4-22-2019

An Experimental Evaluation of the Sensitivity of Coastal Marine Fishes to Acidification, Hypoxia, and Warming

Christopher S. Murray
University of Connecticut - Storrs, christopher.murray@uconn.edu

Follow this and additional works at: https://opencommons.uconn.edu/dissertations

Recommended Citation
https://opencommons.uconn.edu/dissertations/2115
Ocean acidification (OA) during the coming century will impact marine ecosystems in profound ways. Laboratory studies have played a pioneering role in identifying biological vulnerabilities and have documented deleterious effects in taxonomically diverse fauna. The early life-stages of marine fish may be particularly sensitive to OA, thereby constituting a global threat to some of the world’s most important marine resources. Despite the rapid accumulation of experimental evidence, considerable uncertainty remains in estimating the scope of OA impacts. To date, most studies have relied on relatively short-term exposures to estimate effects of elevated $p$CO$_2$, while few have considered longer term OA effect across multiple life-stages. Additionally, while single-factor CO$_2$ experiments are a necessary first step in identifying OA sensitivities, there is a growing understanding that OA will proceed concurrently with warming and deoxygenation, yet multi-stressor factorial experiments remain rare. Furthermore, meta-analyses of existing research have highlighted the large, and thus far unexplained variability in OA responses among taxa and populations. One promising mechanism which could explain this variability involves the role of local adaptation to existing $p$CO$_2$ fluctuations that characterizes marine habitats to different degrees. This framework remains untested in fish. The primary goal of this dissertation was to apply state-of-the-art experimental techniques to address the aforementioned knowledge gaps. By using two ecological important forage species, the Atlantic silverside (*Menidia menidia*) and Northern sand lance (*Ammodytes dubius*) with contrasting life-history characteristics, this dissertation provides novel insights into potential near-future climate impacts on fish. Chapter 1
summarizes a long-term OA experiment on *M. menidia* finding that elevated $pCO_2$ exposure resulted in small but significant reductions in offspring size and condition factor. Chapter 2 tested $CO_2 \times$ temperature effects in *M. menidia* offspring and found complex growth and survival responses. Chapter 3 reports on $CO_2 \times$ temperature trials on offspring of *A. dubius* and documented precipitous reductions to survival and growth. Chapter 4 describes two $CO_2 \times$ dissolved oxygen trials on *M. menidia* offspring that demonstrated a negative synergistic effect on embryonic survival. Together, this dissertation provides much needed baseline data and novel insights into climate effects in forage fish.
An Experimental Evaluation of the Sensitivity of Coastal Marine Fishes to Acidification, Hypoxia, and Warming

Christopher S. Murray

B.S., University of Richmond, 2010
M.S., Stony Brook University, 2014

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Connecticut

2019
An Experimental Evaluation of the Sensitivity of Coastal Marine Fishes to Acidification, Hypoxia, and Warming

Presented by
Christopher S. Murray, B.S., M.S.

Major Advisor
Hannes Baumann

Associate Advisor
Hans Dam

Associate Advisor
Eric Schultz

Associate Advisor
Christopher Chambers

Associate Advisor
Christopher Gobler

University of Connecticut
2019
ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to my advisor Dr. Hannes Baumann for his guidance and generosity over the last seven years. I am grateful for the opportunities that he has afforded me. I hope we continue to produce great science together for years to come.

I am grateful to my committee members Drs. Hans Dam, Eric Schultz, Christopher Chambers, and Christopher Gobler for their generous support during the production of this dissertation.

I express my gratitude to my lab mates, current and former, including Julie Pringle, Dr. Emma Cross, Jacob Snyder, and Callie Concannon. I am grateful for your support in the lab, but also for your friendship.

I am grateful to Charlie Woods, John Hamilton, Dennis Arbige, Gary Grenier, and Bob Dziomba for their technical expertise in the development of the experimental system. Their generosity and technical guidance have been invaluable to this dissertation and served as an additional education for me.

I would like to thank the army of undergraduate volunteers who have assisted me through the years including Molly Hughes, Megan Barry, Wes Huffman, Elizabeth Karamavros, Tyler Clouthier, James Harrington, Rafeed Hussain, Rainer Moy-Huwyler, Charles Dyke, Isaiah Mayo, and Lucas Jones. Without their dedication and hard work none of this science would have been possible.

I would like to also thank the staff at UConn Avery Point including Deb Schuler, Pat Evans, Janet Laflamme, Liz Rawlinson, and Elise Hayes for their support.

It has been a pleasure to be a part of the UConn Avery Point community. The faculty and graduate student body are an amazing group of people from whom I have learned so much about science but also what it means to be a community. I have thoroughly enjoyed my time here.

I am grateful to the NOAA team at Stellwagen Bank Marine Sanctuary including Dr. David Wiley, Michael Thompson, Peter Hong, Dave Slocum, Amy Meloski, and Dr. Tammy Silva as well as the USGS team of Dr. Page Valentine and Dan Blackwood. Without their dedication and generosity the sand lance project would not have succeeded.

I am especially grateful to my family; my sisters Shannon and Ryann and my parents Sharon and Robert whose love, commitment, and generosity has afforded me with endless opportunities. I love you.

And of course my dog Bear whose loyalty and love has been, without hyperbole, the single most important factor in my success. I love you boy.

I would like to thank my funding sources, the National Science Foundation (NSF-OCE #1536165) and Northeast Regional Sea Grant Consortium (RNE16-CTHCE-I).
# TABLE OF CONTENTS

1. Title page .......................................................................................................................... i
2. Copyright page ................................................................................................................... ii
3. Approval page ..................................................................................................................... iii
4. Acknowledgments .............................................................................................................. iv
5. Table of contents ............................................................................................................... v
6. Introduction ....................................................................................................................... 1
7. Chapter 1: Consequences of Elevated CO$_2$ Exposure across Multiple Life Stages in a Coastal Forage Fish ............................................................................................................ 14
8. Chapter 1: Appendix .......................................................................................................... 46
10. Chapter 2: Appendix ........................................................................................................ 81
11. Chapter 3: High Sensitivity of the Northern Sand Lance (*Ammodytes dubius*) to Ocean Acidification and Warming ........................................................................................................... 82
12. Chapter 3: Appendix ....................................................................................................... 109
13. Chapter 4: A Factorial Evaluation of CO$_2$ × Dissolved Oxygen Effects in Atlantic Silverside Offspring ......................................................................................................................... 114
14. Chapter 4: Appendix ....................................................................................................... 137
15. Summary ......................................................................................................................... 138
Introduction

The ongoing anthropogenic rise in atmospheric and surface ocean carbon dioxide (CO$_2$) has no geological analog over the past 66 million years (Hönisch et al., 2012; Zeebe et al., 2016). In a process termed ‘ocean acidification’ (OA), up to half of all anthropogenic CO$_2$ has dissolved into the ocean where it readily reacts with seawater resulting in wholesale changes to CO$_2$ partial pressures ($p$CO$_2$), pH, and carbonate saturation states (Sabine et al., 2004; Orr et al., 2005; Feely et al., 2008a) that will challenge the physiological tolerances of marine organisms in complex ways (Doney et al., 2009; Waldbusser et al., 2015). To date, laboratory studies have played a pioneering role in identifying vulnerable species and life-stages (Hendriks et al., 2010; Kroeker et al., 2010; Wittmann and Pörtner, 2013; Przeslawski et al., 2015). Negative responses have been documented in an array of taxa, including single-cell plankton (Riebesell et al., 2000; Beaufort et al., 2011), copepods (Fitzer et al., 2012; Thor and Dupont, 2015), calcifying mollusks (Talmage and Gobler, 2009; Waldbusser et al., 2015), corals (Hoegh-Guldberg et al., 2007; Anthony et al., 2008), cephalopods (Rosa and Seibel, 2008; Kaplan et al., 2013), and fish (Baumann et al., 2012; Frommel et al., 2012).

Adult fish were assumed to be largely tolerant of predicted $p$CO$_2$ conditions given their well-developed cardiovascular and osmoregulatory systems that are proficient at buffering metabolically produced acidosis and excreting acid-equivalents from body fluids (Ishimatsu et al., 2008; Esbaugh et al., 2012; Heuer and Grosell, 2014). However, with the understanding that early life-stages lack these mature acid-base regulatory tissues, laboratory experiments shifted their focus to embryos and early larvae. These experiments have detected a range of negative responses to future $p$CO$_2$ conditions including reduced survival and growth as well as changes to metabolism and behavior in a diverse group of species (Cattano et al., 2018; Esbaugh, 2018). In fact, meta-
analyses have concluded that fish offspring are among the most OA-sensitive groups of taxa tested thus far (Wittmann and Pörtner, 2013). While these negative responses are notable, just as many studies have documented neutral responses of elevated $p$CO$_2$. In fact, experiments have demonstrated divergent OA responses between similar species and even populations (Cattano et al., 2018). Thus, despite a rapid accumulation of empirical evidence, considerable uncertainty remains in estimating the scope of OA impacts on fish (Heuer and Grosell, 2014; Cattano et al., 2018; Esbaugh, 2018).

The primary goal of this dissertation was to apply novel experimental techniques to address four important knowledge gaps in the understanding of OA effects in marine fish. First, most OA experiments have used relatively short-term exposures on small sample sizes (<50) to record acute responses to elevated $p$CO$_2$ conditions. However, for most species these short-term exposures constitute just a fraction of their overall lifespan and thus may fail to adequately capture the full extent of OA effects. Elevated $p$CO$_2$ conditions can elicit a range of acclimation responses, including the differential expression of key acid-base regulatory enzymes (Tseng et al., 2013) and the uptake and maintenance of elevated bicarbonate in extracellular fluids (Esbaugh et al., 2012). These pathways are potentially energetically expensive (Melzner et al., 2009; Heuer and Grosell, 2014; Heuer and Grosell, 2016) yet few studies have considered how the long-term effects of elevated $p$CO$_2$ will impact survival, metabolism, growth, and other important fitness-related, carry-over effects from early-life exposures in later life-stages (Pechenik, 2006; McCormick and Gagliano, 2008). Furthermore, the majority of OA studies on fish have derived conclusions from relatively small sample sizes that inherently limits the ability of statistical tests to detect subtle shifts in response-trait distributions. Thus, short-term experiments using few individuals may lead to underestimated effects. There is a need for long-term exposure experiments using large sample
sizes to detect how the cumulative effects of elevated $p\text{CO}_2$ exposure impact fish across multiple life-stages.

The second and third types of knowledge gaps addressed here involved quantifying how exposure to OA conditions might interact with the other major climate stressors. These were addressed by applying multi-stressor factorial experimental designs. While single-factor $p\text{CO}_2$ experiments are a necessary first step in identifying sensitivities, there is a growing appreciation that OA will proceed concurrently with warming and deoxygenation (Doney et al., 2012; Bopp et al., 2013). Coastal systems are particularly at risk as eutrophication and warming have combined to stimulate intense heterotrophic consumption of dissolved oxygen (DO) which simultaneously releases CO$_2$ (Cai et al., 2011; Wallace et al., 2014; Baumann et al., 2015). Thus, marine organisms that rely on near-shore habitats may already experience the co-occurring stressors of OA, warming, and hypoxia at levels not predicted to occur in the open ocean for several hundred years (Rabalais et al., 2009; Doney, 2010; Gruber, 2011). Theoretical frameworks of ectotherm physiology indicate that OA acclimation may compromise the capacity of fish to tolerate thermal and hypoxic stress (Perry and Gilmour, 2006; Berenbrink et al., 2011; Pörtner, 2012; Lefevre, 2016). In fish, the earliest life-stages are likely to be most sensitive to combined climate effects (Pörtner and Peck, 2010) yet multi-stressor exposure experiments still remain rare (Kroeker et al., 2013; Gobler and Baumann, 2016). Thus, there is an urgent need for experiments to utilize multi-stressor factorial designs that test for between stressor interactions that could not be interpolated from single-stressor designs (Gobler et al., 2014).

The final knowledge gap addressed by this dissertation involves elucidating the underlying environmental and physiological mechanisms that have facilitated the complex OA responses observed in marine fish offspring (Cattano et al., 2018; Esbaugh, 2018). One promising mechanism
involves the role of local adaptation to existing \( p\text{CO}_2 \) variability which may preadapt organisms to future OA conditions (Pespeni et al., 2013; Hofmann et al., 2014; Vargas et al., 2017). Early-life tolerance to acidification is likely adaptive for organisms that spawn in nearshore systems where large \( p\text{CO}_2 \) fluctuations are frequently the result of upwelling (Feely et al., 2008b), river input (Salisbury et al., 2008), and most importantly fluctuations in biological productivity (Duarte et al., 2013; Wallace et al., 2014; Baumann et al., 2015). By contrast, fish that spawn in the open ocean or during winter where \( p\text{CO}_2 \) levels are relatively low and stable, likely experience weak selection for CO\(_2\) tolerance during early life. An additional source of divergence may arise from differences in early-life developmental rates (Melzner et al., 2009). Rapid growth in warm-water species requires a robust capacity to buffer against metabolic acidosis, whereas slow development in polar or winter-spawning fish may relax such requirements (Pörtner et al., 2004). This theoretical framework, termed the “Ocean Variability Hypothesis” (Baumann, 2019), has been partially tested in a handful of marine invertebrates (Kelly et al., 2013; Hofmann et al., 2014; Vargas et al., 2017) but remains largely untested in fish. Thus, there is a need for investigations to strategically choose species that utilize spawning habitats and have phenologies characterized by contrasting \( p\text{CO}_2 \) and thermal regimes in order to explicitly test the validity of the OVH in fish.

**Study species**

To address the knowledge gaps highlighted here, two species of forage fish were strategically chosen for experimental evaluations. The forage fish designation encompasses a group of small, schooling, zooplanktivorous species that perform the critical role of channeling biological production from the plankton to larger piscivorous fish, mammals, and sea birds (Pikitch et al., 2012). As such, past depletions of forage fish stocks have resulted in profound ecological consequences (Furness, 2003; Cury et al., 2011; Smith et al., 2011; Essington et al., 2015). Given
their notable ecological and economic importance, understanding climate effects in forage fish is a high scientific priority (Pikitch et al., 2012).

The Atlantic silverside (*Menidia menidia*) is a broadly distributed coastal forage fish found along the North American Atlantic coast from Florida to Nova Scotia (Middaugh, 1981). During the spring and summer, the species is often the most abundant fish in many near shore habitats and serves as the primary food source for several commercially and recreationally important species (Conover and Ross, 1982). The Atlantic silverside has an extensive history as laboratory model (Middaugh et al., 1987) and has been used to demonstrate several landmark evolutionary discoveries that include counter-gradient variation in growth (Conover and Present, 1990), temperature-dependent sex determination (Conover and Kynard, 1981), size-dependent overwintering mortality (Schultz et al., 1998), and fisheries-induced evolution in growth (Conover and Munch, 2002).

The species is a semelparous batch spawner that reproduces fortnightly at spring tides from April to July in shallow estuaries (Conover and Ross, 1982). Thus, *M. menidia* is an ideal study organism given the accessibility of spawning adults and well established spawning and rearing protocols, which enable repeated experimentation within a single spawning season. Furthermore, *M. menidia* have already be evaluated in several OA studies (Murray et al., 2014; DePasquale et al., 2015; Malvezzi et al., 2015; Miller et al., 2016). Given that *M. menidia* use dynamic nearshore systems as nursery habitat, their offspring may be adapted to the contemporary temperature, $pCO_2$, and DO fluctuation. However, as anthropogenic impacts threaten coastal systems, there is a critical need to quantify early-life responses to predicted future extreme conditions.

The second study species used in this dissertation was northern sand lance (*Ammodytes dubius*), a semi-demersal forage fish found on the northwest Atlantic shelf from North Carolina to Greenland.
where bottom habitat consists of sand or fine gravel (Winters, 1983; Nelson and Ross, 1991). Their size, shape, high nutritional content, and proclivity to form dense schools render them an ideal prey for several commercially important fish species like bluefin tuna and cod (Chase, 2002; Richardson et al., 2014), sea birds (Robards et al., 2000), and many marine mammals (Weinrich et al., 1998). Thus, throughout much of its range, including in Stellwagen Bank National Marine Sanctuary, *A. dubius* is considered a keystone species of high ecological and economic importance (Willson et al., 1999). In fact, the seasonal migration of humpback whales to Stellwagen Bank is linked to high *A. dubius* biomass on that site (Friedlaender et al., 2009).

Northern sand lance spawn during late fall when females deposit demersal eggs onto sandy offshore banks where embryos develop slowly under cooling winter temperatures (Smith et al., 1978) and stable $p$CO$_2$ conditions (Salisbury and Jönsson, 2018). As such, selection for $p$CO$_2$-resilient genotypes during early life is likely weaker relative to summer spawning, nearshore species like *M. menidia*. Despite its ecological importance and potential sensitivity to OA, no studies have assessed OA and other climate effects in *A. dubius* offspring. Furthermore, the contrasting life-history characteristics of *M. menidia* and *A. dubius* create an ideal comparison for testing the OVH in fish.

**Dissertation outline**

*Chapter 1* describes the results of a long-term experiment where several thousand *M. mendia* offspring were reared for approximately a third of their lifespan to quantify how continuously elevated $p$CO$_2$ exposure influences length, weight, condition factor, and fatty-acid composition across multiple life-stages.
Chapter 2 summarizes five independent $pCO_2 \times$ temperature trials that exposed *M. menidia* embryos and early larvae to three $pCO_2$ levels crossed with four temperature regimes to test how suboptimal thermal conditions might influence $pCO_2$ effects on early-life survival and growth.

Chapter 3 summarizes two years of $pCO_2 \times$ temperature trials on embryos and early larvae of *A. dubius* that tested how these treatments altered hatching dynamics, survival, and growth.

Chapter 4 describes two $pCO_2 \times$ DO trials on embryos and early larvae of *M. menidia* that tested how elevated $pCO_2$ exposure influenced sensitivity to hypoxia by quantifying treatment effects on hatching dynamics, survival, and growth.
References


Baumann, H. 2019. Experimental assessments of marine species sensitivities to ocean acidification and co-stressors: how far have we come? Canadian Journal of Zoology.


Gruber, N. 2011. Warming up, turning sour, losing breath: ocean biogeochemistry under global change. Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences, 369: 1980-1996.


Silverside, 'Menidia beryllina', Atlantic Silverside, 'M. menidia', Tidewater Silverside, 'M. peninsulae' and California Grunion, 'Leuresthes tenuis'.


Chapter 1
Consequences of Elevated CO₂ Exposure across Multiple Life Stages in a Coastal Forage Fish

Published in *ICES Journal of Marine Science*

Abstract
Ocean acidification may impact the fitness of marine fish, however, studies reporting neutral to moderate effects have mostly performed short-term exposures to elevated CO₂, whereas longer-term studies across life stages are still scarce. We performed a CO₂ exposure experiment, in which a large number (n > 2,200) of Atlantic silverside *Menidia menidia* offspring from wild spawners were reared for 135 days through their embryonic, larval, and juvenile stages under control (500 μatm) and high CO₂ conditions (2,300 μatm). While survival was high across treatments, subtle but significant differences in length, weight, condition factor, and fatty acid composition were observed. On average, fish from the acidified treatment were 4% shorter and weighed 6% less, but expressed a higher condition factor than control juveniles. In addition, the metrics of length and weight distributions differed significantly, with juveniles from the high CO₂ treatment occupying more extreme size classes and the length distribution shifting to a positive kurtosis. Six of 27 fatty acids differed significantly between treatments. Our results suggest that high CO₂ conditions alter long-term growth in *M. menidia*, particularly in the absence of excess food. It remains to be shown whether and how these differences will impact fish populations in the wild facing size-selective predation and seasonally varying prey abundance.
Introduction

Understanding how climate change is affecting the fitness and therefore abundance and distribution of marine organisms constitutes one of the most important, if necessarily complex and challenging tasks of our time (Hoegh-Guldberg and Bruno, 2010; Doney et al., 2012). Marine environments are not only warming, they are also gradually acidifying due to the dissolution of rising atmospheric (Doney et al., 2009) and metabolic carbon dioxide (CO₂), the latter particularly manifesting in coastal regions, where increasing nutrient input often fuels excessive primary production and subsequent microbial respiration (Wallace et al., 2014; Cloern et al., 2016). In recognition of these processes, exploring the sensitivity of marine organisms to high CO₂ environments has become one of the most eagerly pursued research priorities in biological oceanography during the past two decades (Browman, 2016). Most of this research has taken the form of laboratory experiments, which are a first necessary step to distinguish CO₂-sensitive from CO₂-tolerant traits in marine taxa, species, and life-stages (the hypothesized losers vs. winners in a future ocean, respectively), before issues of evolutionary adaptation, potential trade-offs, indirect effects, and the responses of ecosystems can be addressed (Sunday et al., 2014; Malvezzi et al., 2015).

Perhaps unsurprisingly, this recent swell of empirical research has revealed an intriguing complexity of organismal responses to high CO₂ that continues to defy easy answers or generalizations. While meta-analyses have shown a majority of responses to high CO₂ to be negative (Hendriks et al., 2010; Kroeker et al., 2010; Branch et al., 2012), there is abundant evidence for non-linear (Ries et al., 2009), neutral (Munday et al., 2011; Hurst et al., 2013) or even positive effects of exposures to elevated CO₂ (Gooding et al., 2009). Calcifying invertebrates and early life stages of marine species are likely most sensitive to the symptoms of ocean acidification.
(Kleypas et al., 2006; Dupont et al., 2008; Kurihara, 2008; Talmage and Gobler, 2010; Seibel et al., 2012; Waldbusser et al., 2013; Bednaršek et al., 2014), however, contrasting sensitivities have been documented within every taxon (Ries et al., 2009; Hendriks et al., 2010) or even among populations within the same species (Frommel et al., 2012b; Frommel et al., 2013).

As a group, marine fish have shown similar complexity. Juvenile and adult fish tolerate CO$_2$ levels far beyond average climate change predictions (> 2,000 μatm, Ishimatsu et al., 2008). However, fish early life stages, while still developing acid-base competency, might be more vulnerable as some have exhibited reduced survival in response to elevated CO$_2$ levels (Ishimatsu et al., 2008; Baumann et al., 2012; Steffen, 2012; Chambers et al., 2014; Pimentel et al., 2014; Frommel et al., 2016). On the other hand, studies reporting no adverse survival effects are at least as numerous (Franke and Clemmesen, 2011; Munday et al., 2011; Hurst et al., 2012; Frommel et al., 2013; Hurst et al., 2013; Munday et al., 2015). Most studies to date have documented some form of sublethal response to high CO$_2$ exposure including abnormal behavior, reduced orientation (Munday et al., 2009b), predator avoidance (Dixson et al., 2010) and swimming capacity (Pimentel et al., 2014), elevated or depressed metabolism (Munday et al., 2009a; Rummer et al., 2013), skeletal malformations (Chambers et al., 2014), otolith hypercalcification (Checkley et al., 2009; Bignami et al., 2013a), tissue damage (Frommel et al., 2013; Frommel et al., 2016), and increased levels of fatty acids (Díaz-Gil et al., 2015). Collectively, these findings suggest that high CO$_2$ environments can impact the early life stages of many marine fish, even if short-term survival under artificial laboratory settings (e.g. no predators) remains statistically unaffected.

How acidification affects fish growth is another surprisingly difficult question. Growth is an important fitness-relevant trait, given that in the wild growth rate is generally inversely related to survival (Anderson, 1988; Hare and Cowen, 1997). However, laboratory studies have reported
growth responses to high CO$_2$ levels that cover the entire spectrum of negative to neutral (Baumann et al., 2012; Hurst et al., 2013) to positive effects (Munday et al., 2009c), which is often simply attributed to species-specific reaction norms. In addition, the diversity of responses may suggest the existence of confounding experimental factors that hinder cross-study comparisons and the development of a unifying framework. First, most acidification studies to date have provided fish larvae with excess food rations, which is practical to rule out feeding related growth differences, but likely disguises the additional metabolic costs of high CO$_2$ environments, because survivors can simply compensate or overcompensate for those costs by increased consumption. Second, the majority of ocean acidification studies on fish have so far employed relatively short-term experimental designs that spanned days to a few weeks post-hatch and measured growth mostly during one ontogenetic stage (i.e., eggs, larvae, or juveniles). For most fish species, particularly those of commercial value, this amounts to a small fraction of their overall life span and could therefore be insensitive to potential carry-over effects from one life stage to the next. Third, logistical constraints in rearing set-ups and analytical throughput often preclude the assessment of large sample sizes; thus, the majority of studies have so far based their conclusions on comparing response means derived from a limited number of individuals (i.e., n = 5-50 per treatment). The resultant statistical power suffices to detect strongly divergent responses, whereas potential subtle shifts in trait distributions (e.g., range, skewness, kurtosis of length, weight or condition), which may be as important as central tendencies, remain undetectable.

Here we report on a large laboratory rearing experiment to assess the growth consequences of high CO$_2$ environments in the Atlantic silverside (*Menidia menidia*), an ecologically important forage fish that is abundant in nearshore habitats along the North American Atlantic coast (Middaugh et al., 1987). From spawners collected in the wild, we reared several thousand offspring under
contrasting CO₂ conditions from fertilization to approximately four months post-hatch, thereby not only spanning the embryonic, larval, and juvenile stages but also about one third of the life span of this annual, semelparous species. During the late larval and juvenile stages, food was provided in standardized, non-excess rations, and several growth-related traits including length, weight, and condition factor were assessed for over 2,200 survivors at the end of the experiment. We hypothesized that long-term, continuous CO₂ exposure in *M. menidia* incurs additional metabolic costs that result in divergent distribution metrics for length, weight, and condition factor in surviving juveniles. In addition, we measured fatty acid profiles in a smaller subset of individuals to determine if high CO₂ exposure alters the retention of fatty acids from diet.

**Methods**

*CO₂ treatments and measurements:* Following best practices and guidelines for ocean acidification (OA) research (Riebesell et al., 2010) we used gas proportioners (ColeParmer®) to mix air with 100% CO₂ (bone dry grade) that was delivered to the bottom of each replicate rearing container via airstones. Control conditions were achieved by forcing compressed laboratory air through a series of CO₂ stripping units containing granular soda lime (AirGas®), a particle filter (1 µm) and then to each replicate via airstone. Two standardized treatment levels were administered; control (CO₂ stripped air only, ~500 µatm CO₂, pH<sub>NBS</sub> = 8.05) and high CO₂ conditions (air:CO₂ mix, ~2,300 µatm CO₂, pH<sub>NBS</sub> = 7.45). These treatments represent levels commonly used in OA research and conditions experienced seasonally by *M. menidia* offspring in the wild (Murray et al., 2014). Target pH levels were monitored daily using a handheld pH probe (Orion ROSS Ultra pH/ATC Triode and Orion Star A121 pH Portable Meter, Thermo Scientific) calibrated bi-weekly with 2-point pH<sub>NBS</sub> references. During the course of the experiment, each replicate tank was sampled three times for measurements of total alkalinity (*Aₜ*; µmol kg⁻¹). Seawater was siphoned into 300 ml
borosilicate bottles and immediately analyzed for $A_T$ at 17°C using an endpoint titration (Mettler Toledo™ G20 Potentiometric Titrator). Salinity was measured via refractometer and methodological accuracy of alkalinity titrations were verified using Dr. Andrew Dickson’s (University of California San Diego, Scripps Institution of Oceanography) certified reference material for $A_T$ in seawater (Batch 147 = 2,231 $\mu$mol $A_T$ kg seawater$^{-1}$). The partial pressure ($p$CO$_2$; $\mu$atm) and fugacity of CO$_2$ (fCO$_2$; $\mu$atm) as well as dissolved inorganic carbon ($C_T$; $\mu$mol kg$^{-1}$) and carbonate ion concentration ($CO_3^{2-}$; $\mu$mol kg$^{-1}$) were calculated in CO2SYS (V2.1, http://cdiac.ornl.gov/ftp/co2sys) based on measured $A_T$, pH$_{NBS}$, temperature, and salinity using K1 and K2 constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and Dickson (1990) for KHSO$_4$. An overview of the carbonate chemistry is given in Table 1.

**Field sampling and experimental design:** Experiments were performed at University of Connecticut’s Avery Point Campus in the Rankin Laboratory, a seawater facility adjacent to eastern Long Island Sound. Ripe adult *M. menidia* were collected on 1 May 2015 from Mumford Cove (41° 19.25’ N 72° 1.09’W), a shallow embayment dominated by eelgrasses (*Zostera marina*) and open to the Long Island Sound. Adults were sampled with a 30 m × 2 m beach seine, separated by sex, transported live to our laboratory, and held for 48 hours in large aerated tanks (17°C, ambient CO$_2$, no food). On the day of fertilization (3 May 2015), ≥ 20 ripe individuals from each sex were strip-spawned and eggs evenly distributed onto window screens (1 mm fiberglass mesh) submerged in plastic dishes with clear seawater. Strip-spawned adults were measured for total length (TL, to lower 0.5 cm; mean TL$_{females}$ = 9.7 cm, mean TL$_{males}$ = 8.7 cm). Fertilized embryos quickly attach to the screens via chorionic filaments, which facilitates precise enumeration and even allotment to treatments and replicates. Following established protocols for rearing *M. menidia* offspring (Murray et al., 2014), replicate containers (20 l) were filled with filtered (to 1 $\mu$m) and
UV sterilized seawater (31 psu) from Long Island Sound and placed in water baths (~300 l) controlled for temperature and light conditions (17°C, 15h light:9h dark) throughout the duration of the experiment. Within 2h of fertilization, each of four replicates per treatment received exactly 200 embryos to measure early life survival, while four other replicates per treatment each received ~400 offspring for long-term rearing. Larvae hatched ~14 days post-fertilization (dpf) and were immediately provided with excess rations of newly hatched brine shrimp nauplii *Artemia salina* (San Francisco strain, Brine Shrimp Direct) and a commercial larval powder food (first four days, Otohime Marine Weaning Diet, size A, Reed Mariculture®). At 2 days post-hatch (dph), living larvae from survival replicates were counted by gently scooping small groups into replacement containers. Between 1 to 14 days post-hatch (dph), all containers were cleaned daily with partial (10%) water exchange.

At 16 dph, larvae from the survival replicates were counted and a sub-sample (N<sub>control</sub> = 37, N<sub>high</sub> = 33) was preserved in 10% formaldehyde/seawater solution for later total length (TL) measurements (nearest 0.01 mm) via calibrated, digital images (ImagePro Premier® V9.1). All surviving larvae were transferred to larger (50 l) tubs and maintained under the previously described protocol. At 33 dph, larvae from the survival replicates were counted and then all larvae transferred to 50 l tubs fitted with screen-covered holes (1 mm mesh) to promote water exchange from a 300 l seawater bath. Due to space constraints, from 33 to 54 dph larvae from the survival replicates were pooled into a single container per CO₂ treatment. Rations of of nauplii and commercial powder food (Otohime B1, Reed Mariculture®) were standardized to the known number of larvae per replicate. At 54 dph, all juveniles from survival and grow-out replicates were counted and pooled at equal numbers into 300 l circle tanks (two tanks per treatment, ~615 fish per tank). Juveniles were fed standardized rations (to the known number of juveniles per tank) of
newly hatched nauplii and B1 commercial powder food. Tanks were siphoned for waste daily and partial water changes completed twice weekly. Additional sub-samples for length measurements (TL, nearest 0.01 mm) were made at 36 dph \( (N_{\text{control}} = 20, N_{\text{high}} = 20) \), 68 dph \( (N_{\text{control}} = 20, N_{\text{high}} = 20) \) and 100 dph \( (N_{\text{control}} = 28, N_{\text{high}} = 28) \).

At 122 dph, the experiment was terminated and all surviving juveniles were euthanized via an overdose of Tricaine-S (MS 222, Western Chemical®) for preservation. While some juveniles from each treatment were immediately frozen at -80°C for fatty acid analyses; ~75% of the samples were fixed in 10% buffered formaldehyde/seawater solution for TL \( (N_{\text{control}} = 1,025; N_{\text{high}} = 1,100, \text{nearest } 0.01 \text{ mm}) \) and weight measurements \( (N_{\text{control}} = 720; N_{\text{high}} = 786, \text{nearest } 0.01 \text{g}) \).

**Fatty acid analysis:** Individual FA profiles were assessed for 60 *M. menidia* juveniles 122 dph \( (n = 15 \text{ per tank}) \). Ten individuals per tank were chosen randomly, after which an additional five individuals were chosen among the smallest size classes (20-28 mm) to extend the range of sizes examined. The resultant samples spanned almost the entire size range (TL, 0.1 mm) of the entire experimental population. These fish along with samples of the early larval and juvenile diets (*Artemia salina* nauplii, brineshrimpdirect.com and Otohime B1, Reed Mariculture, respectively) were preserved individually at -80°C and subsequently shipped on dry ice to the Fisheries and Mariculture Laboratory at the University of Texas Marine Science Institute for fatty acid analysis.

Concentrations of 27 fatty acids (expressed as % of total fatty acids and mg fatty acid \( g^{-1} \text{ dry weight} \)) were measured using a gas chromatograph (Shimadzu GC-2014 Scientific Instruments; www.ssi.shimadzu.com) set with a Phenomenex ZB-WAX plus capillary column (30 m long; 0.53 mm ID; 1.0 \( \mu \text{m} \) thick; www.phenomenex.com) following the methods of Faulk & Holt (2005). For each sample, lipids were cold-extracted from approximately 50 mg dry mass by homogenizing in
a solution of chloroform–methanol (2:1 v/v) plus a measured amount of tricosanoic acid (23:0) as an internal standard for quantification of mg g⁻¹ dry mass of fatty acids. Fatty-acid methyl esters (FAME) were prepared by saponification in potassium hydroxide, followed by 14% boron trifluoride in methanol. Individual fatty acids were identified by comparison to commercial standards (Supelco, Inc).

**Statistical analysis:** Growth and survival analyses were performed using SPSS (V20, IBM). Percent survival was calculated for both treatments from hatch to 2 dph, 2 to 14 dph, 14 to 33 dph, and 33 to 122 dph. An angular transformation (arcsine of the square root of percentage/100) was applied to percentage data before testing for significance between treatments via independent samples t-test. TL was calculated as treatment means ± SD for each group of sub-sampled offspring. Each group was tested for homogeneity of variance between treatments using Levene’s test and for significance between treatments and tanks using independent samples t-test. At 122 dph, TL and wW distributions from both treatments were significantly non-normal (one-sample Kolmogorov-Smirnov test), and TL distributions expressed unequal variances (Levene’s test); thus non-parametric Mann-Whitney U tests were used to evaluate significance between treatments, and non-parametric Kruskal-Wallis pair-wise comparisons tested for significant tank effects. A non-parametric Levene’s test showed ranked TL data to have equal variances, thus meeting the equality of variance assumption. Condition factor (k) was calculated for 122 dph samples using the model 

\[
k = \frac{wW}{TL^b},
\]

where \(b\) was derived from the fitted relationship \(wW = a*TL^b\) from all 122 dph TL and wW data pooled from both treatments. The pooled TL:wW relationship and model fit can be found in supplemental material Fig. S1. Confidence intervals (CI) for 122 dph TL, wW and k medians, as well as skewness and kurtosis were generated from a bias-corrected accelerated (BCa) bootstrap routine using a sample size of 1,000.
To compare fatty acid profiles, we pooled data for each treatment to compare the concentration of each FA both in absolute (mg g dw⁻¹) and in relative terms (% of total FAs). An angular transformation was applied to relative fatty acid values prior to statistical analyses. We used two sample t-tests to compare FA-specific means and calculated relative differences between treatments ($\Delta FA = (FA_{mean\ ambient} - FA_{mean\ high})/FA_{overall\ mean}$) for visualization. We then performed principal component analysis on relative FA concentrations (% of total FAs) and extracted all principal components (PCs) with eigenvalues > 1 (Systat, version 13). We then explored the relationships between PCs and TL and used t-tests to test for differences between CO₂ treatments for each PC. For PCs that varied with mean fish size, we tested for effects of CO₂ treatment using analysis of covariance.

Results

Survival: Survival was generally high across life stages and CO₂ treatments (Fig. 1). Survival (mean ± SD) at hatch (15 dpf) was high across control (83 ± 8 %) and high CO₂ (87 ± 5 %) treatments. Highest mortality was observed during the early larval stage (hatch to 14 dph) where survival was not significantly different (independent samples t-test, $T_{6} = -1.468, p = 0.193$) in high CO₂ (50 ± 21 %) compared to control treatments (27 ± 20 %). Late larval survival (14 to 33 dph) was similar in control (94 %) and high CO₂ treatments (94 %). Juvenile survival (33 and 122 dph) was similar in control (84 ± 2 %) and high CO₂ (88 ± 1 %) treatments.

Sub-sample lengths: At 16 dph, larvae from the control were not significantly different (8.9 ± 1.2 mm) than high CO₂ individuals (mean ± SD = 8.6 ± 1.1 mm). After 36 dph, larvae from high CO₂ (13.9 ± 2.6 mm) were not significantly different than control samples (13.5 ± 2.1 mm). At 68 dph juveniles from the control (23.3 ± 2.2 mm) were significantly longer ($T_{38} = -2.098, p = 0.043$) than
samples from high CO$_2$ (21.3 ± 3.7 mm). By 100 dph, control juveniles had increased their mean size to 33.1± 5.0 mm, which was significantly longer ($T_{54} = -3.209$, $p = 0.002$) than high CO$_2$ juveniles (28.7 ± 5.4 mm). A summary of growth data can be found in Table 2.

**122 dph distributions of length, weight and condition:** After 122 days of high CO$_2$ exposure juveniles were significantly shorter (Mann-Whitney $U_{2,125} = 6.605$, $p = <0.001$, Fig. 2a, Table 3) and weighed significantly less ($U_{1,506} = 2.963$, $p = 0.003$, Fig. 2b, Table 3) than control fish. Bootstrapped TL and wW results showed no overlap of mean and median 95% CIs (Table 3). However, high CO$_2$ juveniles exhibited a significantly higher k ($U_{1,506} = -9.719$, $p < 0.001$, Fig. 2c, Table 3) across most TL classes (Fig. 3). In addition, high CO$_2$ significantly altered the shape of TL (two-sample Kolmogorov-Smirnov, $D_{2,125} = 2.956$, $p = <0.001$, Fig. 4a) and wW distributions ($D_{1,506} = 1.389$, $p = 0.042$, Fig. 4b). The effect was most prominent on TL, where the high CO$_2$ distribution was more variable, exhibited a larger SD and a greater range (Table 3). Skewness of TL distributions was similar, however, we found a sign change to kurtosis, with the statistic shifting to positive in the high CO$_2$ treatment (Table 3). Bootstrapped 95% CIs of kurtosis showed overlap, but a tendency for high CO$_2$ distribution to be more positive (Table 3).

**Tank Effects:** Significant within treatment tank effects were found for 122 dph TL and k measurements. Control tanks exhibited a similar wW (supplemental material Fig. S2) but differed significantly in TL (Kruskal-Wallis $H_3 = -2.708$, $p = 0.041$, supplemental material Fig. S2) and k ($H_3 = 4.394$, $p < 0.001$, supplemental material Fig. S2). TL and wW from high CO$_2$ tanks were similar; however, k differed significantly ($H_3 = -3.872$, $p = 0.001$, supplemental material Fig. S2 a-c).
**Fatty acid composition:** As expected, the FA profiles of *M. menidia* closely resembled the FA profile of the juvenile diet (Otohime B1, Fig.5A). Juveniles from the ambient treatment tended to have slightly higher amounts of specific fatty acids by weight (mg g⁻¹ DW) than juveniles from the high CO₂ group (Fig.5B,C); however, significant differences (t-test, *p* < 0.05) were detected for only six of the 27 FAs (FA_{ambient} > FA_{high}: 16:2n-4, 18:3n-3, 20:3n-3; FA_{high} > FA_{ambient}: 12:0, 18:0, 20:4n-6) (Fig.5B). In the analysis of relative FA concentrations, six PCs with eigenvalues > 1 were extracted, explaining a cumulative 82.3% of the total variance (46.6, 10.0, 9.5, 6.9, 5.0, and 4.3%, respectively). PC1 was strongly positively correlated with TL (*p* < 0.001), but neither the slope (*p* = 0.16) nor the elevation of the linear regressions of PC1 on TL differed statistically between treatments (*p* = 0.61, ANCOVA df = 1). The only other principal component that was significantly correlated with TL was PC4 for the ambient treatment. There were significant differences between treatments for scores on PC2 (*p* < 0.001) and PC6 (*p* = 0.002). The high CO₂ treatment had lower scores on PC2 and higher scores on PC6 than the ambient treatment, resulting in significant separation of these groups (Fig. 6A). Loadings on these two axes (Fig. 6B) suggested that the CO₂ treatment had higher concentrations of 18:3n-6, 20:2n6, 20:4n3, 20:3n6, 15:1, and 18:4n3 and lower concentrations of 16:2n-4, 20:3n-3, 20:5n3, and 18:3n3 than the ambient treatment. Of these suggested differences, only 18:3n6 was significantly higher than the CO₂ treatment and only 16:2n4, 20:3n3, and 18:3n3 were significantly lower in the CO₂ treatment compared with the ambient treatment (t-test, *p* < 0.05).

**Discussion**

We reared a large cohort (n > 2,200) of *M. menidia* offspring under control (~500 µatm) and high CO₂ (~2,150 µatm) conditions for 4.5 months and found small but significant differences between treatments in length, weight, condition factor, and fatty acid profiles. While survival was not
different across treatments, juveniles reared under high CO$_2$ were on average 4% shorter and weighed 6% less, but expressed a higher condition factor than control juveniles. Furthermore, we detected subtle shifts in the distributions of length and weight. High CO$_2$ juveniles exhibited a more variable TL distribution (greater standard deviation) with a positive kurtosis, indicating more fish populating extreme size classes. Our findings therefore suggest that high CO$_2$ induces a small, but detectible growth reduction in developing $M$. menidia offspring.

In this experiment, early post-hatch survival was unaffected by high CO$_2$ levels, not surprising given our past experiments have demonstrated offspring from Long Island Sound acquire CO$_2$ tolerance by early May (Murray et al., 2014). Past studies, however, did not find any influence of CO$_2$ on early growth of $M$. menidia. In fact, very few studies testing the effects of high CO$_2$ on fish have reported significant growth reductions (Baumann et al., 2012; Bignami et al., 2013b); while most have found neutral (Franke and Clemmesen, 2011; Munday et al., 2011; Hurst et al., 2012; Frommel et al., 2013; Chambers et al., 2014), or even positive effects (Munday et al., 2009c; Hurst et al., 2013; Bignami et al., 2014). Similarly contradictory findings have been reported for CO$_2$-induced changes to metabolic scope. For example, high CO$_2$ increased resting metabolic rates (RMR) in two tropical cardinal fish species, $O$storhinchus doederlein$ and $O$. cyanosoma (Munday et al., 2009a), but reduced RMR in the tropical damselfish Acanthochromis polyacanthus (Rummer et al., 2013). Temperate species including Atlantic cod (Gadus morhua) (Melzner et al., 2009), Atlantic halibut (Hippoglossus hippoglossus) (Gräns et al., 2014) and the Antarctic Notothenia rossi (Strobel et al., 2012) showed minor or no effects on aerobic performance after prolonged CO$_2$ exposure.

The conflicting reports of growth and metabolic responses may reflect species-specific reaction norms, or may highlight the limitations of relatively short-term CO$_2$ exposure experiments to
predict complex metabolic consequences. In this study, high CO₂ had a slight positive effect on late larval growth. Only after two months of exposure, covering three distinct ontogenetic stages, did the effect on growth rate produce significant effects on size. At 100 days post-hatch, high CO₂ juveniles were 13% shorter than the control, which suggested a substantially larger effect than actually found after 122 dph. This may indicate a biased sub-sample at 100 days or compensatory growth in high CO₂ juveniles during the final three weeks of the experiment. In contrast to the present work, most OA studies on fish have experimented with either embryonic or early larval stages, or examined only juvenile or adult stages. Thus, they potentially missed longer-term consequences to growth and important carry-over effects from early-life exposure to adulthood (Pechenik, 2006; McCormick and Gagliano, 2008). For example, reductions to survival in larval *M. beryllina* occurred only if high CO₂ exposure also covered the embryonic stage (Baumann et al., 2012). Likewise, consistent carry-over effects from larvae to adults have been observed in acidification experiments on the Olympia oyster *Ostrea lurida* (Hettinger et al., 2012; Hettinger et al., 2013). In this study, the effects of high CO₂ on length and weight were negative, however, high CO₂ juveniles expressed a significantly higher condition factor than control fish, because they were slightly heavier per unit of length. While counter-intuitive, this is consistent with experiments on Atlantic cod, which showed CO₂-induced increases in total lipid content, but not fatty acid composition (Frommel et al., 2012a). It is thus possible that high CO₂ promotes lipid accumulation, perhaps at the expense of increasing length.

Quantifying carryover metabolic responses to high CO₂ in fish early life stages is often complicated by *ad libitum* feeding regimes. Typically employed to avoid acute mortality when larval fish transition from endogenous to exogenous feeding (May, 1974), as well as precluding feeding related growth effects, excess feeding allows larvae to increase their food consumption
and thus mask any additional metabolic costs associated with high CO₂. Increased food consumption under acidified conditions has been shown in the juvenile anemonefish *Amphiprion melanopus* (Nowicki et al., 2012). It is also consistent with our personal observations that *M. menidia* larvae hatching under high CO₂ often appear to start feeding on nauplii faster than their conspecifics under control CO₂ levels. Perhaps elevated food consumption is driven by a stimulation of gustatory senses (Nowicki et al., 2012), similar to a range of other sensory effects associated with high CO₂ exposure (Munday et al., 2009b; Dixson et al., 2010; Cripps et al., 2011). Alternatively, increased consumption could be an active response to a CO₂-induced increase in metabolic rates. To date, at least two studies have demonstrated smaller oil globules in fish larvae hatching under high CO₂ conditions (Chambers et al., 2014; Munday et al., 2015), suggesting increased metabolic demands or a shift in the use of nutritional resources by fish embryos. Either way, newly hatched larvae may have a shorter period to initiate first feeding before starvation, a critical factor determining early life survival (May, 1974). Fish larvae in the wild experience dispersed and often ephemeral food levels, which may not afford them the opportunity to simply increase feeding and could thus face a metabolic deficit and ultimately starvation. There is now a need for studies to further explore the possible link between food availability and the effect size of CO₂ exposure.

Juveniles from the high CO₂ treatment exhibited a more variable TL distribution with a larger standard deviation, broader range, and positive kurtosis. While the kurtosis statistic was only slightly greater than zero, the bootstrapping confirmed the tendency for the high CO₂ TL distribution to be more positive than the control. A positive kurtosis describes a more peaked distribution, produced by a movement of individuals from the ‘shoulders’ of the distribution to the center and tails (DeCarlo, 1997). This suggests the variability of the high CO₂ distribution is
influenced more by a few individuals in extreme size classes, rather than many individuals only slightly different than the mean. While the effect is modest, the shift in distribution shape suggests that acidified conditions produce more slow-growing, and perhaps fast-growing, *M. menidia* offspring. Given that *M. menidia* is an annual species that faces intense size-selective overwintering mortality (Schultz et al., 1998), even small changes in juvenile length distributions may have important implications for the populations dynamics of spawning adults (Houde and Hoyt, 1987).

While some individuals from the experimental population were heavily affected by CO₂, the majority only exhibited small or negligible effects. The presence of both tolerant and sensitive phenotypes suggests traits associated with CO₂ tolerance are not universally expressed in *M. mendia*. CO₂ tolerance may be inherited seasonally via transgenerational plasticity (Murray et al., 2014), but there is also a significant genetic component to CO₂ tolerance (*h² = 0.20*, Malvezzi et al., 2015). Elevated phenotypic variability is often triggered by environmental stressors and maintained especially if the stressor is of intermittent spatial or temporal frequency (Hoffmann and Hercus, 2000). The preferred spawning and nursery habitat of *M. menidia* are shallow subtropical to temperate estuaries (Conover and Ross, 1982); i.e., coastal systems that often exhibit large seasonal pH variability, driven largely by metabolically produced CO₂ coinciding with warming spring-summer temperatures (Baumann et al., 2014). However, some coastal habitats, like those harboring robust seagrass communities, are better buffered against metabolic CO₂ and often maintain higher pH levels than ambient ocean seawater (Hendriks et al., 2014). Local adaptation in *M. menidia* is thought to be maintained by the continuous selection of locally-suited genotypes (Clarke et al., 2010), evidenced by the countergradient variation of traits such as growth rate (Conover and Present, 1990) and energy allocation (Billerbeck et al., 2000). Thus, a genetic
basis for variability in CO₂-tolerance is likely driven by selective pressures during early life pH exposure, particularly if expressing CO₂-tolerant traits creates tradeoffs that are detrimental in high pH environments (Kelly and Hofmann, 2013). That is, variable pH environments may select for CO₂-tolerant genes, while well buffered systems do not. Even though the wild adults used to fertilize this experiment were collected from a well buffered system in Mumford Cove (Baumann et al. unpublished data) CO₂-tolerant genotypes were probably well represented given the species’ significant population connectivity occurring during their offshore overwinter migration (Clarke et al., 2010). As an annual fish, the strategy of continuous selection for local adaptations allows *M. menidia* to thrive across broad thermal gradients, but likely also across fine-scale differences in pH conditions.

Our analyses of fatty acid profiles revealed that most (19 of 27) fatty acids measured were at somewhat higher levels in fish from the ambient treatment than the CO₂ treatment. However, significantly higher values were confined to three fatty acids on both an absolute (mg g⁻¹ dw) and relative basis (% total FA). On the other hand, three fatty acids were significantly elevated in juveniles from the CO₂ treatment. The subtle differentiation of FA profiles between high and ambient CO₂ environments in *M. menidia* juveniles contrasts with findings for larval red drum (*Sciaenops ocellatus*) reared at comparable CO₂ levels through 23 dph, which showed significant increases in FA concentration in 19 of 27 FAs measured (Díaz-Gil et al., 2015). The reason for the differences between these two studies is unknown, but might be attributable to species-specific differences in response to CO₂ exposure, or an effect of rearing temperature (27°C vs. 17°C, this study) or developmental stage (larvae vs. juveniles, this study).

The use of unnatural foods (*Artemia* nauplii and Otohime B1) likely produced juvenile fish with unnatural FA profiles. If the unnatural diet contains physiologically abnormally high or low levels
of FAs, we might observe physiological responses that do not reflect responses of wild fish. Thus, to better understand how OA may affect FA use in wild animals, natural food rations are needed. Nevertheless, we believe that high CO$_2$ conditions are triggering physiological processes resulting in changes to the use of FAs in juvenile $M$. menidia. The significant effects of high CO$_2$ conditions on some of the long-chain (18- to 22-carbon) highly unsaturated fatty acids observed in $M$. menidia may signify the activation of a stress response. Pro-inflammatory eicosanoids are built from the omega-6 fatty acid arachidonic acid (20:4$n$-6), while anti-inflammatory eicosanoids are built from the omega-3 fatty acid eicosapentaenoic acid (20:5$n$-3) (James et al., 2000). The two long-chain fatty acids (18:3$n$-3 and 20:3$n$-3) that were elevated in juveniles from the ambient treatment are precursors of 20:5$n$-3. The corresponding lower levels of 18:3$n$-3 and 20:3$n$-3 in juveniles from the high CO$_2$ treatment may reflect elevated synthesis of anti-inflammatory eicosanoids. Reduced production of pro-inflammatory eicosanoids in fish from the same treatment might account for the accumulation of 20:4$n$-6 in tissues of juveniles. These suggestions require confirmation by more direct measurements of cortisol and other markers of stress.

In summary, by rearing a large number of $M$. menidia offspring across multiple life-stages, this study demonstrated the existence of subtle but potentially important effects of OA on the growth of this important coastal forage fish. It demonstrated the importance of evaluating CO$_2$ exposure over multiple life-stages to capture long-term changes in metabolic processes. Multi-life-stage or multi-generational studies using large sample sizes are needed to critically address the potential for intra- or inter-generational carry-over effects of OA exposure. Additionally, the potential interaction between restricted food and acidified environments certainly warrants further examination and may be of particular importance to better understand whole ecosystem consequences of OA.
Acknowledgements

We are grateful to J. Snyder, M. Hughes, and C. Woods for assistance in the lab, and to C. Faulk for measuring fatty acid profiles. This work was funded by NSF OCE #1536165.
References


Dickson, A. G. 1990. Standard potential of the reaction: AgCl (s) + 12H2 (g) = Ag (s) + HCl (aq), and and the standard acidity constant of the ion HSO₄⁻ in synthetic sea water from 273.15 to 318.15 K. The Journal of Chemical Thermodynamics, 22: 113-127.


Table 1: Mean (±s.d.) pHNBS and temperature (°C) from daily handheld probe measurements. Mean (±s.d.) salinity, total alkalinity (AT; μmol kg⁻¹), dissolved inorganic carbon (CT; μmol kg⁻¹), partial pressure of CO₂ (pCO₂; μatm), fugacity of CO₂ (fCO₂; μatm), and carbonate ion concentration (CO₃²⁻; μmol kg⁻¹) measured from three seawater samples of each replicate tank. Salinity was measured via refractometer and was 31 psu for all tanks. AT was measured by endpoint titrations. CT and pCO₂, fCO₂ and CO₃²⁻ were derived in CO2SYS.

<table>
<thead>
<tr>
<th>Tank</th>
<th>CO₂</th>
<th>pHNBS</th>
<th>Temp</th>
<th>AT</th>
<th>CT</th>
<th>pCO₂</th>
<th>fCO₂</th>
<th>CO₃²⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>High</td>
<td>7.42±0.11</td>
<td>17.5±0.4</td>
<td>2,102±10</td>
<td>2,138±13</td>
<td>2,295±65</td>
<td>2,287±65</td>
<td>31.3±0.6</td>
</tr>
<tr>
<td>B</td>
<td>High</td>
<td>7.43±0.12</td>
<td>17.5±0.4</td>
<td>2,123±27</td>
<td>2,158±24</td>
<td>2,283±95</td>
<td>2,275±94</td>
<td>32.2±1.8</td>
</tr>
<tr>
<td>C</td>
<td>Control</td>
<td>8.06±0.13</td>
<td>17.3±0.3</td>
<td>2,112±7</td>
<td>1,958±7</td>
<td>500±7</td>
<td>498±7</td>
<td>116.8±1.6</td>
</tr>
<tr>
<td>D</td>
<td>Control</td>
<td>8.07±0.12</td>
<td>17.2±0.6</td>
<td>2,110±1</td>
<td>1,956±1</td>
<td>499±7</td>
<td>497±7</td>
<td>116.6±1.4</td>
</tr>
</tbody>
</table>

Table 2: Summary of total lengths (mm) from sub-sampled M. menidia larvae and juveniles from control (500 µatm) and high CO₂ (2,300 µatm) treatments at 17°C. Samples taken 16, 36, 68 and 100 dph. Significance tests (p < 0.05) generated from independent samples t-test.

<table>
<thead>
<tr>
<th>Age (dph)</th>
<th>CO₂ treatment</th>
<th>N</th>
<th>Mean TL (mm)</th>
<th>s.d.</th>
<th>Min</th>
<th>Max</th>
<th>df</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Control</td>
<td>37</td>
<td>8.9</td>
<td>1.2</td>
<td>6.5</td>
<td>11.3</td>
<td>68</td>
<td>1.281</td>
<td>0.204</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>33</td>
<td>8.6</td>
<td>1.1</td>
<td>6.2</td>
<td>10.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>Control</td>
<td>20</td>
<td>13.5</td>
<td>2.1</td>
<td>11.0</td>
<td>17.1</td>
<td>38</td>
<td>-0.553</td>
<td>0.584</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>20</td>
<td>13.9</td>
<td>2.6</td>
<td>7.0</td>
<td>17.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>Control</td>
<td>20</td>
<td>23.3</td>
<td>2.2</td>
<td>19.3</td>
<td>27.4</td>
<td>38</td>
<td>2.098</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>20</td>
<td>21.3</td>
<td>3.7</td>
<td>14.1</td>
<td>28.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>Control</td>
<td>28</td>
<td>33.1</td>
<td>5.0</td>
<td>26.0</td>
<td>45.0</td>
<td>54</td>
<td>3.209</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>28</td>
<td>28.7</td>
<td>5.4</td>
<td>19.0</td>
<td>39.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Summary statistics of total length (TL, mm), wet weight (wW, mg), and condition factor (k) distributions for *M. menidia* juveniles reared for 122 dph at ambient and high CO$_2$ conditions. Intervals in brackets represent 95% confidence intervals [low/high] based on 1,000 bootstraps.

<table>
<thead>
<tr>
<th>Trait</th>
<th>CO$_2$</th>
<th>Mean</th>
<th>Median</th>
<th>s.d.</th>
<th>Range</th>
<th>Skewness (±s.e)</th>
<th>Kurtosis (±s.e)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Ambient</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TL (mm)</td>
<td></td>
<td>41.8</td>
<td>42.3</td>
<td>5.6</td>
<td>25.0-57.9</td>
<td>-0.31±0.08</td>
<td>-0.06±0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[41.4/42.2]</td>
<td>[41.9/42.7]</td>
<td>[5.4/5.9]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>High</strong></td>
<td>40.0</td>
<td>40.6</td>
<td>6.3</td>
<td>18.3-59.4</td>
<td>-0.31±0.07</td>
<td>0.13±0.15</td>
</tr>
<tr>
<td></td>
<td>CO$_2$</td>
<td>[39.7/40.4]</td>
<td>[40.3/41.1]</td>
<td>[6.0/6.6]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Ambient</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wW (mg)</td>
<td></td>
<td>307</td>
<td>300</td>
<td>115</td>
<td>50-730</td>
<td>0.42±0.09</td>
<td>-0.03±0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[299-316]</td>
<td>[109/121]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>High</strong></td>
<td>289</td>
<td>280</td>
<td>119</td>
<td>30-750</td>
<td>0.42±0.09</td>
<td>0.03±0.17</td>
</tr>
<tr>
<td></td>
<td>CO$_2$</td>
<td>[281-297]</td>
<td>[113/125]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Ambient</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>k</td>
<td></td>
<td>0.60</td>
<td>0.60</td>
<td>0.07</td>
<td>0.36-0.95</td>
<td>0.20±0.09</td>
<td>1.73±0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[0.59/0.60]</td>
<td>[0.59/0.60]</td>
<td>[0.06/0.07]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>High</strong></td>
<td>0.63</td>
<td>0.63</td>
<td>0.07</td>
<td>0.33-0.89</td>
<td>0.10±0.09</td>
<td>1.17±0.17</td>
</tr>
<tr>
<td></td>
<td>CO$_2$</td>
<td>[0.63/0.64]</td>
<td>[0.63/0.64]</td>
<td>[0.07/0.08]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 1:** Mean (±s.d.) survival of *M. menidia* offspring reared at control (grey, 500 μatm) and high CO₂ (dark grey, ~2,300 μatm) for hatchlings (fertilization-2 dph), early larvae (2-14 dph), late larvae (14-33 dph) and juveniles (33-122 dph). No s.d. calculated for late larval period as larvae from survival experiment were temporarily pooled into a single replicate.
**Figure 2:** TL (mm), wW (mg) and k of juvenile *M. menidia* reared at control (grey, 500 μatm) and high CO₂ (dark grey, 2,300 μatm) for 122 dph. Black lines represent treatment medians. Filled boxes encompass 25th and 75th percentiles. Circles represent individuals outside 10th and 90th percentiles.
**Figure 3:** Distributions of condition factor per 2mm TL interval for juvenile *M. menidia* reared for 122 dph at ambient (A) and high CO$_2$ conditions (B). Thick and thin black lines correspond to the 10$^{th}$/90$^{th}$ and 25$^{th}$/75$^{th}$ percentiles, respectively, while the red line depicts the median. Data below the 10$^{th}$ and above the 90$^{th}$ percentiles are depicted by black dots. Underlying grey bars show relative frequencies for each 2 mm TL class. Black and grey numbers correspond to numbers of individuals measured for both TL and wW, or for TL only, respectively.
Figure 4: Cumulative frequency distributions of TL and wW in juvenile *M. menidia* reared for 122 dph at control (black lines) and high CO$_2$ (red lines) conditions.
Figure 5: Fatty acid (FA) profiles of juvenile *M. menidia* reared in the laboratory to 4.5 months post-fertilization under contrasting CO$_2$ conditions. A: average ± 1 sd concentrations of 27 FAs (mg FA per g dry weight of fish) with black squares depicting FA-concentrations in the juvenile diet (Otohime B1); B: average difference in FA concentrations (mg g$^{-1}$) between high and ambient CO$_2$ treatment scaled by the overall FA-specific mean; C: average difference in FA concentrations (% of total FA content) between high and ambient CO$_2$ treatment scaled by the overall FA-specific mean. Purple and red bars correspond to higher and lower FA-content, respectively, in the ambient relative to the high CO$_2$ treatment. Asterisks correspond to p-values < 0.05 (*), <0.01(**), and <0.001(***, (t-test, SPSS).
Figure 6: Principal component analysis of fatty acid profiles (% FA data). A: Scores on principal components 2 and 6 for the high CO$_2$ and ambient treatments with confidence ellipsoids ($\pm$ 2 sd). B: Loadings on principal components 2 and 6.
Appendix for Chapter 1: Consequences of Elevated CO₂ Exposure across Multiple Life Stages in a Coastal Forage Fish

Figures

**Fig. S1**: Total length (TL, mm): wet weight (wW, mg) relationship of juvenile Atlantic silversides *M. menidia* reared at control (circles, N=720, 500 μatm) and high CO₂ conditions (squares, N=786, 2,300 μatm CO₂). Data pooled from both treatments (N = 1,506) fitted with a power function.

\[
wW = 0.011 \times TL^{2.735}
\]

\[R^2 = 0.929\]

\[N = 1,506\]
Fig. S2: Total length (TL, mm), wet weight (wW, mg) and condition (k) of juvenile *M. menidia* reared in two control (grey, 500 μatm) and high CO₂ (dark grey, 2,300 μatm) tanks for 122 dph. Black lines represent treatment medians. Filled boxes encompass 25th and 75th percentiles. Circles represent individuals outside 10th and 90th percentiles. Tanks labeled with different letters denote significant pair-wise differences (Kruskal-Wallis tests, *p* < 0.05).
Chapter 2

You Better Repeat It: Complex CO₂ × Temperature Effects in Atlantic Silverside Offspring Revealed by Serial Experimentation

Published in Diversity


Abstract

Concurrent ocean warming and acidification demand experimental approaches that assess biological sensitivities to combined effects of these potential stressors. Here, we summarize five CO₂ × temperature experiments on wild Atlantic silverside, Menidia menidia, offspring that were reared under factorial combinations of CO₂ (nominal: 400, 2200, 4000, and 6000 µatm) and temperature (17, 20, 24, and 28°C) to quantify the temperature-dependence of CO₂ effects in early life growth and survival. Across experiments and temperature treatments, we found few significant CO₂ effects on response traits. Survival effects were limited to a single experiment, where elevated CO₂ exposure reduced embryo survival at 17 and 24°C. Hatch length displayed CO₂ × temperature interactions due largely to reduced hatch size at 24°C in one experiment but increased length at 28°C in another. We found no overall influence of CO₂ on larval growth or survival to 9, 10, 15 and 13–22 days post-hatch, at 28, 24, 20, and 17°C, respectively. Importantly, exposure to cooler (17°C) and warmer (28°C) than optimal rearing temperatures (24°C) in this species did not appear to increase CO₂ sensitivity. Repeated experimentation documented substantial inter- and intra-experiment variability, highlighting the need for experimental replication to more robustly constrain inherently variable responses. Taken together, these results demonstrate that the early life stages of this ecologically important forage fish appear largely tolerant to even extreme levels of CO₂ across a broad thermal regime.
Introduction

The current anthropogenic increase in atmospheric and therefore oceanic carbon dioxide (CO₂) concentrations has been unparalleled over the past 66 million years (Zeebe et al., 2016). Resultant changes in ocean pH and carbon chemistry (ocean acidification, OA) are likely to have major impacts on marine ecosystems (Doney et al., 2009) by changing species abundances, interactions and trophic dynamics, all of which depend ultimately on the CO₂ sensitivities of individual organisms (Harley et al., 2006; Fabry et al., 2008; Wootton et al., 2008). Laboratory experiments have played an important role in quantifying these CO₂ sensitivities, suggesting that they are greater in sessile, calcifying invertebrates than in active, non-calcifying vertebrates, and greater in early life stages than adults (Dupont et al., 2010; Kroeker et al., 2010; Wittmann and Pörtner, 2013). The latter has been particularly well documented for marine fish, where adults are largely tolerant of acute high-CO₂ levels far exceeding predicted OA conditions (Ishimatsu et al., 2008; Melzner et al., 2009). By contrast, fish early life-stages (embryos and early larvae) that are still developing effective acid-base regulation have exhibited reduced survival (Baumann et al., 2012; Stiasny et al., 2016), reduced growth (Chambers et al., 2014; Pimentel et al., 2014), defective development (Frommel et al., 2012; Pimentel et al., 2014), otolith over-calcification (Checkley et al., 2009; Bignami et al., 2013), and behavioral abnormalities in response high-CO₂ conditions in the laboratory (Munday et al., 2009b; Nilsson et al., 2012). Experiments showing no discernible CO₂ effects are also common (Franke and Clemmesen, 2011; Frommel et al., 2013; Hurst et al., 2013; Crespel et al., 2017; Lonthair et al., 2017). This complexity of empirical evidence remains challenging to reconcile (Cattano et al., 2018), but is consistent with the emerging consensus of species- and population-specific CO₂ sensitivities, particularly for fish adapted to high CO₂ and pH variability in their habitats (Vargas et al., 2017).
To date, experimental approaches have largely been guided by open-ocean predictions for administering CO$_2$ treatments (see Riebesell et al., 2010). It is now recognized, however, that many marine organisms experience considerable diel and seasonal pH/CO$_2$ fluctuations in their habitats (Maas et al., 2012; Pespeni et al., 2013; Hofmann et al., 2014; Vargas et al., 2017). Short-term pH/CO$_2$ variability can be attributed to ephemeral upwelling (Feely et al., 2008), river input (Salisbury et al., 2008), and metabolic processes that dominate CO$_2$ variability in coastal habitats (Wallace et al., 2014) and in oxygen minimum zones (Paulmier et al., 2011). The seasonal intensification of community respiration in highly productive coastal systems (e.g. saltmarshes and mangrove lagoons) can increase both average and extreme CO$_2$ levels to nearly double the open-ocean OA predictions for the next 300 years (Baumann et al., 2015). Given the thermal sensitivity of microbial respiration rates, metabolically driven acidification is generally most extreme during peak summer temperatures (Baumann and Smith, 2017). Hence, to better understand climate change effects on coastal species, experiments should implement CO$_2$ and temperature conditions that reflect the range of modern and predicted conditions of their source ecosystems, rather than relying on average global predictions.

While single-factor CO$_2$ experiments are a necessary initial step, it is now widely recognized that OA proceeds in concert with ocean warming and deoxygenation. Experiments are needed to address species sensitivities to multiple stressors of marine climate change (Pörtner, 2012; Bopp et al., 2013). Warming may be the primary driver of ecological disruption, as there is already evidence of shifting fish distributions and phenologies (Perry et al., 2005; Poloczanska et al., 2013), which likely reflect the need for ectotherms to maintain environments within their scope of physiological optima (Pörtner, 2002). The capacity of organisms to maintain performance at temperatures approaching or exceeding their thermal tolerance is a key metric in determining
climate sensitivity (Pörtner and Knust, 2007). Elevated environmental CO$_2$ may increase energetic costs associated with acid-base regulation (Heuer and Grosell, 2016) and could compromise the functional capacity of other vital processes (Pörtner et al., 2005) and therefore increase an organisms sensitivity to thermal extremes (Pörtner, 2012). Thus, CO$_2$ × temperature experiments are not only more realistic, they may also discover important stressor interactions that elude single-stressor approaches (Pörtner and Farrell, 2008).

The majority of studies evaluating CO$_2$ × temperature effects in fish have focused on stenothermal taxa from polar (Strobel et al., 2012; Enzor et al., 2013; Flynn et al., 2015; Leo et al., 2017; Davis et al., 2018) or tropical habitats (Munday et al., 2009a; Nowicki et al., 2012; Domenici et al., 2014; Bignami et al., 2016). These fish are presumably adapted to their relatively stable thermal environments and may thus show limited acclimation capacity to combined climate stressors (Peck, 2005; Tewksbury et al., 2008). By contrast, temperate species are often eurythermal, i.e., capable of acclimating to broad seasonal temperature fluctuations. However, they are still adapted to specific thermal regimes (Pörtner, 2002) and often show narrower thermal requirements during CO$_2$ sensitive early life stages (Pörtner and Peck, 2010; Pimentel et al., 2014).

Many fitness-relevant traits such as growth or survival are highly variable in nature during fish early life stages, thus producing variable outcomes even under most meticulously controlled experimental conditions (Gobler et al., 2018; Snyder et al., 2018). Variations in offspring due to parentage, food quality and quantity, or water sources can introduce additional variability, hence underscoring the risks of generalizing results from single experiments to population or species characteristic such as CO$_2$ or temperature sensitivity. More robust depictions of CO$_2$ and temperature sensitivity are likely to emerge if experiments are replicated and analyzed together, but this approach is still underutilized in studies of climate change effects on marine organisms.
Here we report on five factorial CO$_2$ \( \times \) temperature experiments conducted on offspring of wild Atlantic silversides, *Menidia menidia*, an ecologically important and abundant coastal forage fish with a broad distribution along the east coast of North America (Middaugh et al., 1987). Wild silverside offspring are amenable to experimental manipulations and have thus become a widely used model in OA experiments (Murray et al., 2014; DePasquale et al., 2015; Malvezzi et al., 2015; Lopes et al., 2016; Miller et al., 2016; Murray et al., 2017; Snyder et al., 2018). Over the course of three years, we repeatedly reared Atlantic silverside offspring at different factorial combinations of CO$_2$ and temperature to quantify the temperature-dependence of CO$_2$ effects in growth and survival. We hypothesized that negative responses to high-CO$_2$ levels would largely occur at the species lower and upper thermal limits, while predicting fewer or no CO$_2$ effects at optimal thermal conditions.

**Methods**

*Field sampling and experimental designs:* Collections of wild, spawning ripe Atlantic silverside were made during high tide 1-3 days prior to full or new moons following the species’ semi-lunar spawning periodicity during spring and early summer (Murray et al., 2014). Adults were caught with a 30 × 2 m beach seine from local salt marshes and transported live to our laboratory facilities. For the 2014 experiment (experiment 1), adults were collected from Poquot Beach (40° 56.85’ N, 73° 6.15’ W), and the experiment took place at Stony Brook University’s Flax Pond Marine Laboratory. During 2016 and 2017 (experiments 2 – 5), spawning adults were collected from Mumford Cove (41°19’25”N 72°01’07”W), and experiments were conducted in the Rankin Seawater Facility at University of Connecticut’s Avery Point campus. Ripe adults were held overnight at 20°C in aerated tanks at low densities with no food and strip-spawned the next day. Fertilization dates for each experiment are reported in Table 1.
Strip-spawning protocols maximized fertilization success, while enabling random distribution of embryos across replicates (Murray et al., 2014; Malvezzi et al., 2015). For each experiment, eggs from 12+ running-ripe females were gently mixed into shallow plastic dishes lined with 1-mm plastic window screening. Milt from each of 20+ males was collected and pooled into 500-ml glass beakers, mixed with seawater, stirred, then gently poured into spawning dishes and mixed with eggs for ~15 minutes. The number of spawners used for each experiment and their length measurements are reported in Table S1. In this species, fertilized embryos uncoil chorionic filaments, which readily attach to screening. Window screens were cut into smaller sections where embryos were counted under low magnification with high accuracy.

Experiments were initiated within 2 hours of fertilization when replicate rearing-containers (20-L cylindrical polyethylene buckets) received precisely 100 embryos. Rearing-containers were filled with clean seawater (filtered to 1 µm and UV sterilized). Optimal salinity (27-31) and light conditions (15 h light:9 h dark) for rearing *M. menidia* were maintained across experiments (Middaugh et al., 1987). The number of CO₂ × temperature treatments and replicates varied between experiments (see Table 1). Starting four days post-fertilization (dpf), each rearing-container was checked for hatched larvae. On the morning of first observed hatch, larvae were immediately provided with equal rations of powdered weaning diet (Otohime Marine Fish Diet, size A1, Reed Mariculture®) to stimulate feeding and *ad libitum* levels of newly hatched brine shrimp nauplii (*Artemia salina*, San Francisco strain, brineshrimpdirect.com). Larvae were fed daily *ad libitum* rations of newly hatched nauplii for the remainder of the experiment. To quantify survival to hatch, one day post-hatch larvae were counted by gently scooping small groups into replacement rearing-containers. For initial hatch standard length (SL, nearest 0.01 mm) measurements, random sub-samples (*N* = 10) from each replicate were preserved in 5%
formaldehyde/freshwater solution buffered with saturated sodium tetraborate. The timing of hatch
sub-samples varied slightly among experiments and temperatures (see Table 3). Rearing-
containers were siphoned of waste daily, and treatment water was partially exchanged with new
seawater every other day. Levels of ammonia waste were monitored daily (API® Saltwater
Ammonia Test Kit) to maintain uncritical levels below 0.25 ppm. All experiments were terminated
when larvae reached ~10 mm SL within temperature treatments (determined by visual estimates).
Using body size rather than set time intervals allowed comparing CO₂ effects on offspring during
the same developmental period (i.e., fertilization to ~10 mm SL) across temperature treatments.
Experiment durations ranged from 14 to 36 days (Table 3). At termination, all survivors were
counted and measured for SL (nearest 0.01 mm) via calibrated digital images (Image Pro Premier®
V9.0).

**CO₂ and temperature levels:** We applied a target CO₂ level of 400 µatm (~8.15 pH) for control
treatments, a level characteristic of the open ocean and of coastal systems at the onset of the
silverside spawning season (spawning typically begins early April and extends through July) (Fig.
1, Murray et al., 2014). The target level for high CO₂ was 2,200 µatm (~7.50 pH), a level that is
commonly experienced by silverside offspring in late spring and summer (Fig. 1), but also
represents the maximum prediction of average OA for the next 300 years (Caldeira and Wickett,
2005) and therefore a common benchmark in many OA studies (Murray et al., 2014; Malvezzi et
al., 2015; Murray et al., 2017). The target level for the extreme CO₂ treatment was 6,000 µatm
(~7.15 pH) during experiment 1, but was reduced to 4,200 µatm (~7.20 pH) for experiments 3 and
5. These represent extreme CO₂ conditions rarely reached in contemporary coastal systems, but
may become more common under future climate and eutrophication scenarios (Wallace et al.,
2014).
We administered four temperature treatments over the course of the five experiments; 17°C, 20°C, 24°C and 28°C. The first three temperatures represent local conditions found during the onset (late-April), peak (early-June), and end (July) of the silverside spawning season, respectively (Fig. 1). At the latitude of our source populations (~41°N), silverside spawning habitats rarely reach temperatures of 28°C, however, these conditions may become more common given a projected increase of 2°-3°C in global mean ocean temperature (Stocker et al., 2014). The optimal culturing temperature for *M. menidia* from northern latitudes is ~24°C; thus, 20°C and 24°C treatments were considered near-optimal temperatures, while 17°C and 28°C treatments represented sub-optimal thermal conditions (Middaugh et al., 1987).

**CO₂ × temperature manipulations and measurements:** All experiments followed established best practices and guidelines for seawater acidification in OA research (Riebesell et al., 2010). For 2 × 2 and 3 × 2 factorial designs (see Table 1 for overview of experiments and designs), replicate rearing-containers were placed into large temperature-controlled water baths. Elevated CO₂ levels were achieved via gas proportioners (ColeParmer®) mixing air with 100% CO₂ (bone dry grade) that was delivered continuously to the bottom of each replicate rearing-container via airstone. To counteract metabolic CO₂ accumulation, control CO₂ conditions were achieved by forcing compressed laboratory air through a series of CO₂-stripping units containing granular soda lime (AirGas®), a particle filter (1 µm), and then to each rearing-container via airstone. Target pH levels were monitored daily using a handheld pH probes (Orion Ross Ultra pH/ATC Triode with Orion Star A121 pH Portable Meter; Intellical PHC281 pH Electrode with Hach® HQ11D Handheld pH/ORP Meter) calibrated bi-weekly with National Institute of Standards and Technology (NIST) certified 2-point pH references. Continuous bubbling maintained dissolved oxygen (DO) saturation (>8 mg/L DO) in rearing vessels. Target treatment temperatures were controlled by
thermostats (Aqualogic®) which powered chillers (DeltaStar®) or glass submersible heaters to maintain water bath temperatures.

For 3 × 3 factorial experiments, we constructed an automated acidification system composed of nine discrete recirculation-units designed for larval fish rearing. Each recirculating-unit consists of a sump (90 L), a header tank (40 L) and a main tank (240 L) that holds up to five replicate rearing-containers (20 L) fitted with screened overflow holes (100 µm). In these units, seawater continuously circulates from the sump through a UV sterilizer into the header tank, where it is gravity fed to the bottom of each rearing-container, from which it overflows in the main tank and back into the sump. We designed a LabView (National Instruments®) based program to fully automate the control of seawater chemistry. The software interfaces with the recirculating-units via a data-acquisition module (NI cDAQ-9184, National Instruments®), which controls nine sampling-pumps (one per tank) and a series of gas and water solenoid valves, while receiving input from a central pH electrode (Hach pHD® digital electrode calibrated weekly using NIST 2-point pH references) and DO probe (Hach LDO® Model 2). The software sequentially assesses the pH conditions in each recirculating-unit (once per hour) by pumping water for ~7.5 min through the housing of the central pH probe, comparing measured pH levels to set-points and then adjusting levels by bubbling standardized amounts 100% CO₂ (bone dry grade, AirGas®) or CO₂-stripped air into the sump of each tank. The software also maintains DO saturation (>8 mg/l) by bubbling in CO₂-stripped air. LabView logs current pH, temperature, and DO conditions before cycling to the next unit. Temperatures were controlled by thermostats (Aqualogic®) that powered submersible heaters or in-line chillers (DeltaStar®).

Actual treatment CO₂ levels were determined based on measurements of pH, temperature, salinity, and total alkalinity (AT). Treatment tanks were sampled three times per experiment for
measurements of $A_T$ ($\mu$mol kg$^{-1}$). Seawater was siphoned and filtered (to 10 $\mu$m) into 300-ml borosilicate bottles. Salinity was measured at the time of sampling using a refractometer. Bottles were stored at 3°C and measured for $A_T$ within two weeks of sampling using an endpoint titration (Mettler Toledo® G20 Potentiometric Titrator). Methodological accuracy (within ±1%) of alkalinity titrations were verified and calibrated using Dr. Andrew Dickson’s (University of California San Diego, Scripps Institution of Oceanography, https://www.nodc.noaa.gov/ocads/oceans/Dickson_CRM/batches.html) certified reference material for $A_T$ in seawater. The partial pressure and fugacity of CO$_2$ ($p$CO$_2$, $f$CO$_2$; μatm) as well as dissolved inorganic carbon ($C_T$; μmol kg$^{-1}$) and carbonate ion concentration (CO$_3^{2-}$; μmol kg$^{-1}$) were calculated in CO2SYS (V2.1, http://cdiac.ornl.gov/ftp/co2sys) based on measured $A_T$, pH, temperature, and salinity using K1 and K2 constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and Dickson (1990) for KHSO$_4$. An overview of pH and carbonate chemistry measurements for each experiment is given in Table 2.

Response traits and statistical analysis: For all replicates in each experiment we quantified time (d) to first-hatch (day of fertilization to day of first-hatch), % embryo survival (fertilization to hatch), % larval survival (hatch to end of experiment), SL at hatch, and post-hatch growth rate ((mean final SL – mean hatch SL)/number days reared post-hatch). For experiment 1, only survival traits were quantified. Time to first-hatch was invariant between CO$_2$ levels and thus was not analyzed statistically. Proportional survival data were logit transformed [$=\log_{10}$(survival/(1-survival))] prior to analysis (Warton and Hui, 2011). Grubb’s test (Grubbs, 1969) was used to identify potential outlying replicates, resulting in the removal of three replicates throughout the dataset for low embryo survival (Grubb’s test, p < 0.05).
Statistical analyses were computed using SPSS (V20, IBM). As a first step, we used linear mixed effects models incorporating data from all experiments to test for significant effects ($\alpha < 0.05$) of CO$_2$, temperature, their interaction (fixed factors) and experiment (random factor) for each response trait:

Response trait = CO$_2$ + temperature + CO$_2$ × temperature + experiment + $\varepsilon$ (error).

Response trait data were checked for variance homogeneity and assumption of normality using Levene’s and Shapiro-Wilk tests ($\alpha < 0.05$), respectively. If linear mixed effects models identified traits with significant CO$_2$ or CO$_2$ × temperature effects, we used two-way analysis of variance (ANOVA) to test for significant effects of CO$_2$, temperature, and their interaction within each experiment:

Response trait = CO$_2$ + temperature + CO$_2$ × temperature + $\varepsilon$ (error).

This approach was implemented to characterize how CO$_2$ effects differed between experiments. If two-way ANOVAs detected significant ($\alpha < 0.05$) CO$_2$ or CO$_2$ × temperature interactive effects, we used one-way ANOVAs to test for significant CO$_2$ effects within temperature treatments. Where necessary, least-significant-difference (LSD) post-hoc tests were used for multiple comparisons. We conducted two- and one-way ANOVAs on experiment 1 separately because the extreme CO$_2$ level implemented there (~6,000 $\mu$atm) was higher than in experiments 3 and 5 (~4,200 $\mu$atm). ANOVA groups were checked for variance homogeneity and assumption of normality using Levene’s and Shapiro-Wilk tests ($\alpha < 0.05$), respectively.

Linear mixed effects models indicated that response traits were highly variable between experiments. Hence, we implemented an additional approach to better describe CO$_2$ effects across temperature treatments. We quantified the temperature-specific CO$_2$ effect sizes for each trait (T)
for each experiment by calculating the log-transformed CO₂ response ratio (lnRR) to high and extreme levels of CO₂ exposure. LnRRs evaluate the average proportional change in a trait relative to control treatments, with negative lnRRs indicating negative CO₂ effects. LnRRs have become a common metric for evaluating CO₂ effects in meta-analyses when comparing variable responses across studies (Hedges et al., 1999; Kroeker et al., 2010; Cattano et al., 2018). LnRRs were calculated as:

\[ \text{lnRR}(T) = \ln(T_{\text{high or extreme CO}_2}) - \ln(T_{\text{control CO}_2}). \]

Overall temperature-specific CO₂ responses were calculated as the mean lnRR(T) across experiments.

**Results**

*Embryo survival:* Among-replicate time to first hatch was 5, 6, 10, and 13 dpf at 28°, 24°, 20°, and 17°C, respectively. CO₂ level had no influence on time to hatch (Table 3). Across experiments, mean embryo survival ranged from 46-96% (mean 76%, Fig. 2A-D, Table 3). A linear mixed effect model found no influence of CO₂, temperature, or their interaction on embryo survival (Table 4). However, there was a significant effect of experiment \( (F_{4,118} = 33.581, p < 0.001) \), because in experiment 1, high CO₂ reduced embryo survival (two-way ANOVA, \( F_{2,28} = 18.965, p < 0.001 \)) by 11% at 17° (LSD, \( p = 0.003 \)) and 24°C (LSD, \( p = 0.027 \)) relative to control treatments. This effect was not observed in subsequent experiments with less extreme CO₂ treatments.

*Hatch length:* Across experiments mean hatch SL ranged from 4.54-5.62 mm, but within experiment variation was small (±0.4 mm) (Fig. 2F-I, Table 3). A linear mixed effects model indicated significant temperature \( (F_{3,90} = 19.518, \ p < 0.001) \), CO₂ × temperature \( (F_{6,90} = 3.021, p = 0.010) \), and experiment effects \( (F_{3,90} = 75.361, p < 0.001) \) (Table 4). The CO₂ × temperature interaction was driven by divergent CO₂ × temperature responses between experiments 3 and 5.
During experiment 3, a two-way ANOVA found hatch lengths were significantly affected by CO$_2$ ($F_{2,44} = 7.600, p = 0.002$), temperature ($F_{2,44} = 14.857, p < 0.001$), and their interaction ($F_{4,44} = 5.522, p = 0.001$). Hatch lengths were similar at 17°C, but exposure to extreme CO$_2$ significantly reduced offspring size at 20°C relative to the control treatment (one-way ANOVA, $F_{2,14} = 5.947, p = 0.016$). The CO$_2$-induced reduction in hatch length was largest at 24°C (one-way ANOVA, $F_{2,14} = 13.342, p = 0.001$), with exposure to high and extreme CO$_2$ reducing larval size by 3% (LSD, $p = 0.006$) and 5% (LSD, $p < 0.001$), respectively, compared to controls. During experiment 5, a two-way ANOVA found hatch lengths were significantly affected by CO$_2$ ($F_{2,28} = 5.222, p = 0.013$) and temperature ($F_{1,28} = 25.544, p < 0.001$). Contrary to experiment 3, there was no influence of CO$_2$ at 24°C. However, at 28°C, high and extreme CO$_2$ significantly increased hatch length (one-way ANOVA, $F_{2,14} = 5.942, p = 0.016$) by 3% (LSD, $p = 0.014$) and 4% (LSD, $p = 0.010$), respectively, compared to the control treatment.

**Larval survival:** Larval survival was highly variable both within and between experiments, with treatment means ranging from 9-82% (mean 42%, Fig. 2J-N, Table 3). Across all experiments, a linear mixed effects model found significant effects of temperature ($F_{3,121} = 9.918, p < 0.001$) and experiment ($F_{4,121} = 12.798, p < 0.001$), but no influence of CO$_2$ or CO$_2 \times$ temperature (Table 4). Within temperature mean (±SD) larval survival was highest at 20°C (78±14%), with reduced survival observed at 24° (46±25%), 17° (38±22%), and 28°C (19±19%).

**Larval growth rate:** Growth rates ranged from 0.16-0.48 mm d$^{-1}$ (mean 0.33 mm d$^{-1}$, Fig. 2O-R, Table 3). A linear mixed effects model identified significant temperature ($F_{3,92} = 80.189, p < 0.001$) and experiment ($F_{3,92} = 3.838, p = 0.012$) effects, but no influence of CO$_2$ or CO$_2 \times$ temperature (Table 4). Across experiments, growth rates increased similarly across CO$_2$ levels with increasing
temperatures. Within-temperature means (±SD) ranged from 0.19±0.03, 0.33±0.02, 0.36±0.05, and 0.42±0.06 mm d⁻¹ at 17°, 20°, 24°, and 28°C, respectively.

**Overall CO₂ effect size (LnRR):** The overall CO₂ effect on embryo survival was small in response to high CO₂ conditions (within ±0.06) and similar across temperature treatments (Fig 3A). For offspring exposed to extreme CO₂, all responses were negative (-0.04 to -0.30), but there was no apparent trend with temperature (Fig. 3B). For hatch size, overall effects were small both at high and extreme CO₂ treatments (±0.03), again with no apparent temperature dependency (Fig. 3C-D). The overall effect of high CO₂ conditions on larval survival was highly variable (-0.64 to 0.55) and overall neutral across temperatures (Fig. 3E). Interestingly, the effect of extreme CO₂ conditions on larval survival was positive at 17°C (0.42), but became increasingly negative with increasing temperatures (-0.18 at 24° and -0.44 at 28°C, Fig. 3F). Average CO₂ effects for growth rate were small (within ± 0.10), but exhibited a dome-shaped response across temperatures at both CO₂ levels, with negative growth responses at sub-optimal rearing temperatures (Fig. 3G-H).

**Discussion**

We conducted five factorial experiments to evaluate the sensitivity of *M. menidia* early life traits to high (2,000-2,800 μatm) and extreme (4,000-6,200 μatm) CO₂ conditions across four temperatures (17°, 20°, 24°, and 28°C) that encompassed contemporary and potential future conditions in nearshore silverside spawning habitats. The experiments showed few significant CO₂ effects on response traits. Significant reductions in embryo survival occurred at 17° and 24°C in a single experiment and at the most extreme CO₂ treatment (≈6,000 μatm). Effects on hatch length showed evidence for CO₂ × temperature interactions, given that elevated CO₂ reduced hatch length at 24°C during one experiment, while increasing hatch length at 28°C during another. There were no significant effects of CO₂ on larval survival or growth rate. Together, these findings suggest
that *M. menidia* offspring can tolerate high to extreme CO$_2$ levels across most of the species’ thermal range.

The apparent CO$_2$ resilience of *M. menidia* offspring may reflect the pH/CO$_2$ variability typical of their nursery habitat. Atlantic silversides spawn in shallow subtropical to temperate estuaries (Conover and Ross, 1982) where seasonal acidification elicits increasingly large diel pH fluctuations while progressively reducing daily mean and minimum pH levels (Baumann et al., 2015). Such patterns of seasonal pH/CO$_2$ variation appear to be common in shallow nearshore habitats (Baumann and Smith, 2017). As a batch-spawning fish, silversides spawn fortnightly from late April to early July (at ~41°N) which coincides with the period of most rapid habitat acidification (Murray et al., 2014) Thus, a single female will deposit subsequent batches of embryos into a progressively more pH variable and acidic environment. In a previous study, we found offspring CO$_2$ tolerance closely tracked temporal trends in habitat acidification (Murray et al., 2014). Transgenerational plasticity is a possible explanation for this rapid shift, by which adults experiencing a progressively more acidic environment augment offspring phenotypes to better match current environmental conditions (Salinas et al., 2013). An additional source of CO$_2$ tolerance may arise from local adaptation. Despite being an annual fish with high population connectivity, Atlantic silversides exhibit local adaptation for traits involved in growth and environmental sex determination (Conover, 1998), which are likely maintained through the continuous selection of locally suited genotypes (Clarke et al., 2010). We have previously demonstrated that early life survival under high CO$_2$ conditions does have an additive genetic component in this species and related offspring respond more similarly to high CO$_2$ conditions, suggesting high CO$_2$ tolerance is a heritable trait (Malvezzi et al., 2015). Local adaptation to acidified habitats through the selection and maintenance of CO$_2$-tolerant traits has been
demonstrated in other taxa (Pespeni et al., 2013). For Atlantic silversides that spawn in habitats prone to acidification, adaptations that enable high-CO$_2$ tolerance are likely well represented in wild populations and would explain the observed CO$_2$ tolerance. Importantly, we found that exposure to ~6,000 µatm pCO$_2$ did reduce embryo survival during experiment 1, while offspring were largely tolerant to ~4,200 µatm in subsequent experiments. While 6,000 µatm is an extreme, likely unrealistic CO$_2$ level for silverside spawning habitats, it may represent a tolerance threshold for *M. menidia*. Identifying such thresholds are necessary to accurately assign an organisms’ sensitivity to future climate change (Pörtner, 2008).

Maternal provisioning of eggs through modifications of energy content or fatty acid composition may further influence offspring CO$_2$ sensitivity (Snyder et al., 2018). Such differences may have contributed to CO$_2$ effects on hatch length documented during experiments 3 and 5. In Atlantic cod, CO$_2$ induced reductions in hatch size were not accompanied by increased utilization of yolk reserves during embryogenesis (Dahlke et al., 2017). The authors suggest that yolk utilization was already maximized, and increased demands on acid/base regulation resulted in a shift of endogenous energy use away from somatic growth. Conceivably, CO$_2$ reductions in hatch size during experiment 3 were the result of a similar mechanism. Conversely, differences in maternal provisioning of embryos from experiment 5 may have stimulated yolk utilization under elevated CO$_2$, leading to increased embryonic growth and hatch size (Chambers et al., 2014). Fish embryos passively experience their environment with fixed energy reserves (Rombough, 2011) but are likely most sensitive to elevated CO$_2$ (Baumann et al., 2012). Further investigations are needed into how CO$_2$ × temperature combinations influence embryo energetics.

Atlantic silverside offspring have been shown to be tolerant to combined climate stressors which contrasts with the growing evidence for compounding effects of near future OA and warming in
the early life stages of other fish species. For example, combined treatments synergistically decreased embryo survival in Antarctic dragon fish (*Gymnodraco acuticeps*) (Flynn et al., 2015) and compromised temperature acclimation and aerobic performance in emerald rockcod (*Trematomus bencachii*) (Davis et al., 2018). As extreme stenotherms, polar species appear particularly vulnerable to combined climate effects (Somero, 2010), but eurythermal temperate species have demonstrated similar sensitivities. For example, exposure to acidification and warming reduced hatch size and larval survival in the Senegalese sole (*Solea senegalensis*) (Pimentel et al., 2014) and Atlantic cod (*Gadus morhua*) (Dahlke et al., 2017). In the congeneric *M. beryllina*, a large reduction in survival was found when offspring were simultaneously exposed to high-CO$_2$ and 29°C (Gobler et al., 2018). The CO$_2$ × temperature tolerance demonstrated by *M. menidia* offspring is likely a manifestation of conditions widely experienced by wild silverside early life stages. The acidification of their near shore nursery habitat is largely driven by seasonal changes in community respiration that generally peak with seasonally maximum water temperatures (Baumann and Smith, 2017). Thus, simultaneous occurrence of potentially stressful temperature and CO$_2$ levels are a regular feature of *M. menidia* spawning habitat. Furthermore, because seasonal habitat changes are of the same direction and similar magnitude to climate projections, existing phenotypic or genetic variation already present in silverside populations may confer some degree of tolerance to future marine climate change (Reusch, 2014).

While *M. menidia* early life stages appear resistant to elevated CO$_2$ across a broad thermal regime, the addition of other stressors could potentially be detrimental. For example, temperature-dependent metabolic processes that drive coastal acidification simultaneously consume oxygen; hence, warming, acidification, and hypoxia co-occur in *M. menidia* nursery habitats (Melzner et al., 2012; Baumann and Smith, 2017). Given their co-occurrence in nature, physiological responses
to elevated CO$_2$ and low DO are likely connected. Intermediate CO$_2$ exposure can elicit important adaptive responses which may mediate sublethal effects of low DO (Melzner et al., 2012), yet more extreme exposures may act synergistically to elevate stressor sensitivity (Gobler and Baumann, 2016). Thus, factorial CO$_2$ × DO × temperature experiments would be insightful for more robust characterizations of coastal climate effects on fish early life stages.

Whole lifecycle effects of elevated CO$_2$ exposure remain critically understudied in fish (Cattano et al., 2018). While acclimation to chronic hypercapnia likely has small metabolic costs (Esbaugh et al., 2016), over longer timescales tradeoffs associated with increased acid/base regulation could compromise other physiological processes (Heuer and Grosell, 2016). In a previous study, we documented small but significant size reductions in *M. menidia* reared under ~2,200 µatm CO$_2$ and 17°C for approximately a third of their lifespan (Murray et al., 2017). Importantly, differences in length were only detected after two months of continuous high-CO$_2$ exposure. In the present study, CO$_2$ effect sizes calculated for growth rates displayed dome-shaped response curves, with more negative responses at sub-optimal rearing temperatures. For offspring reared under 17°C and high CO$_2$, the average growth effect size was -0.08 (i.e., -8%), a response of similar magnitude to previously documented growth reductions under the same conditions after four months (Murray et al., 2017). Importantly, that study used large sample populations (>2,000 individuals) providing the necessary power to statistically confirm a CO$_2$ effect. Arguably, many early-life experiments with smaller sample sizes lack the power to robustly detect small effects (Baumann et al., 2018). Thus, it is possible that small or undetectable CO$_2$ reductions in early-life growth accrue and become detectable during long-term exposures. Even minor changes to early life development may have important carry-over effects to later life stages and ultimately impact fitness (Pechenik, 2006). As an annual fish, juvenile growth during summer is critically important for *M. menidia*, as
larger individuals have higher overwintering survival (Schultz et al., 1998). How warming temperatures may interact with CO₂ over longer time-scales is presently unknown and represents a serious gap in our understanding of how combined climate stressors will impact fish (Kroeker et al., 2013).

Across experiments, CO₂ responses were highly complex, consistent with previous OA studies on silverside offspring (Murray et al., 2014; Gobler et al., 2018; Snyder et al., 2018). Experiments produced functionally different outcomes within equivalent treatment conditions despite meticulously controlled experimental conditions. For all traits but growth rate, inter-experiment variation was more substantial than variability driven by CO₂ or temperature level. A portion of this variability could be elicited by small differences in food quantity or quality, water source, or realized CO₂ levels, but parentage likely constitutes the largest source of variation in offspring, mediated through genetic or phenotypic inheritance and maternal provisioning. A limitation of the present study was the use of embryos from a single group of spawning adults for each experiment. This prohibited the incorporation of parentage into the statistical analysis, thereby potentially underestimating an important source of variation as previously reported in M. menidia (Snyder et al., 2018). Plastic offspring responses to CO₂ × temperatures conditions are likely adaptive in species like M. menidia that spawn in highly dynamic systems (Green, 2008). Thus, this inherent plasticity precludes broad generalizations based on single, short-term experiments, but understanding such plasticity is fundamental in assigning potential risks to ongoing climate change (Hoffmann and Sgrò, 2011; Reusch, 2014). Highly variable CO₂ × temperature responses are common across taxonomic groups (Kroeker et al., 2013; Lefevre, 2016), thus experimental replication and inter-experiment statistical comparisons are necessary for robust evaluations of climate sensitivities in marine organisms.
In summary, we analyzed five CO$_2$ × temperature experiments together to robustly characterize CO$_2$ × temperature responses of important fitness related traits in *M. menidia* offspring. While individual experiments demonstrated some negative CO$_2$ effects, overall responses were largely neutral. Importantly, we found sub-optimal rearing temperatures did not increase sensitivity to even extreme CO$_2$ levels. Repeated experimentation documented substantial inter- and intra-experiment variability, highlighting the need for experimental replication to accurately describe inherently variable response traits.

**Acknowledgements**

We are grateful to J. Snyder, M. Hughes, E. Karamavros, J. Pringle, and C. Woods for assistance in the lab. This work was funded by NSF OCE #1536165.
References


Dickson, A. G. 1990. Standard potential of the reaction: AgCl (s) + 12H2 (g) = Ag (s) + HCl (aq), and and the standard acidity constant of the ion HSO$_4^-$ in synthetic sea water from 273.15 to 318.15 K. The Journal of Chemical Thermodynamics, 22: 113-127.


Table 1: Summary of five CO$_2 \times$ temperature experiments on *M. menidia* offspring. Treatment levels for $p$CO$_2$ (µatm) and temperature (°C) represent target conditions, actual measured values are presented in Table 2. Trait are abbreviated as embryo survival (ES), hatch length (HL), larval survival (LS), and growth rate (GR).

<table>
<thead>
<tr>
<th>Exp num</th>
<th>Fertilization date</th>
<th>Target treatment levels</th>
<th>Number of replicates</th>
<th>Measured traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5/5/2014</td>
<td>400,2200,6000</td>
<td>17,24</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>4/22/2016</td>
<td>400,2200</td>
<td>17,24</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>5/19/2016</td>
<td>400,2200,4000</td>
<td>17,20,24</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>4/28/2017</td>
<td>400,2200</td>
<td>24,28</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>5/26/2017</td>
<td>400,2200,4000</td>
<td>24,28</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 2: Mean (±SD) pH and temperature (°C) from daily measurements. Mean (±SD) salinity, total alkalinity (AT; μmol kg⁻¹), dissolved inorganic carbon (CT; μmol kg⁻¹), partial pressure of CO₂ (pCO₂; μatm), fugacity of CO₂ (fCO₂; μatm), and carbonate ion concentration (CO₃²⁻; μmol kg⁻¹) measured from replicated seawater samples of each treatment. Salinity was measured via refractometer and AT from endpoint titrations. CT and pCO₂, fCO₂, and CO₃²⁻ were calculated in CO2SYS.

<table>
<thead>
<tr>
<th>Exp num</th>
<th>Target temp</th>
<th>Measured temp</th>
<th>Target pCO₂</th>
<th>Measured pH</th>
<th>Salinity</th>
<th>AT</th>
<th>CΤ</th>
<th>pCO₂</th>
<th>fCO₂</th>
<th>CO₃²⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
<td>17.5±0.1</td>
<td>400</td>
<td>8.24±0.02</td>
<td>26</td>
<td>251±17</td>
<td>2302±12</td>
<td>433±29</td>
<td>431±29</td>
<td>168±8</td>
</tr>
<tr>
<td></td>
<td>17.5±0.1</td>
<td>2200</td>
<td>7.49±0.05</td>
<td>26</td>
<td>2539±22</td>
<td>2581±5</td>
<td>2564±94</td>
<td>2556±94</td>
<td>38±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.5±0.1</td>
<td>6000</td>
<td>7.14±0.05</td>
<td>26</td>
<td>2492±33</td>
<td>2680±11</td>
<td>5753±277</td>
<td>6733±276</td>
<td>17±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.0±0.2</td>
<td>400</td>
<td>8.20±0.06</td>
<td>26</td>
<td>2501±7</td>
<td>2258±11</td>
<td>474±27</td>
<td>472±27</td>
<td>191±7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.0±0.2</td>
<td>2200</td>
<td>7.47±0.05</td>
<td>26</td>
<td>2474±81</td>
<td>2504±8</td>
<td>2881±172</td>
<td>2872±172</td>
<td>42±2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.0±0.2</td>
<td>6000</td>
<td>7.14±0.05</td>
<td>26</td>
<td>2472±49</td>
<td>2634±13</td>
<td>6195±378</td>
<td>6174±378</td>
<td>20±1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>16.9±0.3</td>
<td>400</td>
<td>8.17±0.12</td>
<td>30</td>
<td>2038±17</td>
<td>1851±8</td>
<td>368±18</td>
<td>367±18</td>
<td>135±6</td>
</tr>
<tr>
<td></td>
<td>16.9±0.3</td>
<td>2200</td>
<td>7.49±0.13</td>
<td>30</td>
<td>2031±12</td>
<td>2058±21</td>
<td>2037±188</td>
<td>2030±188</td>
<td>32±2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.0±0.2</td>
<td>400</td>
<td>8.13±0.09</td>
<td>30</td>
<td>204±11</td>
<td>1838±16</td>
<td>427±29</td>
<td>426±29</td>
<td>150±7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.0±0.2</td>
<td>2200</td>
<td>7.49±0.12</td>
<td>30</td>
<td>2041±11</td>
<td>2048±7</td>
<td>2190±277</td>
<td>2183±276</td>
<td>54±5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>17.4±0.2</td>
<td>400</td>
<td>8.22±0.01</td>
<td>31</td>
<td>2054±8</td>
<td>1838±26</td>
<td>322±12</td>
<td>321±12</td>
<td>153±2</td>
</tr>
<tr>
<td></td>
<td>17.6±0.3</td>
<td>2200</td>
<td>7.51±0.01</td>
<td>31</td>
<td>2047±20</td>
<td>2066±21</td>
<td>1952±39</td>
<td>1945±39</td>
<td>35±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.4±0.2</td>
<td>4200</td>
<td>7.20±0.02</td>
<td>31</td>
<td>2053±24</td>
<td>2174±16</td>
<td>4056±204</td>
<td>4042±203</td>
<td>18±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>19.7±0.2</td>
<td>400</td>
<td>8.20±0.02</td>
<td>31</td>
<td>2048±29</td>
<td>1833±3</td>
<td>345±15</td>
<td>345±15</td>
<td>160±6</td>
</tr>
<tr>
<td></td>
<td>19.6±0.3</td>
<td>2200</td>
<td>7.51±0.03</td>
<td>31</td>
<td>2031±14</td>
<td>2039±10</td>
<td>1964±109</td>
<td>1957±108</td>
<td>38±2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.7±0.2</td>
<td>4200</td>
<td>7.21±0.02</td>
<td>31</td>
<td>2058±6</td>
<td>2153±37</td>
<td>4066±227</td>
<td>4063±226</td>
<td>20±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.0±0.2</td>
<td>400</td>
<td>8.22±0.02</td>
<td>31</td>
<td>2044±9</td>
<td>1798±8</td>
<td>331±14</td>
<td>330±14</td>
<td>185±5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.0±0.2</td>
<td>2200</td>
<td>7.49±0.02</td>
<td>31</td>
<td>2048±20</td>
<td>2050±25</td>
<td>2157±92</td>
<td>2151±92</td>
<td>42±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.0±0.2</td>
<td>4200</td>
<td>7.20±0.02</td>
<td>31</td>
<td>2059±1</td>
<td>2140±8</td>
<td>4339±169</td>
<td>4325±169</td>
<td>22±1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>23.6±0.3</td>
<td>400</td>
<td>8.19±0.03</td>
<td>31</td>
<td>209±63</td>
<td>1842±64</td>
<td>368±38</td>
<td>367±38</td>
<td>180±10</td>
</tr>
<tr>
<td></td>
<td>23.7±0.3</td>
<td>2200</td>
<td>7.51±0.03</td>
<td>31</td>
<td>2124±51</td>
<td>2122±44</td>
<td>2155±83</td>
<td>2148±82</td>
<td>45±4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.1±0.2</td>
<td>400</td>
<td>8.22±0.03</td>
<td>32</td>
<td>2164±88</td>
<td>1860±85</td>
<td>356±35</td>
<td>355±34</td>
<td>216±11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27.9±0.4</td>
<td>2200</td>
<td>7.52±0.03</td>
<td>32</td>
<td>2164±117</td>
<td>2146±113</td>
<td>2217±134</td>
<td>2210±133</td>
<td>54±6</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>24.3±0.4</td>
<td>400</td>
<td>8.19±0.02</td>
<td>32</td>
<td>2137±3</td>
<td>1897±13</td>
<td>389±23</td>
<td>388±23</td>
<td>175±8</td>
</tr>
<tr>
<td></td>
<td>24.1±0.2</td>
<td>2200</td>
<td>7.50±0.04</td>
<td>32</td>
<td>2151±14</td>
<td>2156±27</td>
<td>2265±228</td>
<td>2258±227</td>
<td>43±4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.2±0.3</td>
<td>4200</td>
<td>7.21±0.02</td>
<td>32</td>
<td>2130±27</td>
<td>2230±25</td>
<td>4432±180</td>
<td>4418±179</td>
<td>23±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.2±0.2</td>
<td>400</td>
<td>8.23±0.02</td>
<td>32</td>
<td>2157±24</td>
<td>1857±29</td>
<td>350±19</td>
<td>348±19</td>
<td>215±4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.1±0.2</td>
<td>2200</td>
<td>7.48±0.02</td>
<td>32</td>
<td>2176±50</td>
<td>2172±48</td>
<td>2439±84</td>
<td>2431±83</td>
<td>49±2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.2±0.3</td>
<td>4200</td>
<td>7.20±0.03</td>
<td>32</td>
<td>2155±20</td>
<td>2244±18</td>
<td>4720±217</td>
<td>4714±204</td>
<td>26±1</td>
<td></td>
</tr>
</tbody>
</table>
**Table 3**: Summary of all measured *M. menidia* response traits across five experiments; embryo survival (%), hatch length (mm), larval survival (%), and larval growth rate (mm d\(^{-1}\)) represented as treatments means ± SD. CO\(_2\) levels are shown as control (C), high (H), and extreme (E) (see Table 2 for values). Hatch (rep) and larval (rep) show the number of replicates used for each treatment’s average hatch and larval responses. Sample times are given as days post fertilization (dpf).

<table>
<thead>
<tr>
<th>Exp num</th>
<th>Temp (°C)</th>
<th>Treatment CO(_2)</th>
<th>Days to first hatch</th>
<th>Age at hatch sample (dpf)</th>
<th>Embryo survival (%)</th>
<th>Hatch length (mm)</th>
<th>Age at larval sample (dpf)</th>
<th>Larval survival (%)</th>
<th>Growth rate (mm d(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>C</td>
<td>13</td>
<td>14</td>
<td>68 ± 4</td>
<td>26</td>
<td>34 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>13</td>
<td>14</td>
<td>74 ± 3</td>
<td>26</td>
<td>50 ± 22</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>13</td>
<td>14</td>
<td>56 ± 6</td>
<td>26</td>
<td>43 ± 28</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>6</td>
<td>7</td>
<td>65 ± 4</td>
<td>16</td>
<td>44 ± 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>6</td>
<td>7</td>
<td>65 ± 3</td>
<td>16</td>
<td>53 ± 13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>6</td>
<td>7</td>
<td>56 ± 8</td>
<td>16</td>
<td>37 ± 28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>C</td>
<td>13</td>
<td>15</td>
<td>92 ± 3</td>
<td>5.32 ± 0.05</td>
<td>30</td>
<td>21 ± 8</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>13</td>
<td>15</td>
<td>87 ± 11</td>
<td>5.29 ± 0.05</td>
<td>30</td>
<td>11 ± 7</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>6</td>
<td>7</td>
<td>88 ± 7</td>
<td>5.30 ± 0.14</td>
<td>16</td>
<td>32 ± 33</td>
<td>0.41 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>6</td>
<td>7</td>
<td>76 ± 6</td>
<td>5.35 ± 0.06</td>
<td>16</td>
<td>26 ± 7</td>
<td>0.40 ± 0.05</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>C</td>
<td>13</td>
<td>15</td>
<td>93 ± 5</td>
<td>5.37 ± 0.05</td>
<td>36</td>
<td>32 ± 8</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>13</td>
<td>15</td>
<td>95 ± 5</td>
<td>5.42 ± 0.12</td>
<td>36</td>
<td>56 ± 21</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>13</td>
<td>15</td>
<td>89 ± 6</td>
<td>5.42 ± 0.11</td>
<td>36</td>
<td>59 ± 14</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>10</td>
<td>11</td>
<td>96 ± 5</td>
<td>5.55 ± 0.11</td>
<td>25</td>
<td>82 ± 10</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>10</td>
<td>11</td>
<td>95 ± 5</td>
<td>5.62 ± 0.09</td>
<td>25</td>
<td>77 ± 14</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>10</td>
<td>11</td>
<td>94 ± 7</td>
<td>5.42 ± 0.08</td>
<td>25</td>
<td>75 ± 22</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>6</td>
<td>7</td>
<td>95 ± 5</td>
<td>5.51 ± 0.09</td>
<td>16</td>
<td>72 ± 8</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>6</td>
<td>7</td>
<td>95 ± 6</td>
<td>5.32 ± 0.05</td>
<td>16</td>
<td>74 ± 9</td>
<td>0.35 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>6</td>
<td>7</td>
<td>92 ± 9</td>
<td>5.22 ± 0.11</td>
<td>16</td>
<td>69 ± 14</td>
<td>0.37 ± 0.05</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>C</td>
<td>6</td>
<td>6</td>
<td>62 ± 9</td>
<td>4.98 ± 0.07</td>
<td>16</td>
<td>33 ± 10</td>
<td>0.33 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>6</td>
<td>6</td>
<td>51 ± 7</td>
<td>4.98 ± 0.10</td>
<td>16</td>
<td>36 ± 32</td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>5</td>
<td>5</td>
<td>46 ± 5</td>
<td>4.76 ± 0.04</td>
<td>14</td>
<td>31 ± 35</td>
<td>0.48 ± 0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>5</td>
<td>5</td>
<td>49 ± 3</td>
<td>4.62 ± 0.09</td>
<td>14</td>
<td>40 ± 27</td>
<td>0.44 ± 0.08</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>C</td>
<td>6</td>
<td>6</td>
<td>74 ± 13</td>
<td>4.78 ± 0.07</td>
<td>16</td>
<td>41 ± 27</td>
<td>0.33 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>6</td>
<td>6</td>
<td>83 ± 12</td>
<td>4.90 ± 0.16</td>
<td>16</td>
<td>37 ± 20</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>6</td>
<td>6</td>
<td>55 ± 3</td>
<td>4.83 ± 0.10</td>
<td>16</td>
<td>29 ± 28</td>
<td>0.33 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>5</td>
<td>5</td>
<td>80 ± 13</td>
<td>4.54 ± 0.10</td>
<td>14</td>
<td>14 ± 7</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>5</td>
<td>5</td>
<td>67 ± 9</td>
<td>4.69 ± 0.10</td>
<td>14</td>
<td>14 ± 9</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>5</td>
<td>5</td>
<td>72 ± 13</td>
<td>4.70 ± 0.05</td>
<td>14</td>
<td>9 ± 6</td>
<td>0.38 ± 0.08</td>
</tr>
</tbody>
</table>
Table 4: Summary statistics for linear mixed models testing the effects of CO\textsubscript{2}, temperature and their interaction (fixed factors) and experiment (random factor) on four response traits; embryo survival (ES), hatch length (HL), larval survival (LS), and growth rate (GR) of \textit{M. menidia} offspring. Significant ($\alpha < 0.05$) factors are denoted by \textit{p}-values in bold.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Factor</th>
<th>$F$</th>
<th>df</th>
<th>\textit{p}</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES</td>
<td>CO\textsubscript{2}</td>
<td>2.992</td>
<td>2</td>
<td>0.058</td>
</tr>
<tr>
<td></td>
<td>Temp</td>
<td>1.140</td>
<td>3</td>
<td>0.336</td>
</tr>
<tr>
<td></td>
<td>CO\textsubscript{2}×Temp</td>
<td>0.677</td>
<td>6</td>
<td>0.669</td>
</tr>
<tr>
<td></td>
<td>Experiment</td>
<td>33.581</td>
<td>4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HL</td>
<td>CO\textsubscript{2}</td>
<td>1.895</td>
<td>2</td>
<td>0.156</td>
</tr>
<tr>
<td></td>
<td>Temp</td>
<td>19.518</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>CO\textsubscript{2}×Temp</td>
<td>3.021</td>
<td>6</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>Experiment</td>
<td>75.361</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LS</td>
<td>CO\textsubscript{2}</td>
<td>0.296</td>
<td>2</td>
<td>0.756</td>
</tr>
<tr>
<td></td>
<td>Temp</td>
<td>9.429</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>CO\textsubscript{2}×Temp</td>
<td>0.759</td>
<td>6</td>
<td>0.614</td>
</tr>
<tr>
<td></td>
<td>Experiment</td>
<td>12.385</td>
<td>4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GR</td>
<td>CO\textsubscript{2}</td>
<td>0.457</td>
<td>2</td>
<td>0.595</td>
</tr>
<tr>
<td></td>
<td>Temp</td>
<td>77.964</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>CO\textsubscript{2}×Temp</td>
<td>0.515</td>
<td>6</td>
<td>0.838</td>
</tr>
<tr>
<td></td>
<td>Experiment</td>
<td>3.330</td>
<td>3</td>
<td>0.012</td>
</tr>
</tbody>
</table>
**Figure 1:** Average mean (+/− minimum/maximum) monthly temperature and pH conditions during the spawning and growing season of Atlantic silversides in (A) Flax Pond, Long Island, New York and (B) Mumford Cove, Connecticut. The sites provided wild spawners for experiment 1 (A) and experiments 2-5 (B). Long-term averages were derived from monitoring data collected in 15 min intervals by (A) USGS station #01304057 between 2008 and 2018 and (B) the Baumann lab in Mumford Cove between 2015-2018.
**Figure 2:** *M. menidia.* Offspring responses to control (blue), high (red), and extreme (green) CO$_2$ conditions at four temperatures across five CO$_2 \times$ temperature factorial experiments. Traits include embryo survival (A-E), hatch length (F-I), larval survival (J-M) and larval growth rate (O-R). Individual replicates are represented by small faded circles. Treatment means (±SD) are depicted by large, bold circles and connected by dotted lines. Note: different scales used for hatch length measurements due to differences in sample timing; panels F and G use 1dph length Y axis (left) while panels H and I use hatch length Y axis (right).
**Figure 3:** *M. menidia.* CO₂ effect sizes using log-transformed response ratios (lnRR) of high (light grey) and extreme (dark grey) CO₂ exposure across four rearing temperatures. Response traits include embryo survival (A-B), hatch length (C-D), larval survival (E-F), and growth rate (G-H). Circles represent lnRRs of each experiment, while black lines represent lnRRs averaged across experiments at each rearing temperature. Negative (positive) values indicate a trait decrease (increase) at elevated CO₂ levels compared to control CO₂ conditions.
Appendix for Chapter 2: You Better Repeat It: Complex CO$_2$ × Temperature Effects in Atlantic Silverside Offspring Revealed by Serial Experimentation

Tables

Table S1: *M. menidia*. Collection and length data of adult silversides used to fertilize embryos for five CO$_2$ × temperature experiments. Adult lengths (cm) are shown as total lengths (TL, mean ± SD)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Collection date</th>
<th>Collection site</th>
<th># Female spawners</th>
<th>Female TL (cm)</th>
<th># Male spawners</th>
<th>Male TL (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5/4/2014</td>
<td>Setauket, NY</td>
<td>29</td>
<td>9.0±1.5</td>
<td>24</td>
<td>8.0±1.0</td>
</tr>
<tr>
<td>2</td>
<td>4/20/2016</td>
<td>Mumford Cove, CT</td>
<td>12</td>
<td>10.5±1.5</td>
<td>33</td>
<td>8.0±1.0</td>
</tr>
<tr>
<td>3</td>
<td>5/18/2016</td>
<td>Mumford Cove, CT</td>
<td>32</td>
<td>10.5±1.5</td>
<td>40</td>
<td>8.5±1.0</td>
</tr>
<tr>
<td>4</td>
<td>4/27/2017</td>
<td>Mumford Cove, CT</td>
<td>37</td>
<td>7.0±1.0</td>
<td>48</td>
<td>7.5±1.0</td>
</tr>
<tr>
<td>5</td>
<td>5/25/2017</td>
<td>Mumford Cove, CT</td>
<td>24</td>
<td>9.0±1.0</td>
<td>26</td>
<td>8.5±1.0</td>
</tr>
</tbody>
</table>
Chapter 3

High Sensitivity of the Northern Sand Lance (*Ammodytes dubius*) to Ocean Acidification and Warming

Abstract

Fish early life stages are potentially vulnerable to the combined effects of ocean acidification (OA) and warming, but existing empirical evidence shows considerable variability in responses among taxa and even populations. In part, this variation may reflect adaptations to existing $p\text{CO}_2$ fluctuations that differ among marine habitats. Furthermore, CO$_2$ tolerance may be a function of offspring developmental rates; species with rapid growth may require the development of robust acid-base regulatory capacity during early-life, whereas slow developing species may not. Over two years, we conducted several factorial $p\text{CO}_2 \times$ temperature exposure experiments on offspring of Northern sand lance *Ammodytes dubius*, an ecologically important forage fish that spawns on the northwest Atlantic shelf in early winter, when embryos develop slowly under relatively low and stable $p\text{CO}_2$ conditions. Wild, spawning-ripe adults were collected from Stellwagen Bank National Marine Sanctuary, and fertilized embryos were reared at three $p\text{CO}_2$ conditions (400, 1,000, and 2,100 µatm) crossed with three temperatures (5°, 7°, and 10°C). Across trials, exposure to future $p\text{CO}_2$ conditions consistently resulted in severe reductions in embryo survival. Sensitivity to elevated $p\text{CO}_2$ was highest at 10°C, resulting in up to 20-fold differences in embryo survival between control and predicted end-of-century $p\text{CO}_2$ conditions. Moreover, elevated $p\text{CO}_2$ conditions delayed hatching, reduced remaining endogenous energy reserves at hatch, and in combination with higher temperatures reduced embryonic growth. The severity of these responses likely place *A. dubius* among the most CO$_2$-sensitive fish species tested to date. Furthermore, these findings indicate that life history, spawning habitat, phenology, and developmental rates mediate early life CO$_2$ tolerance.
Introduction

The threat of ocean acidification (OA) to marine organisms remains one of the most intensely pursued research priorities in modern oceanography (Browman 2016). After nearly two decades of empirical research, recent meta-analyses have concluded that high partial pressures of CO$_2$ ($p$CO$_2$) elicit overall negative effects in marine organisms, while also highlighting the large unexplained variability in responses among taxa and even populations (Wittmann & Pörtner 2013; Przeslawski et al. 2015). This complexity is particularly apparent within marine fishes. In some species, for example, offspring show increased mortality, slower development, and altered metabolism under future $p$CO$_2$ (Baumann et al. 2012; Chambers et al. 2014; Dahlke et al. 2017), whereas most other tested species have shown neutral survival and growth responses (Cattano et al. 2018). Further complexity is introduced by the potential for interactive effects with other climate stressors such as ocean warming (Hofmann & Todgham 2010). Given their shared anthropogenic driver, the combined effects of acidification and warming have received considerable experimental attention, but for fish early life-stages the evidence has remained largely inconclusive (Lefevre 2016). The pursuit to elucidate principles of CO$_2$ × temperature sensitivities in fish likely requires a better understanding of mediating eco-physiological processes.

One such principle of increasing interest involves the existing $p$CO$_2$ fluctuations that characterize marine habitats to different degrees. River input (Salisbury et al. 2008), upwelling (Feely et al. 2008), and net community metabolism (Baumann & Smith 2017) all induce considerable $p$CO$_2$/pH variability on small spatial and short temporal scales. Particularly in highly productive, nearshore systems, diel $p$CO$_2$ fluctuations can exceed levels not predicted for the average open-ocean for hundreds of years (Duarte et al. 2013). The ubiquity of nearshore $p$CO$_2$ variability (Baumann & Smith 2017) suggests that organisms in these habitats may be adapted to these conditions.
(Hofmann et al. 2014) and thus largely tolerant of experimental $p$CO$_2$ levels based on average open-ocean predictions (Vargas et al. 2017). Conversely, organisms inhabiting more CO$_2$-stable, offshore environments may be more CO$_2$-sensitive. Differences in CO$_2$ tolerance may also arise from species differences in early life developmental rates. Rapid growth in offspring of summer-spawning or tropical species likely requires the fast development of acid-base regulatory capacity to mitigate metabolic acidosis (Melzner et al. 2009). By contrast, slow metabolic rates in winter-spawning or cold-water species may relax such demands (Pörtner et al. 2004).

A positive relationship between thermal tolerance thresholds and thermal ranges (i.e., Climate Variability Hypothesis, Stevens 1989) is well established in marine ectotherms (Compton et al. 2007; Sunday et al. 2012; Woolsey et al. 2015). A similar framework involving $p$CO$_2$ variability (Ocean Variability and Stage Duration Hypothesis, Baumann 2018) has been tested for marine invertebrates (Kelly et al. 2013; Hofmann et al. 2014; Vargas et al. 2017) but has not yet been specifically applied to fish. Negative effects of elevated $p$CO$_2$ in highly stenothermic Antarctic fish (Flynn et al. 2015; Davis et al. 2018) as well as coastal shelf species (Chambers et al. 2014; Pimentel et al. 2014) appear to support the OVSDH. Furthermore, the $p$CO$_2$ tolerances of two congeneric rockfishes was higher in the species with offspring that develop in a system characterized by greater $p$CO$_2$ variability (Hamilton et al. 2017). However, a more critical evaluation requires strategically expanding the tested fish species beyond common models within tropical reef or nearshore taxa. To date, slow developing species from stable $p$CO$_2$ environments (e.g., winter, open-ocean, or high-latitude spawners) are understudied with respect to OA-sensitivity (Cattano et al. 2018).

One promising candidate species may be northern sand lance (Ammodytes dubius), a semi-demersal, schooling forage fish of key ecological importance in temperate to polar shelf waters of
the north Atlantic (Nelson & Ross 1991) and a member of the Ammodytidae family that has yet to be assessed for CO₂ sensitivity. Adults of *A. dubius* spawn during late fall, depositing eggs onto offshore sandbanks such that embryos develop slowly under cooling temperatures and relatively stable pCO₂ conditions (Salisbury & Jönsson 2018). Across the Northwest Atlantic shelf and particularly at Stellwagen Bank National Marine Sanctuary (SBNMS) seasonal immigrations of megafauna like bluefin tuna, humpback whales, and a variety of sea birds rely on *A. dubius* as prey (Willson et al. 1999), thus underscoring the impetus to characterize the climate sensitivity of this species.

Over the course of two years, we experimentally quantified the effects of current to future pCO₂ (400, 1,000 and 2,100 µatm) and temperature conditions (5°, 7°, and 10°C) on the offspring of wild *A. dubius* from SBNMS. Given the low likelihood that *A. dubius* accumulates high or variable levels of environmental or metabolically produced CO₂ during early development, we hypothesized that *A. dubius* would be more CO₂-sensitive than in-shore or summer spawning species. The study was designed to answer three specific questions: (1) Does exposure to future pCO₂ and temperature conditions reduce embryo survival and prolong hatching? (2) Do future conditions reduce embryonic growth and alter consumption of endogenous energy? (3) Are post-hatch survival and growth rates affected by future conditions?

**Methods**

_**Field sampling and fertilization:**_ This study complied with the ethical guidelines under IACUC protocol #A17-043. Spawning-ripe sand lance were collected from SBNMS (42° 9’ 58.26” N, 70° 18’ 44.19” W, Fig. 1) on 2 December 2016 and 22 November 2017, using a 1.3 × 0.7 m beam trawl (6 mm mesh) towed at 3 knots for 15 min. On deck, adults were checked for ripeness and sorted
by sex. Experimental embryos were produced from three separate fertilizations (Table S1), two of which were completed at sea (2016, 2017: sea), while a third used adults transported to the lab (Rankin Seawater Facility, University of Connecticut Avery Point) and strip-spawned after being held for 2 d in 400-l circular tanks at 10°C without food (2017: lab). In each case, we used at least 10 spawners per sex ranging 14 – 19 cm in total length (TL, mean = 16 cm, Table S1). Strip-spawning protocols were adapted from early experimental work on the congener A. americanus (Smigielski et al. 1984). Embryos fertilized at sea were maintained in coolers at 10°C during transport to the lab. Embryos spawned in the lab (2017: lab) were treated with 40 g l⁻¹ of diatomaceous earth (food grade, Root Naturally®) to prevent clumping (Smigielski et al. 1984).

**Experimental CO₂ and temperature conditions:** We tested factorial combinations of three pCO₂ × three temperature levels. The target for pCO₂ controls was 400 μatm (~8.15 pH₉₀₀₀), a level characteristic of the average open ocean and of the A. dubius spawning habitat in late fall (Salisbury & Jönsson 2018). As contrasts, we chose 1,000 μatm (~7.78 pH) and 2,100 μatm (~7.48 pH), which correspond to predicted average ocean pCO₂ levels by the years 2100 and 2300, respectively (Caldeira & Wickett 2005). The three experimental temperatures were 5°C, 7°C, and 10°C, encompassing current thermal conditions on Stellwagen Bank during fall (Salisbury & Jönsson 2018) and winter as well as potential warming scenarios over the next century (2-3°C, Stocker et al. 2014).

We developed a LabView (National Instruments®) program to automate pCO₂ manipulations in treatment tanks as described in detail by Murray & Baumann (2018). The pH and dissolved oxygen conditions of each tank were monitored by a central pH electrode (Hach pHD®, calibrated weekly using 2-point pH₉₀₀₀ references to nearest 0.01) and an optical DO probe (Hach LDO® Model 2). DO levels were maintained at ~100% saturation. Temperatures were controlled by thermostats.
(Aqualogic®) that powered in-line chillers (DeltaStar®). Realized treatment $p$CO$_2$ conditions were calculated based on measurements of pH, temperature, salinity, and total alkalinity ($A_T$). For measurements of $A_T$ ($\mu$mol kg$^{-1}$), treatment tanks were sampled three times during each experiment by filtering (to 10 µm) treatment seawater into 300 ml borosilicate bottles. Salinity was measured at the time of sampling using a refractometer. Bottles were stored at 3°C and measured for $A_T$ within two weeks of sampling using an endpoint titration (Mettler Toledo® G20 Potentiometric Titrator). Methodological accuracy (within ±1%) of alkalinity titrations were verified and calibrated using Dr. Andrew Dickson’s certified reference material for $A_T$ in seawater (University of California San Diego, Scripps Institution of Oceanography, Batch Nr. 162 & 164). Unmeasured carbonate parameters were calculated in CO2SYS (V2.1, http://cdiac.ornl.gov/ftp/co2sys) as described in Murray & Baumann (2018). Treatment levels and measurements of carbonate chemistry for this study are reported in Table S2.

*Experimental design:* Sand lance embryos and larvae were reared in a purpose-built system consisting of nine recirculating units that each housed five replicate rearing containers (20-L polyethylene buckets). Embryos developed in customized baskets (2-L polyethylene cups) that were floated inside each rearing container. Seawater flowed continuously past the incubating embryos at 4 L h$^{-1}$. All offspring were reared under a light cycle of 11H:13D and a salinity of 31.

In total, we report on three distinct, albeit not strictly independent $p$CO$_2 \times$ temperature trials (Table 1); a pilot trial (#1) with embryos fertilized at sea in fall 2016, and two subsequent trials in 2017 using embryos fertilized at sea (#2) and in the lab (#3). For the pilot trial, equal amounts of fertilized eggs were randomly distributed into replicate rearing containers per $p$CO$_2$ treatment ($n = 2$ for 400; 2,100 µatm; $n = 1$ for 1,000 µatm) and temperature (5, 10°C) within 9 hr post fertilization. Fertilization success was quantified later as the number of clearly developing embryos.
(to stage 3, Smigielski et al. 1984) at 130 degree-days post-fertilization (degree-days = rearing temperature * days, ddpf) after examination via dissecting microscope (8× mag). Thereafter, embryo baskets were monitored daily and hatched larvae were counted and immediately preserved in a 5% formaldehyde/freshwater solution saturated with sodium tetraborate buffer. Standard length at hatch (SL, nearest 0.01 mm) was measured via calibrated microscope images using Image Pro Premier (V9.0, Media Cybernetics®). The pilot trial was terminated after 400 ddpf to fully encapsulate the potential hatching period.

For trial 2 (2017: sea), equal amounts of fertilized embryos (2.5 ml or ~3,000 embryos) were again randomly placed into each of two replicate rearing containers per factorial $p$CO$_2$ (400; 1,100; 2,100 µatm) and temperature combination (5, 7, 10°C) within 9 hr of fertilization. Daily checks for hatchlings started at 100 ddpf, and hatchlings were counted and then either preserved or moved to larval rearing containers. Subsamples (n > 20) for morphometric measurements were taken on the first day when 20 or more larvae hatched in a given treatment (Table S3). Daily monitoring of 7°C treatments was discontinued after initial subsampling and allotments for larval rearing, but continued for all 5°C and 10°C treatments until 400 ddpf. Subsampled hatchlings were measured for two body size metrics; SL, somatic body area (nearest 0.01 mm$^2$), and two measures of remaining endogenous energy reserves; yolk sac area (nearest 0.001 mm$^2$), and oil globule area (nearest 0.001 mm$^2$) as illustrated in Fig. S1.

To quantify post-hatch survival and growth, larvae from trial 2 were collected for 5 d after first hatch occurred in each treatment and distributed among up to five replicates. Due to differences in hatching success, starting larvae per rearing container varied from 57 to 200 (Table S4). Low survival precluded post-hatch rearing for the 2,100 µatm $p$CO$_2$, 10°C treatment. Larvae were immediately provided with daily rations of L-type rotifers (7-10 rotifers ml$^{-1}$, Brachionus plicatilis,
Reed Mariculture®) and even amounts of microalgae (*Nannochloropsis, Tetraselmis* Rotifer Diet, Reed Mariculture®). Newly hatched brine shrimp nauplii (*Artemia salina*, San Francisco strain, brineshrimpdirect.com, 3-5 nauplii ml⁻¹) were added to the rotifer and microalgae diet 70 degree-days after larval rearing started. We replaced 10% of treatment seawater per day to maintain uncritical ammonia levels (< 0.25 ppm, monitored daily). Rearing containers were siphoned twice weekly to remove uneaten food and waste. After 150 degree-days, all surviving larvae were counted and preserved for final SL measurements (nearest 0.01 mm).

For trial 3 (2017: lab), 300 eggs were randomly distributed into each of three replicate rearing containers per factorial $pCO_2$ (400; 1,100; 2,100 µatm) and temperature combination (5, 10°C) within 2 hr of fertilization. Starting at 100 ddpf, embryo baskets were again monitored daily until 400 ddpf and hatchlings counted and immediately preserved. After termination, remaining eggs were examined with a dissecting microscope to distinguish potentially unfertilized from clearly developed but dead embryos, i.e., those at stages 3-5 (Smigielski et al. 1984) without a heartbeat. No unhatched embryo survived to 400 ddpf. Hatched larvae were again measured for hatch SL, somatic body area, yolk sac area, and oil globule area.

**Response traits:** Treatment-specific embryo survival was calculated in trials 1 and 3 as the proportion of hatchlings relative to the number of fertilized embryos (%), while in trial 2 the total number of hatchlings was used as a proxy for embryo survival. In trial 3, fertilization success (%) was calculated as the sum of hatched larvae + arrested embryos divided by 300. Treatment-specific daily hatch frequencies (=daily treatment hatch/total hatch within temperature treatment) were calculated for trials 2 and 3. Larval growth rates (mm d⁻¹, trial 2) were calculated by dividing the length differential (mean final larval length - mean hatch length) by the days of the larval rearing period.
**Statistical analysis:** We tested for significant treatment effects on offspring survival and growth for trials 2 (post-hatch larval survival and growth rate) and 3 (fertilization success and embryo survival), while simply reporting the pilot trial data for comparison. Proportional data were logit transformed \( \log_{10}(\text{value}/(1 - \text{value})) \) prior to analysis, with zero values replaced with 0.0001 (Warton & Hui 2011). General linear models (GLM) were used to test for significant \( (\alpha < 0.05) \) treatment effects on fertilization success, embryo survival, larval survival, and growth rate:

\[
\text{Response trait} = \text{CO}_2 + \text{temperature} + \text{CO}_2 \times \text{temperature} + \text{error}.
\]

Test groups were checked for assumptions of variance homogeneity and normality using Levin’s and Shapiro-Wilk tests \( (\alpha < 0.05) \), respectively, and Bonferroni corrections were applied for multiple comparisons. Where GLMs found significant \( p\text{CO}_2 \) or interactive effects, one-way ANOVAs were used to test for \( p\text{CO}_2 \)-effects within temperature treatments.

Morphometric measurements were separately analyzed for trials 2 and 3, but in the latter, only the 5°C treatments had sufficient numbers of hatchlings. Because morphometric traits were intercorrelated, we performed principal component (PC) analyses preceded by evaluating sampling adequacy (Kaiser-Meyer-Olkin measure, >0.5) and Bartlett’s test of sphericity \( (p < 0.001) \). We extracted rotated PCs (oblimin procedure) with eigenvalues > 1 and used the component scores assigned to each larva in nonparametric Kruskal-Wallis one-way ANOVAs to test for \( p\text{CO}_2 \) and temperature effects, with Dunn-Bonferroni tests used for multiple comparisons. Pearson bivariate correlations were used to evaluate associations between PCs and \( p\text{CO}_2 \) and temperature. All statistical tests were conducted in SPSS (V20, IBM).
Results

*Embryo survival:* Across years and trials, we found a strong and consistent reduction of embryo survival with increasing \( p_{CO_2} \) and temperature conditions. In the pilot trial, overall embryo survival was higher at 5°C (12±14%) than at 10°C treatments (1±1%). At 5°C, survival declined from 23% (400 μatm) to 16% (1,000 μatm) to 1% (2,100 μatm), whereas at 10°C survival was 2%, 1%, and 0% at 400, 1,000, and 2,100 μatm \( p_{CO_2} \), respectively (Fig. 2). During trial 2, three times more larvae hatched at the 5°C (n = 6,819) than 10°C treatments (n = 2,086). At 5°C, total hatch was maximal at 400 μatm \( p_{CO_2} \) (n = 3,683) but reduced by >50% at both 1,000 μatm (n = 1,575), and 2,100 μatm (n = 1,515, Fig. 3A). At 10°C, total hatch was again highest at 400 μatm \( p_{CO_2} \) (n = 1,496), but three times lower at 1,000 μatm (n = 453) and over 10 times lower under 2,100 μatm (n = 129, Fig. 3B).

During trial 3, fertilization success ranged from 13 to 20% (overall mean 17 ± 3%) and was unaffected by treatment conditions (Table 2). Across treatments, embryo survival ranged from 1 to 94%, with significant \( p_{CO_2} \), temperature, and \( p_{CO_2} \times \) temperature effects (GLM, \( p < 0.05 \), Table 2). Mean ± s.d. embryo survival was significantly higher at 5°C (55±34%) than 10°C (31±42%, Bonferroni, \( p < 0.001 \)). At 5°C, survival significantly declined from 94±3% at 400 μatm to 17±6% at 1,000 μatm or to 53±11% at 2,100 μatm \( p_{CO_2} \) (one-way ANOVA, \( F_{2,6} = 66.8, \ p < 0.001 \), Fig. 3C), with a significant difference in survival between 1,000 and 2,100 μatm (Bonferroni, \( p = 0.013 \)). The \( p_{CO_2} \times \) temperature interaction detected by the GLM was caused by an even stronger survival reduction with increasing \( p_{CO_2} \) at 10°C (one-way ANOVA, \( F_{2,6} = 14.2, \ p = 0.005 \), Fig. 3D). Survival declined more than 20-fold from 87±7% at 400 to 4±3% at 1,000 and 1±1% at 2,100 μatm (Bonferroni, \( p < 0.001 \)).
In addition to hatching success (i.e., embryo survival), $pCO_2$ conditions affected hatching patterns. Across trials, embryos reared at 400 µatm $pCO_2$ mostly hatched in one major peak shortly after first hatch, whereas embryos developing at 1,000 and 2,100 µatm hatched gradually over a protracted period of time (Fig. 3E-H). This was most pronounced in trial 2 at 10°C, where 99% of embryos at 400 µatm emerged within the first 3 days of hatching compared to only 5% of embryos in 2,100 µatm $pCO_2$ treatment. In the latter, it took 8-12 days for the majority of hatchlings to emerge (Fig. 3F).

**Morphometrics:** Treatment means ($\pm$ s.d.) for all morphometric measurements can be found in Table S3. PCAs on trial 2 and 3 hatchlings suggested reductions in hatch size and endogenous energy reserves with increasing $pCO_2$ and temperature conditions. For trial 2, two PCs were extracted and explained 43% and 39% of the total variance from the extracted loadings, respectively. Body size metrics loaded positively on PC1 (> 0.89), while endogenous energy metrics loaded positively on PC2 (> 0.89). For trial 3, two PCs were extracted that explained 52% and 34% of the total variance from the extracted loadings, respectively. Endogenous energy metrics loaded highly on PC1 (> 0.93), whereas body size metrics loaded highly on PC2 (> 0.89).

In trial 2, scores of both body size and endogenous energy reserves decreased with increasing $pCO_2$ depending on temperature conditions (Pearson correlation, $p < 0.05$, Table S5). At 5°C, body size scores were unaffected by $pCO_2$ but were significantly lower at 7°C and 10°C under 2,100 µatm (Kruskal-Wallis, $p < 0.05$, Fig. 4, Table 3). Endogenous energy scores significantly decreased with increasing $pCO_2$ levels across all temperatures (Kruskal-Wallis, $p < 0.05$, Fig. 4, Table 3). Overall, endogenous energy scores were significantly lower at 10°C relative to 5°C (Kruskal-Wallis, $X^2 (2) = 8.780$, $p = 0.012$). For trial 3, scores from both PCs were negatively correlated with $pCO_2$ (Pearson correlation, $p < 0.05$, Table S5). A Kruskal-Wallis test showed body
size and endogenous energy scores for hatchlings from the 2,100 µatm $pCO_2$ treatment were significantly lower than those from 400 µatm ($p < 0.05$, Fig. S2, Table 3).

**Post-hatch survival and growth:** Larval survival was highly variable within and between treatments (range = 2 - 26%, mean 9 ± 9%, Table S4) and sensitive to temperature (GLM, $F_{2,27} = 3.544$, $p = 0.043$), but statistically unaffected by $pCO_2$ (Table S4). Average length ranged from 6.30 to 7.36 mm (overall mean 6.87 ± 0.63 mm, Table S4) and was independent of $pCO_2$ or temperature treatments. Growth rate ranged from 0.03 to 0.09 mm d$^{-1}$ (overall mean 0.06 ± 0.03 mm d$^{-1}$, Table S4) and was significantly affected by temperature (GLM, $F_{2,21} = 4.229$, $p = 0.029$) but not $pCO_2$ (Table S4).

**Discussion**

We conducted the first comprehensive evaluation of early life $CO_2 \times$ temperature sensitivities in Northern sand lance, a member of the globally important but so far unstudied forage fish family Ammodytidae. We found that exposure to future $pCO_2$ conditions consistently resulted in severely reduced embryo survival. The lethality of elevated $pCO_2$ increased when combined with elevated rearing temperatures. To date, lethal effects of 1,000-2,100 µatm $pCO_2$ in fish early life-stages have been documented in some, but not most species (Cattano et al. 2018). Notable examples include reductions in survival of up to 74% in inland silversides (*M. beryllina*) (Baumann et al. 2012; Gobler et al. 2018), 48% in summer flounder (*Paralichthys dentatus*) (Chambers et al. 2014), and 47% in Atlantic cod (*Gadus morhua*) (Dahlke et al. 2017). In *A. dubius*, the reductions in embryo survival ranged from 27 to 99% under 1,000-2,100 µatm $pCO_2$ (72% on average) relative to contemporary $pCO_2$ conditions, hence making it one of the most $CO_2$-sensitive fish species documented to date (Cattano et al. 2018). However, despite the strong embryonic effects,
survival of post-hatch larvae were not influenced by $pCO_2$ level. Nonetheless, larval survival was relatively low and highly variable, which limited the interpretation of treatment effects.

In addition to effects on survival, exposure to acidified conditions reduced the remaining endogenous reserves of yolk-sac hatchlings. Acclimation to elevated $pCO_2$ may have increased energetic demands through increased rates of ion regulation and protein synthesis and turnover (Melzner et al. 2009; Pan et al. 2015). Embryos exposed to 2,100 µatm $pCO_2$ were significantly smaller at hatch when reared at 7° and 10°C. Under warmer temperatures, endogenous utilization may have already been maximized and further costs of CO$_2$ acclimation resulted in a metabolic tradeoff where embryos prioritized energy to homeostasis over somatic growth (Wieser & Krumshnabel 2001). The observed changes in hatch morphometries would likely be detrimental to *A. dubius* larvae in the wild, as smaller larvae with less endogenous energy generally experience higher cumulative mortality rates (Miller et al. 1988). Furthermore, the observed delay in bulk hatching under acidified conditions may compound these effects. A CO$_2$-induced decoupling of embryonic duration with temperature and other drivers of phenology means that the timing of bulk hatch could be offset from optimal resources and predation pressure (Cushing 1990).

We found that warmer temperatures compounded the negative effects of elevated CO$_2$ on survival, timing of hatch, and development of *A. dubius* embryos, which is consistent with a growing number of studies reporting negative synergistic effects of elevated $pCO_2$ and sub-optimal temperatures in fish early life-stages. (Pimentel et al. 2014; Flynn et al. 2015; Dahlke et al. 2017; Davis et al. 2018; Gobler et al. 2018). Furthermore, the apparent high sensitivity of *A. dubius* embryo survival to acidification and warming aligns with findings from a broader group of marine taxa (Przeslawski et al. 2015). However, not all fish early life stages have demonstrated this CO$_2$ × temperature paradigm (Lefevre 2016; Murray & Baumann 2018). As the oceans continue to
concurrently warm and acidify, a more detailed mechanistic understanding of CO₂ × temperature effects in marine fish is needed (Pörtner et al. 2017; Jutfelt et al. 2018).

What exactly caused the exceptional pCO₂ lethality in A. dubius embryos is unknown. Mortality could have been due to an uncompensated acidosis (Kikkawa et al. 2004), which can impair pH-sensitive vital processes (Pörtner et al. 2005). Intriguingly, post-trial observations of rearing baskets showed that dead embryos from acidified treatments appeared pigmented and near full development, but were seemingly unable to hatch. Thus, an alternative explanation could be that elevated pCO₂ disrupted the hatching process itself. This was further suggested by the relatively prolonged, intermittent hatching observed in acidified conditions. Hatching in fish is largely dependent on the activity of hatching enzymes, which are pH sensitive and generally perform best in more alkaline conditions (Korwin-Kossakowski 2012). Therefore, under high environmental pCO₂ an exacerbated acidification of the perivitelline fluid may reduce the activity of hatching enzymes, leading to a prolonged delay or complete cessation of hatching (Havas & Rosseland 1995). Hatching enzyme expression or activity has not yet been evaluated in the context of ocean acidification, but potentially represents a critical knowledge gap in marine fishes.

With less than 0.5% of all marine fish species tested, CO₂-effects appear to be highly specific to species or even populations (Cattano et al. 2018) thus still precluding the kind of generalizations that would allow extrapolating findings to untested taxa. One promising hypothesis is that CO₂ tolerance of species and populations increases with the level of pCO₂ variability experienced in their natural habitats (Hofmann et al. 2014; Vargas et al. 2017). Consider the well-studied example of the Atlantic silverside (M. menidia), another coastal forage fish that spawns, however, in nearshore, sub-tropical to temperate habitats during spring and summer (Conover & Kynard 1984). These habitats typically undergo seasonal, metabolic acidification that can elicit diel pH
fluctuations exceeding 0.60 units (7.43 – 8.10 pH, Fig. 5A). Years of serial experimentation on the species revealed that wild silverside embryos are weakly affected by elevated $p\text{CO}_2$ early in the season, but become progressively more CO$_2$-tolerant (Baumann et al. 2018) until by early summer they are unaffected by even extreme $p\text{CO}_2$ conditions (>4,000 µatm) (Murray & Baumann 2018). Mechanisms that rapidly enhance offspring CO$_2$ tolerance are likely adaptive in many nearshore species that spawn embryos into highly variable environments (Hoffmann & Hercus 2000) and therefore appear tolerant of future $p\text{CO}_2$ conditions in experimental settings (Vargas et al. 2017).

In the Gulf of Maine, *A. dubius* spawn in late fall and deposit embryos onto offshore sandbanks, where $p\text{CO}_2$ conditions are stable and generally resemble average surface ocean levels (Salisbury & Jönsson 2018). Importantly, pH values never fall below 8.00 (Fig. 5B). In contrast to silversides, *A. dubius* may therefore face weaker selection for CO$_2$-tolerant phenotypes during early life, assuming that tolerance to elevated environmental $p\text{CO}_2$ is metabolically costly and thus not maintained in species that do not need it (Sunday et al. 2014). Furthermore, slow developing offspring like *A. dubius* may have a lower capacity for buffering metabolically produced CO$_2$ than faster developing, more active offspring of other fish species (Melzner et al. 2009). As a result, the sensitivity of *A. dubius* embryos to 2,100 µatm $p\text{CO}_2$ appears to be an order of magnitude higher than in *M. menidia* (Fig. 5C). The diverging responses between the two species with different habitat and life history characteristics are consistent with the Ocean Variability and Stage Duration Hypothesis (Baumann 2018). For marine invertebrates, this hypothesis has been empirically supported (Kelly et al. 2013; Hofmann et al. 2014; Vargas et al. 2017), and our findings now suggest that this framework equally applies to marine fish and comprises a potentially useful tool for predicting early life climate sensitivities.
In summary, we demonstrated exceptional CO$_2$ × temperature sensitivities in embryos of the Northern sand lance, a keystone species along the northwest Atlantic shelf, supporting commercially important fish, sea birds, and marine mammals. This high sensitivity may be a function of the species’ spawning phenology and habitat; slow embryonic development combined with the stable $p_{CO_2}$ conditions may have relaxed selection for CO$_2$-tolerant phenotypes during early life. Ammodytids are important forage fishes in coastal and shelf ecosystems across the northern hemisphere, but the family remains generally understudied (Orr et al. 2015). Therefore the potential that high $p_{CO_2}$ sensitivity is a shared trait among the Ammodytidae with similar winter spawning phenologies constitutes a conservation issue of global concern. Our study is therefore a mandate for follow-up research to understand the mechanisms and prevalence of near-future $p_{CO_2}$ lethality in this ecologically important group of forage fish.

Acknowledgements

This study was funded through the Northeast Regional SeaGrant Consortium (RNE16-CTHCE-I). We are grateful to the crew of the R/V Auk (Dave Slocum, Amy Meloski, Mike Thompson, and Peter Hong) and collaborators at USGS (Page Valentine and Dan Blackwood) for their generous support collecting wild sand lance. We are thankful to Emma Cross, Jacob Snyder, and Julie Pringle (UConn) for collection and laboratory assistance.
References


Baumann H. 2019. Experimental assessments of marine species sensitivities to ocean acidification and co-stressors: how far have we come? Canadian Journal of Zoology.


Gobler CJ, Merlo LR, Morell BK, Griffith AW. 2018. Temperature, acidification, and food supply interact to negatively affect the growth and survival of the forage fish, *Menidia beryllina* (inland silverside) and *Cyprinodon variegatus* (sheepshead minnow). Frontiers in Marine Science 5:86.


Sutton AJ, Christopher L.; Salisbury, Joseph E.; Vandemark, Douglas; Musielewicz, Sylvia; Maenner Jones, Stacy; Dietrich, Colin; Bott, Randy; Osborne, John. 2014. High-resolution ocean and atmosphere pCO₂ time-series measurements from mooring NH_70W_43N National Oceanographic Data Center, NOAA Version 7.7.


**Tables**

**Table 1:** Summary of three $p\text{CO}_2 \times$ temperature trials on *A. dubius* offspring. Treatment levels for $p\text{CO}_2$ (µatm) and temperature (°C) represent target conditions, actual measured values are presented in Table S2. Traits are abbreviated as fertilization success (FS), embryo survival (ES), total hatch (TH), daily hatch frequencies (DHF), hatch length (HL), somatic body area (SA), yolk sac area (YSA), oil globule area (OGA), larval survival (LS), larval length (LL) and growth rate (GR).

<table>
<thead>
<tr>
<th>Trial</th>
<th>Fert.</th>
<th>$p\text{CO}_2$</th>
<th>Temp</th>
<th>Replicates</th>
<th>Response traits</th>
<th>Survival</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2016</td>
<td>400, 1000, 2100</td>
<td>5,10</td>
<td>1-2</td>
<td>ES</td>
<td>HL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2017</td>
<td>400, 1000, 2100</td>
<td>5,7,10</td>
<td>1</td>
<td>TH, DHF, LS</td>
<td>HL, SA, YSA, OGA, LL, GR</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2017</td>
<td>400, 1000, 2100</td>
<td>5,10</td>
<td>3</td>
<td>FS, ES, DHF</td>
<td>HL, SA, YSA, OGA</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2:** *A. dubius*. Summary statistics for GLMs testing $p\text{CO}_2 \times$ temperature effects on trial 3 fertilization success (FS) and embryo survival (ES). Significant effects are denoted by $p$-values in bold.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Factor</th>
<th>df</th>
<th>F</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS</td>
<td>$\text{CO}_2$</td>
<td>2</td>
<td>2.270</td>
<td>0.118</td>
</tr>
<tr>
<td></td>
<td>Temp</td>
<td>1</td>
<td>0.688</td>
<td>0.423</td>
</tr>
<tr>
<td></td>
<td>$\text{CO}_2 \times$ temp</td>
<td>2</td>
<td>3.377</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ES</td>
<td>$\text{CO}_2$</td>
<td>2</td>
<td>29.259</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Temp</td>
<td>1</td>
<td>20.299</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>$\text{CO}_2 \times$ temp</td>
<td>2</td>
<td>5.895</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: *A. dubius*. Summary statistics from Kruskal-Wallis H evaluating within temperature CO₂-effects on morphometric PC scores from trials 2 and 3. Trait abbreviations are BS for body size and EE for endogenous energy reserves. Significant effects are highlighted in bold.

<table>
<thead>
<tr>
<th>Trial</th>
<th>PC</th>
<th>Traits</th>
<th>Temp(°C)</th>
<th>df</th>
<th>Kruskal-Wallis H</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5</td>
<td>BS</td>
<td>7</td>
<td>2</td>
<td>12.194</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td>2</td>
<td>10.008</td>
<td><strong>0.007</strong></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>EE</td>
<td>7</td>
<td>2</td>
<td>28.026</td>
<td>&lt; <strong>0.001</strong></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>11.855</td>
<td>2</td>
<td></td>
<td><strong>0.003</strong></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>EE</td>
<td>22.260</td>
<td>2</td>
<td></td>
<td>&lt; <strong>0.001</strong></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>BS</td>
<td>8.810</td>
<td>2</td>
<td></td>
<td><strong>0.012</strong></td>
</tr>
</tbody>
</table>
Fig. 1: Map of Stellwagen Bank National Marine Sanctuary and the surrounding region. The red circle denotes the sampling location for spawning-ripe *A. dubius*.
Fig. 2: Treatment mean embryo survival (%) of *A. dubius* offspring from trial 1 (pilot fertilization) reared at three CO₂ levels (colored bars) crossed with two temperature treatments. Arranged left to right, bars represent 400 µatm (blue), 1,000 µatm (green), and 2,100 µatm pCO₂ (red), respectively.
Fig 3: Hatching timelines of *A. dubius* offspring from trials 2 (sea fertilization) and 3 (lab fertilization). Embryos were reared at 5°C (blue background) and 10 °C (red background) crossed with three $p$CO$_2$ levels; 400 µatm (blue lines), 1,000 µatm (green lines), and 2,100 µatm (red lines). Traits presented are total hatch (A-B, cumulative number hatched), embryo survival (C-D, %) and daily hatch frequencies (E-H, daily hatch/total final hatch within temperature treatment).
Fig. 4: *A. dubius*. Mean (±s.d) treatment PC scores from of size traits (PC 1) and endogenous energy reserves (PC 2) from trial 2 (sea fertilization) offspring. $p$CO$_2$ treatments, arranged left to right at each temperature, are denoted by colored circles and lines; blue (400 µatm), green (1,000 µatm), and red (2,100 µatm). Significant within-temperature $p$CO$_2$ effects are indicated by differing letters above treatment PC scores (Dunn-Bonferroni, $\alpha < 0.05$).
Fig. 5: Seasonal pH (NIST, blue) and temperature (°C, red) variability from spawning habitats of *M. menidia* (A; Mumford Cove, CT; 41° 19' 26" N, 72° 1' 10" W; DOI: 10.1575/1912/bco-dmo.660079) and *A. dubius* (B; Coastal Western Gulf of Maine Mooring; 43° 1' 12" N, 70° 32' 24" W; Sutton et al., 2014). The Coastal Western Gulf of Maine Mooring is located ~100 km north of our sand lance collection site, approximately 10 km from shore at a depth of 65 m. Faded circles are all individual measurements within datasets, solid lines represent smoothed data and shaded areas denote embryonic and early larval durations. Panel C shows relationship between ΔpH (total pH range during respective spawning seasons) and mean pCO₂ effect size on embryo survival (log-linear response ratio (LnRR) = ln(survival at ~2,100 µatm) – ln(survival at ~400 µatm)). Response variation is shown as 95% bootstrapped (BCa) confidence intervals. Data for *M. mendia* (red circle) was taken from Baumann et al. 2018. For *A. dubius* (black triangle), mean effect size was calculated from all 2,100 µatm treatments during trials 1-3. Negative LnRRs indicate a reduction in trait value, where 95% CIs do not overlap 0 the effect is considered significant (Hedges et al., 1999).
Appendix for Chapter 3: High Sensitivity of an Important Forage Fish (*Ammodytes dubius*) to Ocean Acidification and Warming.

Tables

**Table S1:** Spawning ripe *A. dubius* were collected from Stellwagen Bank National Marine Sanctuary to fertilize embryos used in factorial CO₂ × temperature experiments. Adult lengths are shown as mean total lengths (TL, cm) ± standard deviation.

<table>
<thead>
<tr>
<th>Fertilization</th>
<th>Collection date</th>
<th>Fertilization date</th>
<th># Female spawners</th>
<th>Female TL</th>
<th># Male spawners</th>
<th>Male TL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2016 field</td>
<td>12/02/2016</td>
<td>12/2/2016</td>
<td>13</td>
<td>16.3±1.9</td>
<td>10</td>
<td>16.2±2.2</td>
</tr>
<tr>
<td>2017 field</td>
<td>11/22/2017</td>
<td>11/22/2017</td>
<td>25</td>
<td>16.1±1.0</td>
<td>27</td>
<td>16.0±1.3</td>
</tr>
<tr>
<td>2017 lab</td>
<td>11/22/2017</td>
<td>11/24/2017</td>
<td>14</td>
<td>15.9±1.1</td>
<td>26</td>
<td>15.7±1.0</td>
</tr>
</tbody>
</table>
Table S2: Carbon chemistry and temperature measurements from CO₂ × temperature factorial experiments on *A. dubius* offspring. Mean (±s.d.) pH (NIST) and temperature (°C) are from daily measurements. Mean (±s.d.) salinity, total alkalinity (*A*_T; μmol kg⁻¹), dissolved inorganic carbon (*C*_T; μmol kg⁻¹), partial pressure and fugacity of CO₂ (*p*CO₂; *f*CO₂; μatm), and carbonate ion concentration (CO₃²⁻; μmol kg⁻¹) quantified from replicated seawater samples. Salinity was measured via refractometer and *A*_T from endpoint titrations while *p*CO₂, *C*_T, *f*CO₂ and CO₃²⁻ were calculated in CO2SYS. Salinity was 31 psu for all trials.

<table>
<thead>
<tr>
<th>Trials</th>
<th>Target temp</th>
<th>Temp</th>
<th>Target <em>p</em>CO₂</th>
<th><em>p</em>CO₂</th>
<th><em>A</em>_T</th>
<th><em>C</em>_T</th>
<th><em>f</em>CO₂</th>
<th>CO₃²⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>5.4±0.1</td>
<td>400</td>
<td>8.09±0.2</td>
<td>446±3</td>
<td>2137±17</td>
<td>2032±17</td>
<td>444±3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.4±0.1</td>
<td>1000</td>
<td>7.80±0.01</td>
<td>890±3</td>
<td>2132±8</td>
<td>2108±8</td>
<td>886±3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.3±0.2</td>
<td>2100</td>
<td>7.50±0.03</td>
<td>1828±8</td>
<td>2137±10</td>
<td>2197±10</td>
<td>1821±8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.0±0.8</td>
<td>400</td>
<td>8.09±0.01</td>
<td>453±4</td>
<td>2143±19</td>
<td>2013±18</td>
<td>451±4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.3±0.8</td>
<td>1000</td>
<td>7.81±0.01</td>
<td>909±6</td>
<td>2125±16</td>
<td>2082±15</td>
<td>905±6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.3±0.8</td>
<td>2100</td>
<td>7.51±0.03</td>
<td>1881±2</td>
<td>2131±3</td>
<td>2171±3</td>
<td>1873±2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.9±0.2</td>
<td>400</td>
<td>8.15±0.06</td>
<td>385±11</td>
<td>2198±69</td>
<td>2074±65</td>
<td>384±11</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.9±0.2</td>
<td>1000</td>
<td>7.72±0.04</td>
<td>1103±49</td>
<td>2175±103</td>
<td>2177±103</td>
<td>1099±49</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.8±0.3</td>
<td>2100</td>
<td>7.43±0.07</td>
<td>2180±68</td>
<td>2159±76</td>
<td>2246±79</td>
<td>2171±68</td>
</tr>
<tr>
<td>2-3</td>
<td>7</td>
<td>7.1±0.3</td>
<td>400</td>
<td>8.16±0.05</td>
<td>379±9</td>
<td>2177±64</td>
<td>2039±59</td>
<td>377±9</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7.0±0.3</td>
<td>1000</td>
<td>7.74±0.07</td>
<td>1076±29</td>
<td>2185±71</td>
<td>2174±69</td>
<td>1072±28</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7.2±0.3</td>
<td>2100</td>
<td>7.45±0.08</td>
<td>2155±74</td>
<td>2177±82</td>
<td>2250±84</td>
<td>2146±74</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.0±0.4</td>
<td>400</td>
<td>8.15±0.04</td>
<td>404±9</td>
<td>2219±41</td>
<td>2066±41</td>
<td>402±9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.7±0.4</td>
<td>1000</td>
<td>7.76±0.04</td>
<td>1051±10</td>
<td>2180±22</td>
<td>2153±22</td>
<td>1047±10</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.5±0.4</td>
<td>2100</td>
<td>7.48±0.03</td>
<td>2056±23</td>
<td>2173±34</td>
<td>2228±33</td>
<td>2048±23</td>
</tr>
</tbody>
</table>
Table S3: Ages and sample-sizes (N) of newly-hatched *A. dubius* larvae from trials 1-3. Morphometric measurements shown as treatment mean (± s.d.) hatch length (HL, mm), somatic body area (SA, mm²), yolk sac area (YSA, mm²), and oil globule area (OGA, mm²). Sample age ranges are shown as days post-fertilization (dpf).

<table>
<thead>
<tr>
<th>Trial</th>
<th>Temp (°C)</th>
<th>pCO₂ (µatm)</th>
<th>Age (dpf)</th>
<th>N</th>
<th>HL (mm)</th>
<th>SA (mm²)</th>
<th>YSA (mm²)</th>
<th>OGA (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>400</td>
<td>42</td>
<td>10</td>
<td>5.84 ± 0.32</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>400</td>
<td>35</td>
<td>38</td>
<td>5.47 ± 0.30</td>
<td>1.26 ± 0.16</td>
<td>0.167 ± 0.041</td>
<td>0.050 ± 0.017</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1,000</td>
<td>36</td>
<td>39</td>
<td>5.42 ± 0.34</td>
<td>1.22 ± 0.12</td>
<td>0.124 ± 0.038</td>
<td>0.041 ± 0.014</td>
</tr>
<tr>
<td></td>
<td>2,100</td>
<td>400</td>
<td>27</td>
<td>40</td>
<td>5.24 ± 0.23</td>
<td>1.21 ± 0.10</td>
<td>0.184 ± 0.036</td>
<td>0.047 ± 0.014</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>1,000</td>
<td>29</td>
<td>40</td>
<td>5.22 ± 0.20</td>
<td>1.18 ± 0.10</td>
<td>0.148 ± 0.029</td>
<td>0.038 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>2,100</td>
<td>400</td>
<td>28</td>
<td>48</td>
<td>5.12 ± 0.22</td>
<td>1.13 ± 0.14</td>
<td>0.139 ± 0.049</td>
<td>0.033 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1,000</td>
<td>22</td>
<td>32</td>
<td>5.34 ± 0.23</td>
<td>1.25 ± 0.12</td>
<td>0.114 ± 0.040</td>
<td>0.033 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>2,100</td>
<td>400</td>
<td>27</td>
<td>24</td>
<td>5.23 ± 0.29</td>
<td>1.18 ± 0.13</td>
<td>0.123 ± 0.064</td>
<td>0.037 ± 0.016</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>400</td>
<td>35-66</td>
<td>128</td>
<td>5.51 ± 0.34</td>
<td>1.30 ± 0.17</td>
<td>0.144 ± 0.057</td>
<td>0.041 ± 0.019</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1,000</td>
<td>35-62</td>
<td>28</td>
<td>5.40 ± 0.38</td>
<td>1.27 ± 0.13</td>
<td>0.108 ± 0.056</td>
<td>0.035 ± 0.025</td>
</tr>
<tr>
<td></td>
<td>2,100</td>
<td>35-64</td>
<td>62</td>
<td>5.33 ± 0.36</td>
<td>1.25 ± 0.14</td>
<td>0.107 ± 0.046</td>
<td>0.028 ± 0.017</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>400</td>
<td>18-25</td>
<td>121</td>
<td>5.21 ± 0.07</td>
<td>1.27 ± 0.04</td>
<td>0.176 ± 0.044</td>
<td>0.059 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>22-27</td>
<td>6</td>
<td>5.22 ± 0.35</td>
<td>1.25 ± 0.07</td>
<td>0.185 ± 0.047</td>
<td>0.064 ± 0.024</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,100</td>
<td>21-24</td>
<td>2</td>
<td>5.60 ± 0.34</td>
<td>1.34 ± 0.13</td>
<td>0.148 ± 0.050</td>
<td>0.064 ± 0.021</td>
<td></td>
</tr>
</tbody>
</table>

Table S4: Summary of larval survival (LS, %), larval length (LL, mm), and growth rate (GR, mm d⁻¹) in trial 2 *A. dubius* offspring. The number of starting larvae and response traits are given as treatment means (± s.d.).

<table>
<thead>
<tr>
<th>Treatment temp (°C)</th>
<th>pCO₂ (µatm)</th>
<th>Number of replicates</th>
<th>Starting larvae</th>
<th>LS (%)</th>
<th>LL (mm)</th>
<th>GR (mm d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>400</td>
<td>4</td>
<td>200 ± 0</td>
<td>10 ± 6</td>
<td>7.36 ± 0.75</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>10</td>
<td>400</td>
<td>5</td>
<td>108 ± 4</td>
<td>8 ± 6</td>
<td>6.40 ± 0.55</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>2100</td>
<td>5</td>
<td>127 ± 10</td>
<td>7 ± 7</td>
<td>6.87 ± 0.82</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>7</td>
<td>400</td>
<td>5</td>
<td>142 ± 38</td>
<td>2 ± 2</td>
<td>6.73 ± 0.72</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>5</td>
<td>111 ± 33</td>
<td>8 ± 8</td>
<td>6.97 ± 0.06</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>2100</td>
<td>5</td>
<td>124 ± 19</td>
<td>6 ± 6</td>
<td>7.34 ± 0.51</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>10</td>
<td>400</td>
<td>3</td>
<td>200 ± 0</td>
<td>16 ± 9</td>
<td>6.77 ± 0.56</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>3</td>
<td>108 ± 1</td>
<td>26 ± 14</td>
<td>6.30 ± 0.25</td>
<td>0.06 ± 0.02</td>
</tr>
</tbody>
</table>
Table S5: *A. dubius*. Pearson bivariate correlations between treatment factors and PC scores extracted from trial 2 and 3 morphometric groups. Significant ($\alpha < 0.05$) correlations are highlighted in bold.

<table>
<thead>
<tr>
<th>Trial</th>
<th>PC</th>
<th>CO₂</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pearson correlation</td>
<td>-0.0137</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p$</td>
<td><strong>0.015</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>320</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>Pearson correlation</td>
<td>-0.282</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p$</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>320</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>Pearson correlation</td>
<td>-0.320</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p$</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Pearson correlation</td>
<td>-0.189</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p$</td>
<td><strong>0.005</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>218</td>
</tr>
</tbody>
</table>
**Fig. S1:** *A. dubius.* Schematic of morphometric measurements made on newly hatched larvae from trials 2 and 3. Measurements include hatch length (standard length, nearest 0.00 mm), somatic body area (nearest 0.00 mm$^2$), yolk sac area (nearest 0.00 mm$^2$), and oil globule area (nearest 0.00 mm$^2$).

**Fig. S2:** *A. dubius.* Mean (±s.d) treatment PC scores from endogenous energy reserves (PC 1) and body size (PC 2) traits from trial 3 (lab fertilization). Significant differences between $p$CO$_2$ treatments are indicated by differing letters above PC scores (Dunn-Bonferroni, $\alpha < 0.05$)
Chapter 4

A Factorial Evaluation of the Combined Effects of Acidification and Hypoxia in Atlantic Silverside Offspring

Abstract

Anthropogenic impacts are rapidly altering the physical conditions of marine systems. Under the combined effects of ocean acidification, warming, and eutrophication occurrences of coastal hypoxia and acidification are increasing in frequency and severity. Despite the recognition of this multi-stressor threat, the empirical evidence for combined effects of acidification and hypoxia on fish early life-stages is still scarce. This study reports on two factorial experiments that characterized the individual and combined effects of elevated $p$CO$_2$ (400, 2,200, 4,500 µatm) and low dissolved oxygen (DO; 2.5, 3, 4, 7 mg L$^{-1}$) in Atlantic silverside (Menidia menidia) embryos and early larvae. During both trials, offspring survival and growth consistently declined with DO level, but there were few additional effects of $p$CO$_2$. Mortality rates were high under hypoxic conditions but reduced survival was also detected under more modest DO reductions (~55% saturation). All low DO treatments resulted in significant sub-lethal effects that included smaller hatch sizes and slower post-hatch growth rates. Compared to the effect of low DO, effects of elevated $p$CO$_2$ were small and not significant for most traits. However, at 3 mg L$^{-1}$ DO a negative synergistic effect was detected where embryo survival under elevated $p$CO$_2$ (2,200 – 4,400 µatm) declined by ~30% relative to control $p$CO$_2$ levels (~400 µatm). These results demonstrate that M. menidia offspring are sensitive to DO levels well above operational hypoxia thresholds and conditions already seasonally prevalent in productive coastal habitats. Furthermore, the interactive effect on embryo survival suggests that acidified conditions increase the DO threshold for hypoxia-induced embryonic mortality.
**Introduction**

Occurrences of low dissolved oxygen (DO) in marine systems are increasing in frequency and severity under the combined anthropogenic drivers of global warming and eutrophication (Diaz and Rosenberg, 2008; Vaquer-Sunyer and Duarte, 2008). Hypoxia is predicted to become increasingly common in coastal systems and thereby negatively impact marine fisheries (Breitburg, 1992; Rabalais et al., 2009). Aggressive reduction of nutrient pollution can help prevent episodic or chronic hypoxia, but ecosystem managers are compelled to weigh the ecological benefits of DO restoration against the economic costs of pollution reduction (Diaz and Rosenberg, 2008). Thus, informed policy decisions require detailed knowledge of DO thresholds that trigger lethal and sub-lethal responses in marine fauna (Paerl et al., 1998; Paerl, 2006; Vaquer-Sunyer and Duarte, 2008). Decades of laboratory and field observations have demonstrated a range of negative responses to hypoxia including direct mortality, reduced growth, and altered behaviors and distributions, all of which can impact the abundance and diversity of fish stocks (Pihl et al., 1991; Breitburg, 2002; Miller et al., 2002; Ekau et al., 2010).

However, there is a growing understanding that the heterotrophic processes that drive coastal deoxygenation simultaneously produce elevated $pCO_2$ conditions (Cai et al., 2011; Wallace et al., 2014; Baumann et al., 2015). Until recently, the co-occurrence of hypoxia and acidification as a coupled, multi-stressor symptom of marine climate change has largely been ignored by experimental approaches (Gobler and Baumann, 2016). In highly productive nearshore habitats (e.g., salt marshes, sea grass meadows, and enclosed lagoons) where biologically driven $pCO_2$/DO fluctuations are ubiquitous (Baumann and Smith, 2017), anthropogenic effects threaten to amplify natural fluctuations and exacerbate the duration and severity of extreme events (Gruber, 2011; Doney et al., 2012; McNeil and Sasse, 2016). Furthermore, the advancement of ocean acidification
(OA) will decouple how $pCO_2$ and DO co-vary by increasing baseline acidity levels (Melzner et al., 2012; Pacella et al., 2018).

Species that inhabit dynamic nearshore systems are likely adapted to the local variability in $pCO_2$ and DO (Hoffmann and Hercus, 2000), but future extreme events may push conditions beyond their physiological tolerance limits (Hofmann and Todgham, 2010; Melzner et al., 2012). Furthermore, higher baseline $pCO_2$ conditions may increase hypoxia sensitivity thresholds by reducing the functional capacity of pH-sensitive tissues involved in oxygen acquisition or through metabolic adjustments to support increased acid-base regulation (Pörtner, 2012; Heuer and Grosell, 2014). Factorial experimentation is a powerful method for quantifying multi-stressor effects and their interactions (Wernberg et al., 2012). Multi-stressor exposures can simply produce additive effects (no stressor interaction), but responses can also be antagonistic (i.e., less than the sum of individual responses) or synergistic (i.e., amplifying the overall effect relative to the sum of individual effects) (Gobler et al., 2014). Although few species have been tested to date, interactive effects of $pCO_2 \times$ DO may be common (Gobler and Baumann, 2016). For example, juvenile rockfish (Sebastes spp.) showed acute metabolic and behavioral effects under combined high $pCO_2$/low DO conditions (Davis et al., 2018). In both juvenile Atlantic and inland silversides (Menidia menidia and M. beryllina), acidified conditions increased hypoxia sensitivity by raising the DO threshold for surface respiration and mortality (Miller et al., 2016). In adult woolly sculpin (Clinocottus analis), exposure to acidified conditions increased metabolic rates associated with acid-base regulation, resulting in increased critical oxygen tension and hypoxia sensitivity (Hancock and Place, 2016).

Fish early life-stage are predicted to be the most vulnerable to the direct effects of marine climate change (Pörtner and Peck, 2010). Predicted near-future $pCO_2$ conditions have been shown to elicit
a range of negative survival, metabolic, and behavioral responses in a taxonomically diverse group of fish (Cattano et al., 2018). Oxygen acquisition is likely more sensitive to the downstream effects of high $pCO_2$-acclimation in early offspring that are still developing competent acid/base regulatory and cardiovascular tissues (Pörtner, 2012). Furthermore, embryos and early larvae lack the mobility to simply avoid sub-optimal conditions, and thus must rely on physiological mechanisms to cope with high $pCO_2$/low DO conditions. However, only a single study has so far evaluated fish early-life $pCO_2 \times$ DO effects. Offspring of the sheepshead minnow (Cyprinodon variegatus) appeared resilient to extreme conditions, but negative additive and synergistic effects on survival and growth were documented in both M. beryllina and M. menidia (DePasquale et al., 2015). Given the prevalence of interactive $pCO_2$/DO effects in fish, and the lack of observations from embryos and larvae, there is a critical need to expand our baseline understanding of multi-stressor effects during the early life-stage of fish.

The present study quantified individual and combined effects of acidification and hypoxia on whole-animal response traits in Atlantic silverside offspring. The species is an ecologically important coastal forage fish with a broad latitudinal range along the east coast of North America (Conover and Ross, 1982). It reproduces in spring and summer in shallow nearshore habitats that are highly vulnerable to impacts from OA, warming, and eutrophication (Hughes et al., 2015). Furthermore, two studies have already tested $pCO_2 \times$ DO effects in this species, allowing for a comparison of relative sensitivities across different silverside populations and experimental setups. Such comparisons enable a more robust estimate of stressor effects given the high response variability common in laboratory experiments (Baumann et al., in press). This study consisted of two independent trials that reared offspring under nine combinations of current to future $pCO_2 \times$
DO conditions from fertilization through the end of the early larval stage to document treatment effects on time-to-hatch, embryo survival, hatch size, larval survival, and larval growth rate.

Methods

Field sampling and fertilization: Two collections of spawning-ripe Atlantic silversides were made by beach seine from Mumford Cove, CT (41°19′25″ N 72°01′07″ W) one month apart during the spring of 2017 (Table 1). Adults were transported to the lab (Rankin Seawater Facility at University of Connecticut Avery Point) where they were sorted by sex and held overnight at 20°C without food in 40 L aerated tanks. The next day, 20+ ripe adults of each sex were used to fertilize experimental embryos using well-established strip-spawning protocols (Murray et al., 2014; Malvezzi et al., 2015; Murray and Baumann, 2018). Eggs from females were gently mixed into plastic dishes lined with 1-mm window screening submerged in seawater. Milt from males was collected into 500-ml beakers, mixed with seawater, and gently poured into spawning dishes to soak for ~15 min. Fertilized embryos uncoil chorionic filaments that attach to window screening, allowing unfertilized eggs to be gently rinsed away. Embryos were then disinfected in 100-ppm iodine/seawater solution for ~15 min (Ovadine®, Western Chemical, Inc.). Window screening was then cut into small sections so that embryos could be counted under low magnification with high accuracy for distribution to rearing containers. The number of spawners used for each trial and their length measurements are reported in Table 1.

Experimental pCO$_2$ and DO conditions: Treatment levels were chosen to reflect current and predicted future pCO$_2$ × DO conditions in temperate estuaries (Fig. 1; Wallace et al., 2014; Baumann et al., 2015; Baumann and Smith, 2017; Pacella et al., 2018). The target pCO$_2$ level for
control treatments was 400 µatm (~8.15 pHNIST), a concentration that resembles the average open-ocean and also *M. menidia* spawning habitats during early spring (Murray et al., 2014). The target level for the intermediate \( \text{pCO}_2 \) treatment was 2,200 µatm, a level that is commonly experienced by silverside offspring in late spring and summer (Baumann et al., 2015), but also represents an important benchmark for OA studies as the maximum open-ocean prediction for the next 300 years (Caldeira and Wickett, 2005). The target \( \text{pCO}_2 \) level for the extreme treatment was 4,400 µatm (~7.20 pH), a level that is rarely reached in coastal systems today but may become more common under future climate and eutrophication scenarios (Cai et al., 2011; Wallace et al., 2014; Pacella et al., 2018). Experimental DO treatments were inferred by observations of co-varying, metabolically-driven, \( \text{pCO}_2/\text{DO} \) variations in a coastal system during the silverside spawning season (Baumann et al., 2015). Fully saturated DO treatments were maintained at 7.5 mg L\(^{-1}\) (~100% saturation) while sub-oxic and hypoxic DO conditions were set to 4 mg L\(^{-1}\) (55%) and 2.5 mg L\(^{-1}\) (33%, trial 1), respectively. For trial 2, the extreme DO level was increased to 3.0 mg L\(^{-1}\) (42%) to avoid complete larval mortality observed during trial 1.

Seawater acidification protocols were designed in accordance with the best practices and guidelines for ocean acidification research (Riebesell et al., 2010). We developed a LabView (National Instruments\textsuperscript{®}) program to automate \( \text{pCO}_2 \) and DO manipulations in nine treatment tanks. The program is described in detail by Murray et al. (2018). The system monitored each tank once per hour by energizing a submerged sampling pump that sent treatment water to a central pH electrode (Hach pHD\textsuperscript{®}, calibrated weekly using 2-point pHNIST references) and an optical dissolved oxygen (DO) probe (Hach LDO\textsuperscript{®} Model 2). The probes were allowed to stabilize for six minutes before the system automatically corrected discrepancies between measured and programmed pH and DO conditions by injecting standardized amounts of 100% CO\(_2\) (bone dry grade, AirGas\textsuperscript{®}),
nitrogen gas, or CO2-stripped air into sump tanks. Temperatures were maintained by thermostat controlled submersible heaters (Aqualogic®). Actual treatment pCO₂ conditions were calculated in CO2SYS (V2.1, http://cdiac.ornl.gov/ftp/co2sys) based on measurements of pH, temperature, salinity, and total alkalinity (AT). For a detailed description of AT measurements and carbon chemistry calculations refer to Murray et al. (2018). The pH and carbonate chemistry measurements of this study are reported in Table S1.

**Experimental design:** Nine customized recirculating units were constructed to rear fish early life-stages in factorial experimental designs. Each unit cycled water through three main compartments; a header tank (40 L), a sump (90 L), and a main tank (240 L). Five replicate rearing-containers (20 L polyethylene buckets) were housed in each main tank. Developing embryos were held in customized embryo-baskets (2-l polyethylene cups fitted with 300-µm meshed bottoms) which were floated inside each rearing container. Seawater from header tank was continuously fed into each embryo basket at 4 L hr⁻¹ and then exited replicate rearing-containers through screened overflow holes (100 µm) into the main tank before draining into the sump for re-treatment. Optimal temperature (24°C), salinity (30 psu), light conditions (15h light:9 h dark) for rearing *M. menidia* were maintained throughout the experiment (Middaugh et al., 1987).

Experimental trials were initiated within 2 hr of fertilization when precisely 100 embryos were distributed into embryo-baskets (20 L, N = 5). At day 5 post-fertilization (dpf) and then daily thereafter, replicates were checked every 12 hr for hatchlings, which were counted and moved to the main rearing-container. Sub-samples (N = 10) for initial length measurements were taken on the first day a replicate had 10+ hatchlings. Sub-samples were preserved in a 5% formaldehyde/freshwater solution saturated with sodium tetraborate buffer. Embryo baskets were pulled from rearing containers after 12 dpf. To stimulate initial feeding, newly hatched larvae were
immediately provided with equal rations of powdered weaning diet (Otohime Marine Fish Diet, size A1, Reed Mariculture®) in addition to *ad libitum* levels of newly hatched brine shrimp nauplii (*Artemia salina*, San Francisco strain, brineshrimpdirect.com). Larvae were fed daily *ad libitum* rations of newly hatched nauplii for the remainder of the experiment. Waste and uneaten food were siphoned daily and treatment seawater was exchanged regularly to maintain levels of ammonia waste below 0.25 ppm (Saltwater Ammonia Test Kit, API®). Larvae were reared until 15 d post-hatch (dph) when all survivors were counted and preserved for final length measurements. All length measurements (standard length, nearest 0.01 mm) were made via calibrated microscope images using Image Pro Premier (V9.0, Media Cybernetics®).

**Response traits and statistical analysis:** Five response traits were calculated for each replicate. Time to hatch was calculated from the day of fertilization to the average day of first hatch. Embryo survival (%) was calculated as the number of total hatchlings divided by the initial number of embryos (i.e., 100). Larval survival (%) was calculated as the number of surviving larvae at 15 dph divided by the number of survivors at hatch minus 10 initial sub-samples. Hatch lengths (mm) were averaged for each replicate. Mean growth rates (mm d⁻¹) were calculated by dividing the length differential (mean final larval length – mean hatch length) by 15 d. Treatment means ± standard deviation (s.d.) were calculated for each response trait. To quantify differences in hatch timing, cumulative daily hatching curves (from 5 – 12 dpf) were calculated for each treatment.

All statistical tests were conducted in SPSS (V20, IBM). Prior to analysis, proportional data were logit transformed \([\log_{10}(\text{value}/(1 – \text{value}))]\) with zero and one values replaced with 0.01 and 0.99, respectfully (Warton and Hui, 2011). Two replicates were removed from analysis of embryo survival due to an error in embryo allotment during trial 2, but were retained for tests on all other traits. Two-way linear mixed-effects models (LMEs) were used to test for significant effects (α
< 0.05) of $pCO_2$, DO, their interaction (fixed factors) and trial (random factor) on survival and growth traits using the model:

$$\text{Response trait} = pCO_2 + DO + pCO_2 \times DO + \text{trial} + \text{error}.$$ 

Bonferroni corrected post-hoc tests were used for multiple comparisons. For response traits exhibiting significant $pCO_2 \times DO$ interactive effects, a LMEM using $pCO_2$ as fixed factor and trial as random factor was used to test for significant $pCO_2$ effects within DO treatments. For DO treatments not repeated between trials (2.5 and 3.0 mg L$^{-1}$), nonparametric Kruskal-Wallis one-way ANOVAs was used to test for $pCO_2$ effects. Dunn-Bonferroni tests were applied for multiple comparisons. No statistics were computed for time to hatch which were just reported for comparison.

**Results**

**Survival:** Time to hatch was shortest under saturated DO conditions with larvae first emerging on day 6 post-fertilization. Hatching was delayed to 7 dpf at 3 and 4 mg L$^{-1}$ and 8 dpf under 2.5 mg L$^{-1}$ (Fig. 2). There were only small (<1 d) differences in hatch timing between $pCO_2$ levels within DO treatments (Fig. 2). Overall, embryo survival ranged from 30 to 95% (Fig. 3A-B) and was strongly reduced by declining DO conditions (LMEM, $p < 0.001$, Table 2). Across trials, embryo survival was similar at 7.5 (84±14%) and 4 mg L$^{-1}$ DO (75±19%), but declined significantly at 3 (65±19%) and 2.5 mg L$^{-1}$ (32±8%) relative to oxygen saturated conditions (Bonferroni, $p < 0.01$).

While embryo survival was unaffected by $pCO_2$ conditions at 2.5 and 7.5 mg L$^{-1}$ (Table 2), the LMEM detected a $pCO \times DO$ interaction ($p = 0.037$, Table 2) driven by significant but divergent
\( pCO_2 \) responses at 3 (Kruskal-Wallis, \( X^2 (2) = 10.842, p = 0.012 \) and 4 mg L\(^{-1} \) DO (LMEM, \( F_{5,24} = 6.472, p = 0.005 \)). Within the 4 mg L\(^{-1} \) DO treatments, embryo survival was highest at 2,200 \( \mu \)atm (86±18\%) and declined to 71±21\% under 400 \( \mu \)atm) and was significantly reduced to 66%±11 at 4,400 \( \mu \)atm \( pCO_2 \) (Bonferroni, \( p = 0.006 \), Fig. 3A). However, at 3 mg L\(^{-1} \) DO embryo survival remained high at 400 \( \mu \)atm (86±16\%) but was significantly lower at 2,200 \( \mu \)atm (49±9\%, Dunn-Bonferroni, \( p =0.007 \)) and 4,400 \( \mu \)atm (61±5\%, Dunn-Bonferroni, \( p =0.040 \), Fig. 3B).

Larval survival ranged from 0 to 87\% (Fig. 3C-D) and was significantly affected by DO (LMEM, \( p < 0.001 \)) but not by \( pCO_2 \) conditions (Table 2). Survival was highest under saturated DO conditions (71±16\%) and declined significantly to 43±20\%, 14±16\%, and 0\% at 4, 3, and 2.5 mg L\(^{-1} \) DO, respectively (Bonferroni, \( p < 0.001 \)).

**Growth:** The mean size of newly hatched larvae ranged from 4.14 to 5.30 mm (Fig. 3E-F), and an LMEM detected a significant effect of DO (\( p < 0.001 \)) but not of \( pCO_2 \) (Table 2). Larvae were longest under saturated DO conditions (5.26±0.26 mm), while exposure to 4.0, 3.0, and 2.5 mg L\(^{-1} \) significantly reduced hatch sizes to 4.61±0.13, 4.40±0.10 mm, 4.22±0.10 mm, respectively (Bonferroni, \( p < 0.001 \)). Additionally, hatched larvae were significantly smaller at 2.5 and 3.0 mg L\(^{-1} \) relative to 4 mg L\(^{-1} \) DO (Bonferroni, \( p < 0.001 \)).

Post-hatch growth rates ranged from 0.33 to 0.74 mm d\(^{-1} \) (Fig. 3G-H) and were significantly affected by DO (LMEM, \( p < 0.001 \)) but not by \( pCO_2 \) level (Table 2). Overall, growth was fastest at 7.5 mg L\(^{-1} \) DO (0.68 mm d\(^{-1} \)) and declined significantly to 0.52 and 0.35 mm d\(^{-1} \) at 4 and 3 mg L\(^{-1} \) DO, respectively (Bonferroni, \( p < 0.001 \)). Growth was significantly faster at 4 relative to 3 mg L\(^{-1} \) DO (Bonferroni, \( p < 0.001 \)). No growth rates were calculated for 2.5 mg L\(^{-1} \) DO due to near complete mortality.
Discussion

This study characterized the individual and combined effects of acidification and hypoxia on early-life survival and growth in the ecologically important forage fish *M. menidia*. Across two trials, survival and growth severely declined with DO level, but few additional effects of \( p\text{CO}_2 \) were detected. No offspring survived exposure to the most extreme DO treatment (2.5 mg L\(^{-1}\) DO) while larval survival at 3.0 mg L\(^{-1}\) was five-fold lower than under saturated DO conditions. Reduced survival was also observed in offspring reared at 4 mg L\(^{-1}\) DO, a level commonly reached in silverside spawning habitat (Baumann et al., 2015; Baumann and Smith, 2017). Sub-lethal effects of low DO included delayed hatching, reduced hatch size, and slower post-hatch growth. Relative to DO, the effects of \( p\text{CO}_2 \) were small and not significant for most traits. However, at 3 mg L\(^{-1}\) DO survival of embryos reared under elevated \( p\text{CO}_2 \) was ~30% lower relative to the 400 µatm \( p\text{CO}_2 \) and thus indicative of a negative synergistic effect. These results demonstrate that *M. menidia* offspring are sensitive to DO levels well above the average operational threshold for designating hypoxia (~2.3 mg L\(^{-1}\); Vaquer-Sunyer and Duarte, 2008).

Interactive effects of elevated \( p\text{CO}_2 \) and low DO may be common in marine fauna (Gobler and Baumann, 2016). The observed negative synergistic effect on embryo survival is consistent with both previous studies on *M. menidia* that showed similar survival effects in larvae and juveniles (DePasquale et al., 2015; Miller et al., 2016). Exactly how elevated \( p\text{CO}_2 \) conditions increase hypoxia sensitivity in this and other species is presently unknown. Acclimation to elevated \( p\text{CO}_2 \) may increase basal metabolic rates and thus oxygen requirements of developing embryos, or acidification could directly compromise the functional capacity of tissues involved in oxygen acquisition and allocation (Perry and Gilmour, 2006; Berenbrink et al., 2011; Pörtner, 2012; Zaprudnova et al., 2015). In contrast to 3 mg L\(^{-1}\) DO, there was no influence of \( p\text{CO}_2 \) detected
within 2.5 mg L\(^{-1}\) DO treatments. A potential explanation for this inconsistency is that exposure to 2.5 mg L\(^{-1}\) DO was already lethal for most individuals and the additional effects of \(p\)CO\(_2\) were thus undetectable. Together, these responses suggest that exposure to elevated \(p\)CO\(_2\) can lower the DO threshold for lethal effects, but not necessarily exacerbate the response. Nevertheless, negative synergistic effects of acidification and hypoxia in \textit{M. menidia} offspring have now been observed in three independent studies, thereby highlighting the importance of multi-stressor experiments to accurately quantify climate effects in fish.

The continued reduction of DO will be among the most consequential anthropogenic impacts to marine life (Breitburg, 2002; Diaz and Rosenberg, 2008; Rabalais et al., 2009). Effective policy initiatives aimed at reducing hypoxia in coastal habitats require fundamental information on the DO thresholds that trigger negative responses in marine fauna (Paerl, 2006). In this study, exposure 2.5 mg L\(^{-1}\) DO was highly lethal for \textit{M. menidia} offspring. Of the few embryos that survived to hatch, all emerged severely underdeveloped and perished within days. An increase in DO to 3 mg L\(^{-1}\) modestly improved embryo survival, but larval survival was still five-fold lower than in saturated conditions. Even exposure to 4 mg L\(^{-1}\) DO, a level common in \textit{M. mendia} nursery habitat (Baumann and Smith, 2017), still reduced overall offspring survival by half. All low DO exposures resulted in significant sub-lethal effects; time to first hatch was extended, and both size at hatch and post-hatch growth rates severely declined at lower DO levels. Sub-lethal growth effects during early life could negatively affect recruitment and population dynamics, because smaller, weaker, and slower growing offspring experience higher cumulative mortality rates from starvation or predation (Sissenwine, 1984; Anderson, 1988; Miller et al., 1988). Taken together, these results indicate that \textit{M. menidia} early life-stages are highly sensitive to low DO and lack the compensatory mechanisms exhibited by other nearshore species (Miller et al., 2002; DePasquale et al., 2015;
Davis et al., 2018). Importantly, the high threshold for negative DO responses (> 50% saturation) suggest *M. mendia* early life-stages already live close to their physiological tolerance limits, and further reductions in ambient DO of nearshore habitats would adversely impact this important forage fish.

Overall, low DO conditions were more lethal for larvae than in embryos. Hypoxia tolerance may be adaptive in silverside embryos that are generally fixed to benthic vegetation in shallow estuaries that can be prone to periodic hypoxia (Conover and Ross, 1982; Pihl et al., 1991; Hughes et al., 2015). Acclimation to low DO in embryos is likely achieved by depressing metabolic rates to reduce oxygen demand (Nilsson and Östlund-Nilsson, 2008). Metabolic depression by embryos in this study was evidenced by the 0.6, 0.8, and 1.0 mm reduction in hatch size at 4, 3, and 2.5 mg L\(^{-1}\) DO, respectively. However, the higher rates of mortality observed post-hatch suggest swimming and feeding larvae cannot sufficiently depress metabolism to match declining oxygen supply. Thus, the early larval stage likely serves as a bottleneck for hypoxia tolerance in *M. menidia*. In 3 mg L\(^{-1}\) DO treatments, offspring that were able to survive to 15 dph were largely restricted to swimming in immediate surface waters, likely taking advantage of the higher DO levels at the air-water interface. In the wild, such a behavior response would likely increase rates of predation or reduce foraging ability of developing larvae (Miller et al., 2016). Furthermore, there is accumulating evidence that elevated \(pCO_2\) exposure elicits a range of deleterious behavioral responses in fish (Munday et al., 2009; Jutfelt et al., 2013; Nagelkerken and Munday, 2016). As mobile offspring, fish larvae can best evade the physiological challenges of hypoxia by simply avoiding it (Breitburg, 1992). However, how acidified conditions alter the ability of fish larvae to detect and avoid advancing hypoxia remains completely untested.
The static $p$CO$_2$ × DO treatments applied here were intended to generate much needed baseline information on multi-stressor exposures in an important forage fish. However, anthropogenic effects will not only alter the average conditions of nearshore habitats but also amplify existing $p$CO$_2$/DO variability and increase severity and duration of extreme conditions (Melzner et al., 2012; McNeil and Sasse, 2016; Pacella et al., 2018). Thus, experimental approaches must advance in sophistication to test how organisms will respond to current and predicted future $p$CO$_2$/DO fluctuations (Gobler and Baumann, 2016). Fluctuations might ameliorate the negative effects of extreme levels by allowing organisms to recover while the system oscillates to more favorable conditions (Frieder et al., 2014). By contrast, constant physiological adjustments to match changing environmental conditions might be more stressful for sensitive early life-stages. Furthermore, global warming constitutes the third major anthropogenic stressor afflicting marine habitats (Doney et al., 2012). The extent to which organisms are affected by acidification and hypoxia may depend on temperature, and warming is predicted to increase organismal sensitivity to both stressors (Pörtner et al., 2005; Vaquer-Sunyer and Duarte, 2011; Pörtner, 2012; Clark et al., 2013). However, three-way factorial experiments produce unwieldy designs generally not feasible for studies on fish early life-stages. Thus, to incorporate all major stressors, experimenters should strategically select treatment levels that best reflect future conditions while maintaining reasonable experimental designs.

In summary, this study confirmed that low DO conditions severely reduced early-life performance in $M$. menidia offspring. Lethal and sub-lethal effects were observed well above traditional DO thresholds for identifying hypoxia. While the relative effects of acidification were small, exposure to elevated $p$CO$_2$ increased the DO threshold for hypoxia induced embryonic mortality from 2.5 to 3 mg L$^{-1}$. Future work is needed to quantify how fish early life-stages respond to the
amplification of high-frequency $p$CO$_2$ and DO fluctuations under the warming conditions predicted for the near-future in dynamic coastal environments.

**Acknowledgements**

This study was funded through a National Science Foundation grant to H.B. (NSF-OCE 1536165). We are grateful to the Charlie Woods, Julie Pringle, Jacob Snyder, James Harrington, Charles Dyke, and Isaiah Mayo for laboratory assistance and to Lucas Jones for conducting in larval measurements.
References


Gruber, N. 2011. Warming up, turning sour, losing breath: ocean biogeochemistry under global change. Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences, 369: 1980-1996.


Tables

Table 1: Spawning ripe *M. menidia* collected from Mumford Cove, CT to fertilize embryos used in factorial \(\text{CO}_2 \times \text{DO}\) experiments. Adult lengths are shown as mean total lengths (TL, cm) ± standard deviation.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Collection date</th>
<th>Fertilization date</th>
<th># Female spawners</th>
<th>Female TL</th>
<th># Male spawners</th>
<th>Male TL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5/8/2017</td>
<td>5/9/2017</td>
<td>21</td>
<td>9.5±1.5</td>
<td>39</td>
<td>8.0±1.0</td>
</tr>
<tr>
<td>2</td>
<td>6/8/2017</td>
<td>6/9/2017</td>
<td>28</td>
<td>11.0±1.0</td>
<td>23</td>
<td>9.0±1.0</td>
</tr>
</tbody>
</table>

Table 2: Outputs of linear mixed effect models testing the significance of fixed effects on survival and growth traits on *M. menidia* offspring.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Fixed effects</th>
<th>F</th>
<th>Num. df</th>
<th>Den. df</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo survival</td>
<td>(p\text{CO}_2)</td>
<td>0.837</td>
<td>2</td>
<td>75</td>
<td>0.437</td>
</tr>
<tr>
<td></td>
<td>DO</td>
<td>17.641</td>
<td>3</td>
<td>75</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(p\text{CO}_2\times\text{DO})</td>
<td>4.318</td>
<td>6</td>
<td>75</td>
<td>0.001</td>
</tr>
<tr>
<td>Larval survival</td>
<td>(\text{CO}_2)</td>
<td>0.618</td>
<td>2</td>
<td>77</td>
<td>0.542</td>
</tr>
<tr>
<td></td>
<td>DO</td>
<td>150.752</td>
<td>3</td>
<td>77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(p\text{CO}_2\times\text{DO})</td>
<td>1.823</td>
<td>6</td>
<td>77</td>
<td>0.105</td>
</tr>
<tr>
<td>Hatch length</td>
<td>(p\text{CO}_2)</td>
<td>0.786</td>
<td>2</td>
<td>77</td>
<td>0.459</td>
</tr>
<tr>
<td></td>
<td>DO</td>
<td>211.987</td>
<td>3</td>
<td>77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(p\text{CO}_2\times\text{DO})</td>
<td>1.363</td>
<td>6</td>
<td>77</td>
<td>0.240</td>
</tr>
<tr>
<td>Growth rate</td>
<td>(p\text{CO}_2)</td>
<td>0.073</td>
<td>2</td>
<td>63</td>
<td>0.930</td>
</tr>
<tr>
<td></td>
<td>DO</td>
<td>82.546</td>
<td>2</td>
<td>63</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(p\text{CO}_2\times\text{DO})</td>
<td>0.761</td>
<td>4</td>
<td>63</td>
<td>0.554</td>
</tr>
</tbody>
</table>
Fig. 1: Dissolved oxygen (mg L$^{-1}$, blue line and circles) and pH (NIST, red line and circles) conditions during the spring and summer of 2017 from the source location of spawning $M$. menidia adults (Mumford Cove, CT; 41° 19' 26'' N, 72° 1' 10'' W$^\prime$). Faded circles are all individual measurements, solid lines are smoothed data fits (loess smoothing function, nearest 10%, SigmaPlot®). Publicly available source data were retrieved from DOI: 10.1575/1912/bco-dmo.660079.
**Fig. 2:** Cumulative hatching success (%) of *M. menidia* offspring reared at three *pCO₂* levels (400 µatm, blue lines; 2,200 µatm, green lines; 4,500 µatm, red lines) crossed with four DO concentrations (2.5, 3.0, 4.0, and 7.5 mg L⁻¹). Lines represent treatment mean cumulative hatching success pooled from both trials.
Fig. 3: Treatments mean (±s.d.) embryo survival (%; A-B), larval survival (%; C-D), hatch length (mm, E-F), and growth rate (mm d\(^{-1}\); G-H) from *M. menidia* offspring from two trials reared under three \(p\)CO\(_2\) levels (400 µatm, blue lines; 2,200 µatm, green lines; 4,500 µatm, red lines) crossed with four total DO concentrations (2.5, 3.0, 4.0, and 7.5 mg L\(^{-1}\)).
Appendix for Chapter 4: A Factorial Evaluation of the Combined Effects of Acidification and Hypoxia in Atlantic Silverside Offspring

**Table S1:** Carbon chemistry, dissolved oxygen (DO), and temperature measurements from CO$_2$ × DO factorial experiments on *M. menidia* offspring. Mean (±s.d.) temperature (°C), pH (NIST), and DO (mg/L) are from daily measurements. Mean (±s.d.) total alkalinity ($A_T$; μmol kg$^{-1}$), dissolved inorganic carbon ($C_T$; μmol kg$^{-1}$), partial pressure and fugacity of CO$_2$ ($pCO_2$; fCO$_2$; μatm), and carbonate ion concentration ($CO_3^{2-}$; μmol kg$^{-1}$) quantified from replicated seawater samples. Salinity was measured via refractometer (30 psu for all samples) and $A_T$ from endpoint titrations while $pCO_2$, $C_T$, fCO$_2$ and CO$_3^{2-}$ were calculated in CO2SYS.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Measured Temp</th>
<th>Target DO</th>
<th>Measured DO</th>
<th>Measured $pCO_2$</th>
<th>Measured pH</th>
<th>$pCO_2$</th>
<th>$A_T$</th>
<th>$C_T$</th>
<th>fCO$_2$</th>
<th>CO$_3^{2-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24.7 ± 0.4</td>
<td>8.0</td>
<td>7.8 ± 0.1</td>
<td>400</td>
<td>8.18 ± 0.02</td>
<td>370 ± 1</td>
<td>2001 ± 6</td>
<td>1769 ± 5</td>
<td>369 ± 1</td>
<td>165.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>24.5 ± 0.3</td>
<td>8.0</td>
<td>7.7 ± 0.1</td>
<td>2100</td>
<td>7.56 ± 0.1</td>
<td>1826 ± 7</td>
<td>2005 ± 8</td>
<td>1990 ± 8</td>
<td>1821 ± 7</td>
<td>46.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>24 ± 0.6</td>
<td>8.0</td>
<td>7.7 ± 0.1</td>
<td>4500</td>
<td>7.19 ± 0.07</td>
<td>4368 ± 19</td>
<td>1998 ± 9</td>
<td>2099 ± 9</td>
<td>4354 ± 19</td>
<td>20.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>24.6 ± 0.4</td>
<td>4.0</td>
<td>4.1 ± 0.4</td>
<td>400</td>
<td>8.12 ± 0.04</td>
<td>439 ± 2</td>
<td>1996 ± 7</td>
<td>1793 ± 6</td>
<td>437 ± 2</td>
<td>145.8 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>24.7 ± 0.4</td>
<td>4.0</td>
<td>4.0 ± 0.4</td>
<td>2100</td>
<td>7.46 ± 0.06</td>
<td>2338 ± 5</td>
<td>2002 ± 4</td>
<td>2015 ± 4</td>
<td>2330 ± 5</td>
<td>37.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>24.2 ± 0.5</td>
<td>4.0</td>
<td>4.1 ± 0.3</td>
<td>4500</td>
<td>7.22 ± 0.03</td>
<td>4119 ± 17</td>
<td>2004 ± 8</td>
<td>2093 ± 8</td>
<td>4105 ± 16</td>
<td>21.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>24.9 ± 0.3</td>
<td>2.5</td>
<td>2.7 ± 0.4</td>
<td>400</td>
<td>8.15 ± 0.05</td>
<td>400 ± 1</td>
<td>2004 ± 3</td>
<td>1783 ± 3</td>
<td>399 ± 1</td>
<td>157.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>24.2 ± 0.5</td>
<td>2.5</td>
<td>2.7 ± 0.4</td>
<td>2100</td>
<td>7.48 ± 0.08</td>
<td>2189 ± 4</td>
<td>2010 ± 3</td>
<td>2017 ± 3</td>
<td>2182 ± 4</td>
<td>39.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>24.3 ± 0.4</td>
<td>2.5</td>
<td>2.5 ± 0.3</td>
<td>4500</td>
<td>7.2 ± 0.05</td>
<td>4337 ± 36</td>
<td>2114 ± 17</td>
<td>2112 ± 18</td>
<td>4323 ± 36</td>
<td>21 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>24.4 ± 0.3</td>
<td>8.0</td>
<td>7.8 ± 0.1</td>
<td>400</td>
<td>8.17 ± 0.08</td>
<td>385 ± 2</td>
<td>2062 ± 11</td>
<td>1829 ± 10</td>
<td>384 ± 2</td>
<td>167.5 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>24.7 ± 0.3</td>
<td>8.0</td>
<td>7.8 ± 0.1</td>
<td>2100</td>
<td>7.5 ± 0.07</td>
<td>2173 ± 22</td>
<td>2060 ± 21</td>
<td>2062 ± 21</td>
<td>2166 ± 22</td>
<td>42.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>24.4 ± 0.4</td>
<td>8.0</td>
<td>7.7 ± 0.1</td>
<td>4500</td>
<td>7.19 ± 0.11</td>
<td>4539 ± 55</td>
<td>2064 ± 25</td>
<td>2167 ± 26</td>
<td>4524 ± 55</td>
<td>21.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>24.4 ± 0.4</td>
<td>4.0</td>
<td>4.2 ± 0.3</td>
<td>400</td>
<td>8.07 ± 0.09</td>
<td>505 ± 1</td>
<td>2046 ± 4</td>
<td>1861 ± 4</td>
<td>503 ± 1</td>
<td>137.2 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>24.4 ± 0.3</td>
<td>4.0</td>
<td>4.1 ± 0.4</td>
<td>2100</td>
<td>7.5 ± 0.05</td>
<td>2157 ± 12</td>
<td>2055 ± 12</td>
<td>2057 ± 12</td>
<td>2151 ± 12</td>
<td>42 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>24.4 ± 0.4</td>
<td>4.0</td>
<td>4.1 ± 0.4</td>
<td>4500</td>
<td>7.19 ± 0.07</td>
<td>4512 ± 20</td>
<td>2069 ± 10</td>
<td>2162 ± 10</td>
<td>4498 ± 20</td>
<td>21.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>24 ± 0.4</td>
<td>3.0</td>
<td>3.1 ± 0.5</td>
<td>400</td>
<td>8.06 ± 0.09</td>
<td>520 ± 5</td>
<td>2050 ± 19</td>
<td>1871 ± 18</td>
<td>518 ± 5</td>
<td>133.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>24.1 ± 0.4</td>
<td>3.0</td>
<td>3.0 ± 0.3</td>
<td>2100</td>
<td>7.5 ± 0.05</td>
<td>2151 ± 19</td>
<td>2039 ± 17</td>
<td>2043 ± 18</td>
<td>2144 ± 18</td>
<td>41 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>24.2 ± 0.5</td>
<td>3.0</td>
<td>3.0 ± 0.3</td>
<td>4500</td>
<td>7.19 ± 0.06</td>
<td>4473 ± 64</td>
<td>2053 ± 29</td>
<td>2155 ± 31</td>
<td>4459 ± 64</td>
<td>21 ± 0.3</td>
</tr>
</tbody>
</table>
Summary

Ongoing anthropogenic climate change will profoundly impact marine ecosystems (Hoegh-Guldberg and Bruno, 2010), yet uncertainty regarding how individual species will respond to future conditions still limits robust ecological predictions (Hofmann and Todgham, 2010). This dissertation addresses important knowledge gaps of how climate change will impact marine fish by applying novel experimental approaches. Wild offspring of two ecologically important forage fish species (*Menidia menidia* and *Ammodytes dubius*) from contrasting habitats (i.e., nearshore vs. offshore) served as study organisms in a series of laboratory experiments that tested the individual and combined effects of acidification (OA), hypoxia, and warming on survival and growth traits. While each chapter covered a range of specific response traits, the primary set of questions that were investigated by this dissertation and their conclusions are outlined in Table 1.

While adult fish have the necessary homeostatic capacity to tolerate acute exposures to future OA conditions (Ishimatsu et al., 2008), young offspring that lack regulatory tissues are likely more sensitive (Baumann et al., 2012). The most serious consequence of OA during early life is the direct mortality of embryos and larvae (Baumann et al., 2012; Chambers et al., 2014; Dahlke et al., 2017). Here, evidence that OA conditions can strongly reduce early-life survival was limited (Chapter 3). Exposure to *p*CO₂ levels predicted during the next 100-300 years precipitously reduced embryo survival in *A. dubius* (Table 1). As a consequence, there is now an urgent need for additional studies to elucidate the exact mechanisms that cause *p*CO₂-related mortality in sand lance early life-stages. The possibility that high sensitivity to OA is a conserved trait amongst members of the Ammodytidae that share a winter spawning phenology constitutes a major climate threat to many northern hemisphere ecosystems that are dependent on these important forage fish. By contrast, *M. menidia* offspring were largely tolerant of OA conditions and showed neutral
survival responses to even extreme $p$CO$_2$ levels (>4,000 µatm) (Table 1). While past OA studies have documented reduced survival in this species (Murray et al., 2014) the survival responses documented here are in line with the majority of experiments conducted on *M. menidia* (Baumann et al., 2018).

Larval size is positively related with early-life survival in fish (Anderson, 1988), thus data on how elevated $p$CO$_2$ exposure impacts embryonic and larval development are critically needed. For *M. menidia*, OA conditions had a weak negative effect on size at hatch and no effect on post-hatch growth rate (Table 1). Size effects were mostly absent (Chapter 1, 4). There was a single example of reduced hatch size (Chapter 2), but the effect was only transient as a subsequent trial showed an increase in hatch size under the same conditions. By contrast, embryo development of *A. dubius* was far more sensitive to OA conditions (Table 1). Chapter 3 documented a reduction in sand lance hatch size under OA and warm conditions, but offspring from all elevated $p$CO$_2$ treatments showed increased consumption of endogenous energy, signaling that OA can impose significant metabolic costs. However, no $p$CO$_2$ effect was detected on post-hatch growth. As with survival, the embryo stage appears more sensitive to $p$CO$_2$ effects on growth than post-hatch larvae. This is consistent with the broader consensus that fish embryos are the most OA sensitive life stage in fish (Baumann et al., 2012; Wittmann and Pörtner, 2013; Cattano et al., 2018).

The timing of hatch can be an important determinant of recruitment success in fish that inhabit seasonal ecosystems where physical conditions and food availability can vary over short intervals (Schultz et al., 1991). Across trials (Chapters 1, 2 and 4) OA conditions had no detectable effect on the timing of hatch in *M. mendia* (Table 1). By contrast, hatching by *A. dubius* was strongly influenced by OA conditions (Chapter 3, Table 1). Sand lance offspring reared under present-day $p$CO$_2$ conditions were consistently the first to emerge and mostly hatched in a single large pulse
over two or three days post-first hatch. By contrast, offspring reared under OA conditions hatched gradually over a protracted period of time, with the bulk of hatchlings emerging more than a week after control offspring. As winter spawners, sand lance in the Gulf of Maine likely time their reproduction such that larvae emerge at the beginning of the spring bloom when food resources are increasing but competition is low (Robards et al., 2000). If OA decouples the timing of hatch from important indicators of seasonality (i.e., temperature and day length), sand lance larvae may face increased rates starvation or predation mortality (Cushing, 1990).

The long-term consequences of chronic OA exposure remain understudied in marine fish (Cattano et al., 2018). While many species appear capable of acclimating to acute OA exposures (Ishimatsu et al., 2008), the metabolic costs of long-term acclimation remain largely unquantified. Here, Chapter 1 evaluated the long-term effects of OA in *M. menidia* offspring that were reared under modern and future $pCO_2$ conditions for 135 days post-fertilization, covering approximately a third of their lifespan (Table 1). Importantly, differences in growth between treatments were not detected until after two months when sub-sampled juveniles from elevated $pCO_2$ were significantly shorter than fish from the control group. At the conclusion of the experiment, the population from acidified conditions was on average 4% shorter and weighed 6% less than control juveniles. Interestingly, the OA population had a significantly higher condition factor. Furthermore, the two populations showed differences in their fatty acid profiles, with evidence that elevated $pCO_2$ juveniles were retaining specific fatty acids related to an anti-inflammatory stress response. These results demonstrate that even in OA-tolerant species, the cost of $pCO_2$ acclimation can still result in reduced somatic growth over the long-term. Thus, many experiments that attempt to extrapolate OA effects from short-term exposures may underestimate how elevated $pCO_2$ can impact whole-
life cycle traits. There is now a need for more advanced experimental design that quantify carry-over and long-term OA effects in fish from fertilization to reproduction.

While single-factor experiments provide important baseline responses to elevated $p$CO$_2$ conditions, there is a growing appreciation that OA shares the same underlying anthropogenic drivers as ocean warming and deoxygenation (Doney, 2010; Bopp et al., 2013). Thus, estimating the true climate change sensitivity of marine organisms requires that experiments consider the simultaneous effects of these coupled stressors (Kroeker et al., 2013; Gobler and Baumann, 2016).

I explored $p$CO$_2$ × temperature effects in *M. menidia* and *A. dubius* early-life stages, respectively (Chapters 2 and 3, Table 1). Contrary to expectations, I found no clear evidence for higher $p$CO$_2$ sensitivity in *M. menidia* at sub-optimal rearing temperatures. Offspring appeared fully capable of tolerating even extreme $p$CO$_2$ concentrations (>4,000 μatm) at temperatures that correspond to the thermal extremes experienced during their spawning season at this latitude. By contrast, exposure to warming greatly enhanced the lethality of OA conditions in *A. dubius*. At 10°C, $p$CO$_2$ conditions predicted for the end of this century resulted in the near complete mortality of *A. dubius* embryos. Furthermore, the reduction in body size produced by high $p$CO$_2$ exposure was exacerbated by warming. The Gulf of Maine (where *A. dubius* is a keystone forage fish) is one of the fastest warming regions of the ocean (Pershing et al., 2015). Thus, sand lance offspring may already be experiencing stressful thermal conditions that will be exacerbated by ongoing OA within decades.

Offspring of species that reproduce in productive nearshore estuaries are predicted to increasingly encounter metabolically driven periods of acidification and hypoxia (Doney et al., 2012; Wallace et al., 2014; Gobler and Baumann, 2016). Here, I tested $p$CO$_2$ × dissolved oxygen (DO) effects in *M. menidia* embryos and larvae (Chapter 4, Table 1). All survival and growth traits were highly sensitive to low-DO conditions, including levels well above the traditional threshold for
designating hypoxia (Vaquer-Sunyer and Duarte, 2008). Thus, *M. menidia* offspring may already encounter stressful DO conditions in their natal habitats. While there were few additional effects of OA, a negative synergistic effect was detected on embryo survival that showed elevated $p$CO$_2$ can modestly increase the threshold for lethal hypoxic effects in embryos. This synergistic effect is consistent with past $p$CO$_2$ × DO experiments on this species (DePasquale et al., 2015; Miller et al., 2016) which highlights the importance of factorial experiments that can quantify multi-stressor effects in fish.

In addition to the primary research questions in Table 1, an additional objective of this dissertation was to test of the Ocean Variability Hypothesis in fish (Baumann, 2019). To date, the large and mostly unexplained variability in early-life responses to elevated $p$CO$_2$ has hindered attempts to generalize OA effects in fish. One promising mechanism involves the role of local adaptation to existing $p$CO$_2$ variability of natal habitats. Early-life adaptations to counter acidified conditions are likely common in species that spawn in nearshore systems as demonstrated by the robust CO$_2$ tolerance of *M. menidia* offspring (Chapters 1, 2, and 4, Table 1). By contrast, offspring of the offshore and winter-spawning *A. dubius* were highly sensitive OA conditions (Chapter 3, Table 1). While additional studies are required to robustly confirm the OVH, it provides a useful framework to strategically choose species for experimentation that may be more vulnerable to OA.

To date, experimenters often choose study species based on availability or familiarity. However, robust evaluations of OA sensitivity in the ocean will only be achieved by choosing study species based on concrete theoretical frameworks rather than logistical convenience.

In summary, this dissertation provided much needed baseline data and novel insights on climate effects in forage fish. In reality, laboratory experiments alone cannot predict how a species will cope with future climate scenarios. Instead, the value of these data will be realized when they are
combined with modeling and observational approaches. However, to the dismay of experimenters there appears to be an eagerness for scientists and policy makers alike to shift sparse resources towards modeling approaches at the expense of laboratory experiments. Over the past two decades, laboratory experiments have transformed our understanding of climate effects in the ocean, but perhaps the most important finding is that we still know relatively little about how individual species will respond to the multiple dimensions of marine climate change. Thus, rather than conclude the experiments have nothing left to teach us, there needs to be a concerted effort to strategically increase the complexity of laboratory experiments to meet the increasing needs of modeling approaches. The findings documented by this dissertation emphasize that we have relatively little time left to act.
References


Table 1: A summary of the major findings of this dissertation and what chapters contain the supporting evidence. A black horizontal double arrow (↔) indicates that only a neutral effect was found. A green vertical double sided arrow (↕) that evidence for both negative and positive effects were found within that chapter. A red downward-facing arrow (↓) signifies a negative effect was detected. A horizontal dash (—) indicates the chapter did not address that question.

<table>
<thead>
<tr>
<th>Question</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Does exposure to future $p$CO$_2$ conditions affect early-life survival in forage fish?</td>
<td>↔ ↔ ↓ ↑</td>
</tr>
<tr>
<td>Does exposure to future $p$CO$_2$ conditions alter embryo development and early larval growth in forage fish?</td>
<td>↔ ↑ ↓ ↔</td>
</tr>
<tr>
<td>Does elevated $p$CO$_2$ modify time to hatch in forage fish embryos?</td>
<td>↔ ↔ ↓ ↔</td>
</tr>
<tr>
<td>Does long-term acclimation to elevated $p$CO$_2$ influence cumulative offspring mortality from fertilization through the juvenile stage?</td>
<td>↔ — — —</td>
</tr>
<tr>
<td>Does long-term acclimation to elevated $p$CO$_2$ modify offspring growth over multiple life stages?</td>
<td>↓ — — —</td>
</tr>
<tr>
<td>Do sub-optimal rearing temperatures alter early-life $p$CO$_2$ sensitivity in forage fish?</td>
<td>— ↑ ↓ —</td>
</tr>
<tr>
<td>Does elevated $p$CO$_2$ exposure modify early-life hypoxia tolerance in a forage fish?</td>
<td>— — ↓ —</td>
</tr>
</tbody>
</table>