09:00, 12:00, and 17:00 on experimental days: 0, 10, 20. Samples were taken across a wide range of pH values during each diel-cycling pH treatment. Samples were sterilized with 0.2 mL of 5% $HgCl_2$, to prevent further alterations to the dissolved inorganic carbon (DIC) composition of the sampled water, and therefore pCO_2 . Prior to 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) using a Metrohm 888 TitrandTM autotitrator and 869 compact sample changer with 0.1 mol l⁻¹ HCL as titrant; reproducibility was estimated as typically better than ±6 μmol/kg based on 22 replicate analyses of an in-house standard. CO2SYS software (Lewis et al., 1998) was used to calculate pCO₂ values using known parameters: TA, pH, temperature, and salinity in conjunction with the thermodynamic constants outlined by Millero (1979).

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 lack of DO regulation while pH increased.

Change in Mass As Primary Growth Metric

Contrary to previous studies and general intuition our SGR values exhibited a significant interaction effect with initial weight violating an integral statistical assumption, thus overall change in body mass was the primary metric utilized to analyze treatment effects. Change in body mass of all flounder in each trial between d 0-10 and 10-20 are plotted in figure 2.

Treatment Effects

Dunnett's tests were used to determine growth impacts of various treatment groups relative to concurrent controls. All tests were one-tailed with a significance level of α=0.01 as the test investigated growth detriment due to treatments compared to the concurrent control, not overall change in growth.

All treatment groups subjected to extreme diel-cycling hypoxia, across all pH treatments, exhibited significant growth detriment compared to concurrent controls, based on results of Dunnett's tests (Table 3). Other groups, including moderate diel-cycling hypoxia and static pH, moderate diel-cycling hypoxia and extreme diel-cycling pH, and static DO and moderate diel-cycling pH exhibited growth detriment during the first 10 d of their respective trials, based on results of Dunnett's test (Table 3). No consistent growth detriment was observed outside of the extreme diel-cycling hypoxia treatments (Table 3). Separate Dunnett's tests were conducted using LGR as the metric for growth. These tests did not yield different results compared to the tests using mean change in mass of each treatment as the growth metric.

During the recovery/compensatory growth experiment the group that was subjected to the most extreme treatment (d 0-10) and returned to static levels (d 10-20) yielded values of $p < 0.01$ and $p = 0.99$, respectively, when mean change in mass of treatments were compared to concurrent control using a Dunnett's test. The group that was subjected to the most moderate treatment (d 0-10) and returned to static levels (d 10-20) yielded values of $p = 0.40$ and $p = 0.97$, respectively, when mean change in mass of treatments were compared to concurrent control using a Dunnett's test.

The GCA comparing growth curves of the group subjected to the most extreme treatment for 20 d and the concurrent group that was subjected to the most extreme treatment (d 0-10) and returned to static levels (d 10-20) yielded $p < 0.01$, suggesting a highly significant difference in growth trajectories amongst the two groups. The GCA

comparing growth curves of the group subjected to the most moderate treatment for 20 d and the concurrent group that was subjected to the most moderate treatment (d 0-10) and returned to static levels (d 10-20) yielded $p=0.23$, suggesting no significant difference in growth trajectories amongst the two groups. Growth curves derived during the recovery/compensatory growth experiment depict negligible impact of the most moderate treatments and highlight the presence of recovery growth when juvenile flounder are subjected to the most extreme treatment (d 0-10) and returned to static levels (d 10-20) (Figure 3).

The GCA comparing the extreme diel-cycling hypoxia and static pH group to the concurrent extreme diel-cycling hypoxia and moderate cycling pH group (Figure 2), yielding $p=0.055$. The ANCOVAs analyzing the same groups across days 0-10, 10-20, and, 0-20 yielded $p=0.015$, $p=0.50$, and $p=0.12$, respectively. Given the fact that a number of comparisons are possible when performing a GCA a significance level more stringent than $p=0.05$ is suggested (Mirman, 2014). These analyses therefore suggest that although we observed a difference in growth between the two concurrent extreme diel-cycling treatments during d 0-10 the treatments yielded similar overall results, thus we cannot suggest a difference between the treatments.

Specific Growth Rate

Specific growth rate was calculated for d 0-10 and d 10-20 for each treatment group (Table 4). Specific growth rate varied over the course of each trial period and was affected by some of the treatments, similarly to the effect treatments had on change in

body mass. We observed a decreasing SGR over the course of the three trials that coincided with an increase in initial body size.

During the recovery/compensatory growth experiment the group that was subjected to the most extreme treatment (d 0-10) and was returned to static levels (d 10-20) yielded SGR values of 2.16 and 3.59, respectively. The group that was subjected to the most moderate treatment (d 0-10) and was returned to static levels (d 10-20) yielded SGR values of 3.65 and 3.26, respectively.

Behavioral Observations & Mortality

Summer flounder appeared to exhibit increased gill ventilation rate and stroke volume during DO minima under both moderate and extreme diel-cycling hypoxia treatments. Reduced feeding, during both morning and evening feedings, was observed under all extreme diel-cycling hypoxia treatments. Fish in the most extreme diel-cycling hypoxia treatments exhibited either sluggish behavior, reduced activity when fed, or panicked swimming indicative of an escape response during the morning feeding that coincided with the DO minima. Previously described observations are all qualitative but were consistently exhibited by most fish under the extreme diel-cycling hypoxia treatments during experimentation.

Summer flounder exhibited 100% mortality on d 21 of trial 2, under the most extreme diel-cycling hypoxia and pH treatment. The treatment was replicated and 90% of the fish exhibited mortality on d 15 of trial 3, one individual survived 30 d of extreme

diel-cycling hypoxia and pH. No mortality occurred after 30 d of the most moderate diel-cycling hypoxia and pH treatment.

pCO₂ Values

The pH and $p\text{CO}_2$ of our aquarium systems ranged from 6.85 and 13,706 μatm to 8.03 and 805 μatm (Figure 4). Water samples from Pepper Creek suggest the carbonate chemistry of our laboratory was slightly elevated, but fairly similar to that exhibited in summer flounder nursery habitat as our data from the field yielded values pH and $p\text{CO}_2$ values of 7.35 and $\sim 2,080$ μatm at 08:09, 7.60 and 1080 μatm at 12:03, and 7.79 and ~ 683 μatm at 16:41.

Due to the dynamic pH, and therefore carbonate chemistry of our laboratory, $p\text{CO}_2$ values are best represented in graphical form. Minor discrepancies in sampling time of day during given treatment across multiple sampling days often yields differences in pH, due diel-cycling pH, and therefore $p\text{CO}_2$. Presenting average water chemistry parameters at different but generalized time intervals over the course of 20 d experiments distorts the data, and erroneously suggests unstable water chemistry. A plot with all recorded pH and $p\text{CO}_2$ from all experiments gives the best description of our observed chemical parameters (Figure 4).

Discussion

Extreme Diel-Cycling Hypoxia Negatively Impacted Summer Flounder Growth

Exposure to extreme diel-cycle hypoxia, across all pH treatments yielded significantly reduced growth in juvenile summer flounder compared to fish in concurrent control groups. Our data strongly suggests extreme diel-cycling hypoxia has a significant detrimental effect on juvenile summer flounder growth. No consistent growth detriment was observed at moderate levels of diel-cycling hypoxia, across all pH treatments, thus our data suggests moderate diel-cycling hypoxia has an insignificant impact on growth.

No Independent or Interactive Impact of Diel-Cycling pH on Summer Flounder Growth

Our data does not suggest an independent or interactive effect of diel-cycling pH on juvenile summer flounder growth. It is important to note, our Dunnett's tests yield p-values at least an order of magnitude smaller under the extreme diel-cycling hypoxia treatments at both levels of diel-cycling pH compared to the diel-cycling hypoxia and static pH group (Table 3). However, a GCA and ANCOVAs across multiple temporal intervals suggests no significant overall difference between the extreme diel-cycling hypoxia and static pH group and the concurrent extreme diel-cycling DO and moderate pH group. Bogue et al., (*In prep*) reported similar results, as diel-cycling pH did not have an independent or interactive effect on growth on mummichog growth.

Future studies should concurrently examine extreme diel-cycling hypoxia across all pH treatments to allow for direct comparison amongst the groups. Furthermore, future studies should maximize the allowable number of fish used in given treatments and control groups to maximize the power of statistical analyses.

Growth Rate Acclimation & Recovery Growth

Juvenile summer flounder did not exhibit growth rate acclimation to extreme diel-cycling hypoxia and pH, consistent with results found 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. Given the temporal constraints of the study we cannot suggest juvenile summer flounder are capable of exhibiting compensatory growth when returning water to optimal conditions subsequent to exposure to extreme diel-cycling hypoxia and pH. Bejda et al., (1992) suggests juvenile winter flounder (*Pseudopleuronectes americanus*) exhibit compensatory growth subsequently following ten to eleven weeks of exposure to chronic (2.2 mg O₂ l⁻¹) and diel-cycling hypoxia (2.5 – 6.4 mg O₂ l⁻¹) when water is returned to normoxia (7.2 mg O₂ l⁻¹) for five weeks. Given the temporal discrepancies in our study with that of Bejda et al., (1992) we cannot suggest similar or contradictory results for summer flounder.

Summer flounder did exhibit recovery growth after exposure to extreme diel-cycling hypoxia and pH as indicated by our Dunnett's test and GCA. This suggests ephemeral exposure to extreme diel-cycling hypoxia and pH may not manifest in long-term damage for summer flounder and potentially other estuarine fishes. Growth rate

acclimation, compensatory growth, and growth rate recovery were not analyzed in fish subjected to moderate diel-cycling hypoxia and pH because no initial growth detriment occurred.

Behavioral Observations & Mortality

Behavioral adaptations observed in extreme diel-cycling hypoxia treatments were likely due to increased stress associated with the treatments. Behavioral adaptations were not successful in mitigating growth detriment as increased gill ventilation rate and stroke volume and panicked swimming behavior, indicative of an escape response, are energy intensive. Furthermore, panicked swimming behavior was futile given the experimental design, and thus an extremely inefficient use of energy; perhaps further exacerbating growth detriment under extreme diel-cycling hypoxia. The occurrence of increased gill ventilation rate and stroke volume, in the absence of other observed behavioral adaptations, has an insignificant impact on growth as this behavior was observed during the DO minima by many fish in the moderate diel-cycling hypoxia treatments. The stress associated with the extreme diel-cycling hypoxia treatments yielded reduced feeding rates, likely a contributing factor to the observed growth detriment.

Summer flounder exhibit a primarily sedentary lifestyle, thus lethargic behavior under extreme diel-cycling hypoxia has less scope to decrease routine metabolism and mitigate detrimental growth impacts associated with diel-cycling hypoxia and pH compared to other more active mid-water species (Schurmann & Steffensen, 1994; Crocker & Cech, 1997; Brady et al., 2009; Stierhoff et al., 2009b). A laboratory study by

Dixon et al., (*In Prep*) found that several estuarine species utilize aquatic surface respiration, a behavior summer flounder are physiological incapable of performing, to cope with stress associated with diel-cycling hypoxia. This physiological limitation relative to other estuarine fishes likely increases the susceptibility of summer flounder to growth detriment and mortality.

Juvenile summer flounder exhibit mortality following ~2-3 weeks of exposure to extreme diel-cycling hypoxia and pH. During mortality events, day 21 of trial 2 and day 15 of trial 3, we confirmed the laboratory, including DO and pH regulation, were functioning normally. Dissolved oxygen, pH, and salinity values were normal and ammonia, nitrite, and nitrate levels were ~0 ppm. The DO and pH traces during the most extreme treatments, along with all other treatments across all trials, were highly consistent as exhibited by the small coefficient of variation of DO and pH values across treatments (Table 1). The fish subjected to the most extreme diel-cycling hypoxia and pH in trial 2, 100% mortality on d 21, had a mean mass of 8.15 g at the start of the experiment, while fish subjected to the same treatment in trial 3, 90% mortality on 15 d, had a mean mass of 10.16 g at the start of the experiment. Furthermore, the individual that survived 30 d under the most extreme diel-cycling hypoxia and pH treatment in trial 3 had an initial mass of 6.32 g at the start of the experiment and a final mass of 10.51 g on d 30. A possible explanation may be that the tolerance to extreme diel-cycling hypoxia and pH diminishes through ontogeny, even amongst young-of-year summer flounder. Our study cannot strongly suggest any changes in tolerance to diel-cycling hypoxia and pH throughout early ontogeny but the matter should be further investigated.

Nursery Habitat Quality

Our results suggest that increased levels of productivity, due to severe eutrophication, and therefore, extreme diel-cycling hypoxia and pH reduces estuarine nursery habitat quality for summer flounder. Based on the findings of this study the addition of diel-cycling pH does not exacerbate the growth detriment for juvenile summer flounder over the effects of diel-cycling hypoxia alone. Therefore, appears the fact that growth rates of young summer flounder in the field, calculated from RNA:DNA (Stierhoff et al., 2009a), are lower than would be predicted based on laboratory growth experiments using only diel-cycling hypoxia, is not the result of co-occurring acidification cycles. The most parsimonious explanation for such discrepancies may be attributed to laboratory conditions including *ad libitum* feeding twice daily, absence of predators, lack of incentive and ability to forage, stable optimal temperature, and stable optimal salinity, as such optimal conditions are rarely consistently exhibited in the field.

Most Significant Findings

Juvenile summer flounder exhibit growth detriment when exposed to extreme, but not moderate, diel-cycling hypoxia. Furthermore, 10 d of exposure to extreme diel-cycling hypoxia and pH likely does not manifest in long-term growth detriment and prolonged exposure, ~2-3 weeks, to extreme diel-cycling hypoxia and pH causes mortality. We observed no evidence of an interactive or independent impact associated with diel-cycling pH. However, given the severity of these natural pH cycles (MD DNR, 2012) more work needs to be done investigating the effect of pH cycling across various

estuarine species, particularly calcifying organisms. Our work in conjunction with Bogue et al., (*In prep*) suggests diel-cycling pH may lack the potential to negatively impact estuarine fishes.

Table 1. Diel-cycling DO and pH treatments.

	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	Mid DO, static pH	High DO, static pH
pH= 7.2-7.8	Normoxia, Mid pH	Mid DO, Mid pH	High DO, Mid pH
pH= 6.8-8.1	Normoxia, High pH	Mid DO, High pH	High DO, High pH

Table 2. Treatment information. Dissolved oxygen and pH minima and maxima information, parenthesis denote coefficient of variation.

	Static	Moderate Cycling	Extreme Cycling
DO mg l ⁻¹ Treatment	7.43 (0.019)	3.13 (0.094) – 8.28 (0.093)	1.16 (0.081) – 10.01 (0.096)
pH Treatment	7.49 (0.002)	7.22 (0.001) – 7.78 (0.001)	6.87 (0.007) – 7.99 (0.005)

Table 3. Results of Dunnett's test comparing mean change in mass of each treatment to concurrent control. Values on the left of each treatment group correspond to d 0-10 of each experiment, and values on the right of each treatment group correspond to d 10-20 of each experiment. Values are color coded using key on the top left corner of the table.

<p>p<0.01</p> <p>p<0.1</p> <p>p>0.1</p>	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	p<0.01 – p=0.15	p=0.07 – p=0.02
pH: 7.2-7.8	p=0.08 – p=0.91	p=0.84 – p=0.99	p<0.01 – p<0.01
pH: 6.8-8.1	p=0.99 – p=0.41	p<0.01 – p=0.54	p<0.01 – p<0.01

Table 4. *SGR values*. Values on the left of each treatment group represent SGR from days 0-10 and values on the right represent SGR from days 10-20 of each trial. Each concurrent trial is color coded using key on the top left corner of the table.

Trial 1 Trial 2 Trial 3	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	4.12 – 3.57 3.15 – 3.11 3.79 – 2.29	2.87 – 2.07	3.27 – 2.59
pH: 7.2-7.8	3.72 – 3.67	3.48 – 3.12	2.25 – 2.42
pH: 6.8-8.1	4.04 – 2.97	3.11 – 2.09	2.04 – 2.26

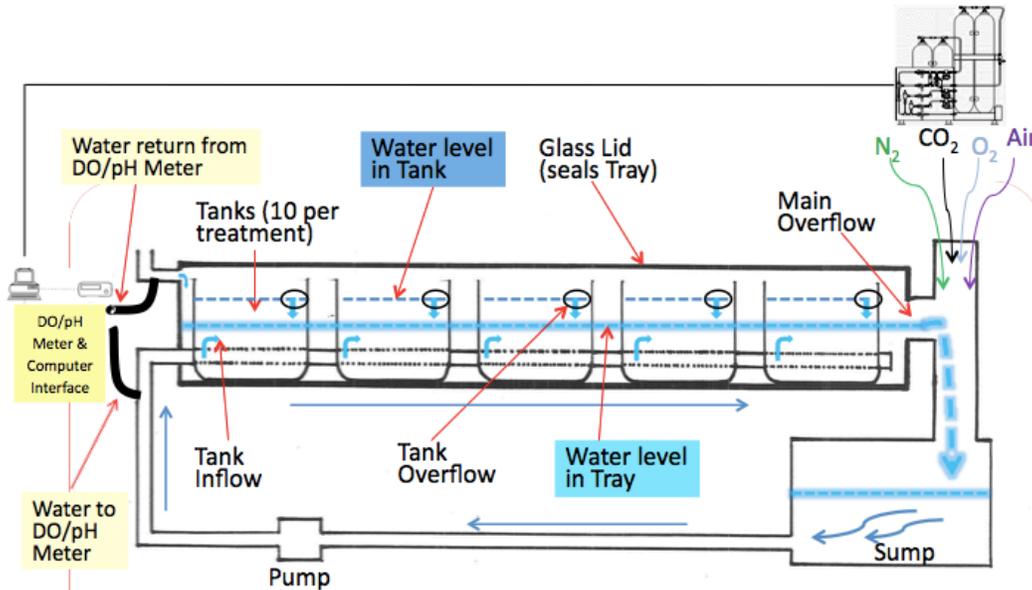


Figure 1: DO and pH treatment aquarium. DO/pH meter and computer interface dictate the amount of N_2 , CO_2 , O_2 , and air that is bubbled into the sump via solenoid-controlled gas manifolds. A portion of the water in the tank supply line is diverted to the DO/pH probes to be monitored, ensuring DO and pH levels are in compliance with treatment regime, and returned to the tank supply line. The computer receives DO and pH data from the Hach sc200 meter that determines the correct amount of gas to be released from the gas solenoids to ensure desired DO and pH levels are maintained. Each system has ten 18 l tanks, holding 1 summer flounder per tank (Note: only 5 tanks are visible in diagram). Water flows through tank inflows and overflows back in to the system where it is once again treated in the sump and pumped back through the tank supply line. Diagram courtesy of P.A. Greca and T.E. Targett.

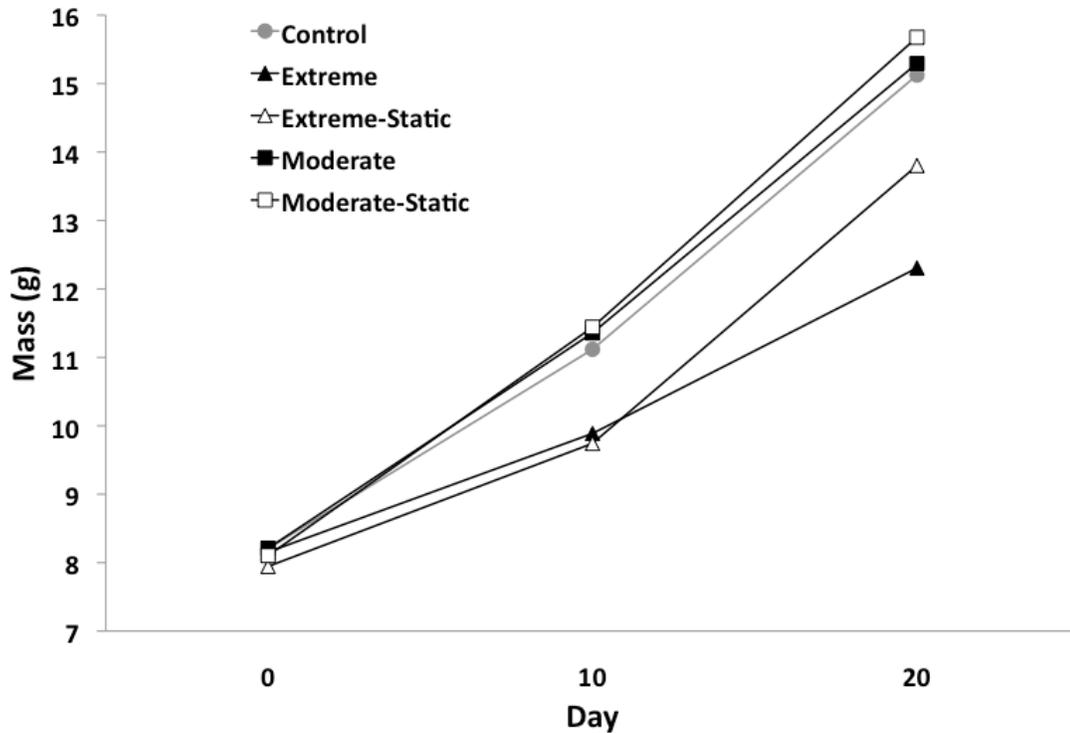


Figure 3: Growth curves from recovery/compensatory growth treatments. Mean size (g) of fish per treatment, denoted by key, are shown at three intervals, days 0, 10, and 20, over the course of the trial. The figure highlights negligible impact of the most moderate treatments and presence of recovery growth when fish are subjected to the most extreme treatment (d 0-10) and returned to static levels (d 10-20).

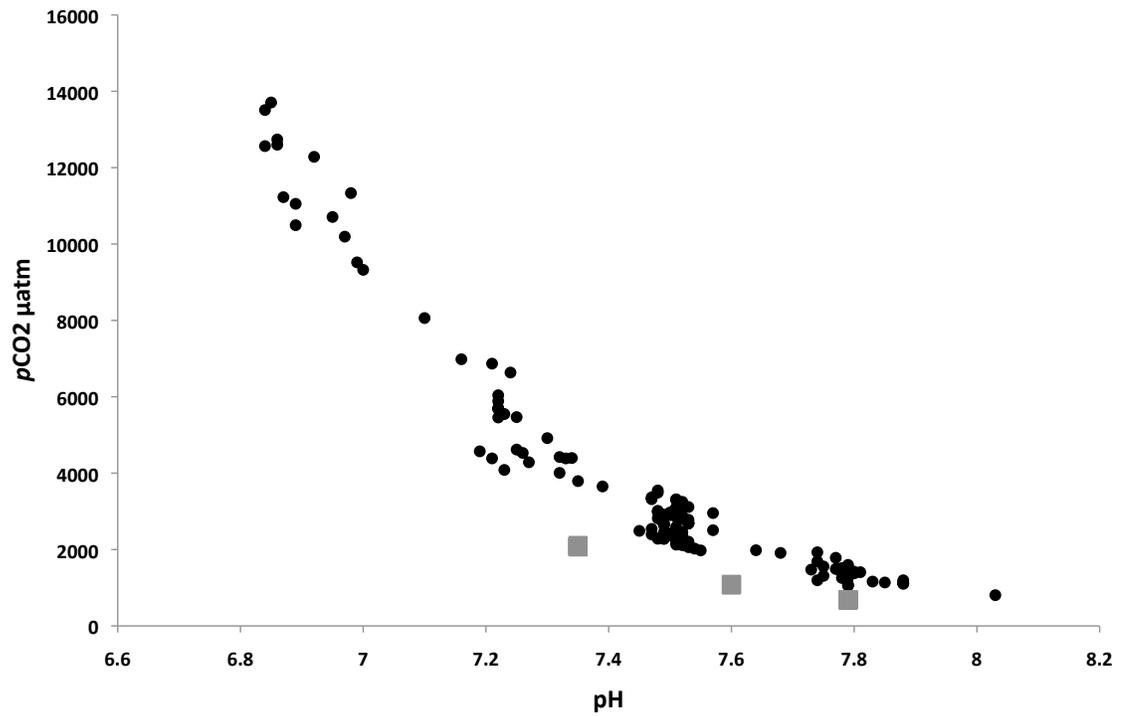


Figure 4: $p\text{CO}_2$ and pH values. Black circles represent $p\text{CO}_2$ and pH values from water samples taken from the aquarium systems during experiments and grey squares represent $p\text{CO}_2$ and pH values from water samples taken from Pepper Creek.

REFERENCES

Able, K.W. (1999) Measures of juvenile fish habitat quality: examples from a National Estuarine Research Reserve. *American Fisheries Society Symposium* 22:134-147.

Able, K.W., M.P. Fahay. 2010. Ecology of Estuarine Fishes: Temperate Waters of the Western North Atlantic. John Hopkins Press.

Beck, M.W., K.L. Heck Jr, K.W. Able, D.L. Childers, D.B. Eggleston, B.M. Gillanders, B. Halpern, C.G. Hays, K. Hoshino, T.J. Minello, R.J. Orth, P.F. Sheridan, and M.P. Weinstein (2001) The Identification, conservation, and management of estuarine and marine nurseries for fish and invertebrates. *BioScience* 51:633-641.

Bejda, A., B.A. Phelan, and A.L. Studholme (1992) The effect of dissolved-oxygen on the growth of young-of-the-year winter flounder, *Pseudopleuronectes americanus*. *Enviro Biol Fish* 34:321-327.

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

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

Brady, D.C. and T.E. Targett (2013) Movement of juvenile weakfish *Cynoscion regalis* and spot *Leiostomas xanthurus* in relation to diel-cycling hypoxia in an estuarine tidal tributary. *Mar Ecol Prog Ser* 491: 199-219.

Brady, D.C., T.E. Targett, and 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 Aquat 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 Ecol* 381: S143-S149.

Breitburg, D.L., N. Steinburg, S. DuBeau, C. Cooksey, E.D. Houde (1994) Effects of low dissolved oxygen on predation on estuarine fish larvae. *Mar Ecol Prog Ser* 104: 235-246.

Cai, W.J., X.P. Hu, W.J., Huang, M.C. Murrell, J.C. Lehrter, S.E. Lohrenz, W.C. Chou, W.D. Zhai, J.T. Hollibaugh, Y.C. Wang, P.S. Zhao, X.H. Guo, K. Gundersen, M.H. Dai and G.C. Gong (2011) Acidification of subsurface coastal waters enhanced by eutrophication. *Nat Geosci* 4:766-770.

Campbell, L.A. and J. A. Rice (2014) Effects of hypoxia-induced habitat compression on growth of juvenile fish in the Neuse River Estuary, North Carolina, USA. *Mar Ecol Prog Ser* 497: 199-213.

Crocker, C. E., and Cech Jr, J. J. (1997). Effects of environmental hypoxia on oxygen consumption rate and swimming activity in juvenile white sturgeon, *Acipenser transmontanus*, in relation to temperature and life intervals. *Environmental Biology of Fishes*, 50(4), 383-389.

Denman, K., J.R. Christian, N. Steiner, H.O. Pörtner, and 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 Enviro Qual* 30:275-28.

Eby, L.A. and 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.

Eby, L. A., Crowder, L. B., McClellan, C. M., Peterson, C. H., & Powers, M. J. (2005). Habitat degradation from intermittent hypoxia: impacts on demersal fishes. *Mar Ecol Progr Ser* 291: 249-262.

Fogarty, M.J., M.R. Sissenwine, and E.B. Cohen (1991) Recruitment variability and the dynamics of exploited marine populations. *Trend Ecol Evol* 6:241-246.

Herbert, N.A. and J.F. Steffensen (2006) Hypoxia increases the behavioural activity of schooling herring: a response to physiological stress or respiratory distress? *Mar Biol* 149:1217-1225.

Gibson, R.N. (1994) Impact of habitat quality and quantity on the recruitment of juvenile flatfishes. *Nether J Sea Research*. 32:191-206.

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

Houde E.D. (1987) Fish early life dynamics and recruitment variability. *American Fisheries Society Symposium* 2:17-29.

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

Howell, P. and D. Simpson (1994) Abundance of marine resources in relation to dissolved oxygen in Long Island Sound. *Estuaries* 17:349-402.

Lewis, E., Wallace, D., & Allison, L. J. (1998). *Program developed for CO2 system calculations* (p. 38). Tennessee: Carbon Dioxide Information Analysis Center, managed by Lockheed Martin Energy Research Corporation for the US Department of Energy.

Lubbers, L., W.R. Boynton, and W.M. Kemp (1990) Variations in structure of estuarine fish communities in relation to abundance of submerged vascular plants. *Mar Ecol Prog Ser* 65:1-14.

Maryland Department of Natural Resources. "Eyes on the Bay." June 6 2012.

< http://mddnr.chesapeakebay.net/newmontech/contmon/archived_results3_2012.cfm>

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

Miller D.C, Poucher S.L, Coiro L (2002) Determination of lethal dissolved oxygen levels for selected marine and estuarine fishes, crustaceans, and a bivalve. *Mar Biol* 140:287–296.

Millero, F. J. (1979). The thermodynamics of the carbonate system in seawater. *Geochimica et Cosmochimica Acta*, 43(10), 1651-1661.

Minello, T.J. (1999) Nekton densities in shallow estuarine habitats of Texas and Louisiana and the identification of essential fish habitat. *American Fisheries Society Symposium* 22:43-75.

Mirman, D. Language & Cognitive Dynamics Laboratory. “Growth Curve Analysis.” April 2 2015. <danmirman.org/gca>

Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., ... & Yool, A. (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature*, 437(7059), 681-686.

Poucher, S. L., and Coiro, L. (1997). Test reports: effects of low dissolved oxygen on saltwater animals. *Memorandum to DC Miller, US Environmental Protection Agency, Atlantic Ecology Division, Narragansett, Rhode Island*.

Rees, B. B., T.E Targett, B. J. Ciotii, C. A. Tolman, S. S. Akkina, and 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). *Academic Press*.

Riebesell, U., V.J., Fabry, L. Hansson, and J.-P. Gattuso (eds)(2010) Guide to best practices for ocean acidification research and data reporting. *Publications Office of the European Union*.

Schurmann, H., & Steffensen, J. F. (1994). Spontaneous swimming activity of Atlantic cod *Gadus morhua* exposed to graded hypoxia at three temperatures. *J Exp Bio* 197(1), 129-142.

Steffensen, J.F., J.P. Lomholt, and K. Johansen (1982) Gill ventilation and O₂ extraction during graded hypoxia in two ecologically distinct species of flatfish, the flounder (*Platichthys flesus*) and the plaice (*Pleuronectes platessa*). *Enviro Biol Fishes* 7:157-163.

Stierhoff, K.L., T.E. Targett, and P.A. Greco (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, and K.L. 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 Ser* 325:255-266.

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

Taylor, C., and J.M. Miller (2001) Physiological performance of juvenile southern flounder, *Paralichthys lethostigma* (Jordan and Gilbert, 1884), in chronic and episodic hypoxia. *J Exp Mar Biol Ecol* 258:195-214.

Thomas, P., Md. S. Rahman, J.A. Kummer, and S. Lawson (2006) Reproductive endocrine dysfunction in Atlantic croaker exposed to hypoxia. *Mar Environ Res* 62:S 249-S252.

Tyler, R.M. and 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.

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

van Ginnekan, V., G. van den Thillart, A. Addink, and C. Erkelens (1996) Synergistic effect of acidification and hypoxia: in vivo ³¹P-NMR and respirometric study in fishes. *Am J Physiol Regul Integr Comp Physiol* 271:1746-1752.

Vaquer-Sunyer, R. and C.M. Duarte (2008) Thresholds of hypoxia for marine biodiversity. *Proceedings of the National Academy of Science* 105:15452-15457.

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

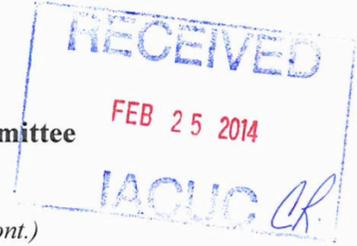
Weinstein, L. (1979) Shallow marsh habitats as primary nurseries for fish and shellfish, Cape Fear River, North Carolina. *Fisheries Bulletin* 77:339-357.

Appendix

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**

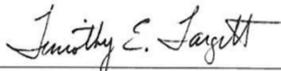


(Please complete below using Arial, size 12 Font.)

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 : <i>Cynoscion regalis</i> , <i>Paralichthys dentatus</i> , <i>Morone saxatilis</i> , <i>Fundulus heteroclitus</i> , <i>Menidia menidia</i> , <i>Anchoa mitchilli</i> , <i>Bairdiella chrysoura</i> , <i>Micropogonias undulatus</i> , <i>Leiostomus xanthurus</i> , <i>Mugilidae sp.</i> , <i>Fundulus majalis</i> , <i>Brevoortia tyrannus</i> , <i>Hemiramphus sp.</i> , <i>Menticirrhus saxatilis</i>	
Pain Category: (please mark one)	
USDA PAIN CATEGORY: (Note change of categories from previous form)	
Category	Description
<input type="checkbox"/> B	Breeding or holding where NO research is conducted
<input type="checkbox"/> C	Procedure involving momentary or no pain or distress
<input type="checkbox"/> D	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
<input checked="" type="checkbox"/> E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

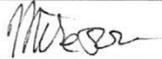
Official Use Only	
IACUC Approval Signature:	
Date of Approval:	4-1-2014

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. <i>(Teaching Protocols Exempt)</i>
12. I understand that by signing, I agree to these assurances.
<div style="display: flex; justify-content: space-between; align-items: flex-end;"> <div style="text-align: center;">  <hr style="width: 80%; margin: 0 auto;"/> <p>Signature of Principal Investigator</p> </div> <div style="text-align: center;"> <p>2-23-2014</p> <hr style="width: 80%; margin: 0 auto;"/> <p>Date</p> </div> </div>

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	
4.	
5.	
6.	
7.	
8.	
9.	
10.	
11.	
12.	
13.	
14.	
15.	

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?

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:

- 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? 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.