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Does the seasonal acidification of spawning habitat influence offspring CO_2 reaction norms through transgenerational plasticity in the coastal fish species, *Menidia menidia*?

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Christopher S. Murray

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Christopher S. Murray

We, the thesis committee for the above candidate for the

Master of Science degree, hereby recommend

acceptance of this thesis.

Dr. Hannes Baumann, Professor – Thesis Advisor
School of Marine and Atmospheric Sciences

Dr. Christopher Gobler, Professor - Second Reader
School of Marine and Atmospheric Sciences

Dr. Janet Nye, Professor – Third Reader
School of Marine and Atmospheric Sciences

This thesis is accepted by the Graduate School

Charles Taber

Dean of the Graduate School

Abstract of the Thesis

Does the seasonal acidification of spawning habitat influence offspring CO_2 reaction norms through transgenerational plasticity in the costal fish species, *Menidia menidia*?

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Experimental assessments of species vulnerabilities to ocean acidification are rapidly expanding, yet the potential for short- and long-term adaptation to high CO_2 by contemporary marine organisms remains poorly understood. We used a novel experimental approach that combined bi-weekly sampling of a wild, spawning fish population (Atlantic silverside, *Menidia menidia*) with standardized offspring CO_2 exposure experiments and parallel pH monitoring of a coastal ecosystem. We assessed whether offspring produced at different times of the spawning season (April-July) would be similarly susceptible to elevated (~1,100 μatm, pH_{NBS} = 7.77) and high CO_2 levels (~2,300 μatm, pH_{NBS} = 7.47). Early in the season (April), high CO_2 levels significantly ($P < 0.05$) reduced fish survival by 54% (2012) and 33% (2013) and 1-10d post hatch growth by 17% relative to ambient conditions. However, offspring from parents collected

later in the season became increasingly CO_2-tolerant until, by mid-May, offspring survival was equally high at all CO_2 levels. This interannually consistent plasticity coincided with the rapid annual pH decline in the species' spawning habitat (mean pH: 1 April/31 May = 8.05/7.67). It suggests that parents can condition their offspring to seasonally acidifying environments, either via changes in maternal provisioning and/or epigenetic transgenerational plasticity (TGP). TGP to increasing CO_2 has been shown in the laboratory but never before in a wild population. Our novel findings of direct CO_2-related survival reductions in *wild* fish offspring and seasonally plastic responses imply that realistic assessments of species CO_2-sensitivities must control for parental environments that are seasonally variable in coastal habitats.

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INTRODUCTION

Fossil fuel combustion has steadily increased atmospheric CO_2 concentrations from a pre-industrial level of ~280 ppm (Solomon 2007) to what now exceeds 400 ppm (Mauna Loa Observatory; NOAA-ESRL). This rate of increase is at least an order of magnitude faster than has occurred in several million years (Doney & Schimel 2007) resulting in a global atmospheric CO_2 level exceeding any concentration the Earth has experienced over the past 800,000 years (Lüthi et al. 2008). Approximately a third of anthropogenic CO_2 has been absorbed by the oceans where it has significantly altered seawater chemistry (Sabine et al. 2004). Dissolved CO_2 reacts with seawater to form carbonic acid in the reaction $(H_2O + CO_2 \leftrightarrow H_2CO_3)$ that disassociates into a proton and a bicarbonate ion $(H_2CO_3 \leftrightarrow HCO_3^- + H^+)$. Bicarbonate ions may further disassociate in the reaction $(HCO_3^- \leftrightarrow CO_3^{2-} + H^+)$, though 90% of dissolved CO_2 remains as bicarbonate ions, while less than 10% remains as carbonate ions (Melzner et al. 2012). The collective effects of these reactions, known as ocean acidification, will increase $[H^+]$ concentrations (measured as a reduction in seawater pH) while decreasing $[CO_3^{2-}]$ concentrations and the saturation states (Ω) of calcite and aragonite (Brewer 1997). Since the onset of the industrial revolution, average ocean surface pH has fallen approximately 0.1 units (Doney et al. 2009) and will continue to decrease for centuries (Stocker et al. 2013). By the end of this century oceanic pH will decrease further by 0.3-0.4 units (Orr et al. 2005) and by 2300, as atmospheric CO_2 eclipses 2,000 ppm, a decline of 0.70 to 0.80 pH units is probable (Caldeira & Wickett 2003, Caldeira & Wickett 2005). At present, the ocean is warmer (+0.7°C), more acidic (-0.1 pH) and less saturated with carbonate (~210 µmol kg^{-1}) than at any other time over the last 420,000 years (Hoegh-Guldberg et al. 2007).

A reduction in the saturation state of biologically useful forms of $CaCO_3$, especially aragonite and calcite, will create adverse conditions for marine calcifying organisms (Kleypas & Langdon 2006, Fabry et al. 2008, Branch et al. 2013). As atmospheric CO_2 levels stabilize at 450ppm, only 8% of existing coral reefs will be surrounded by waters with aragonite saturation states comparable to conditions prior to the industrial revolution (Cao & Caldeira 2008). Calcification rates of warm-water corals are primarily controlled by [$CaCO_3$] but more specifically [CO_3^{2-}], as [Ca^{2+}] is constant in seawater (Kleypas & Langdon 2006). Laboratory experiments have demonstrated that a decrease in [CO_3^{2-}] induced by increases in CO_2 results in reduced calcification rates of several coral species (Ohde & Hossain 2004, Langdon & Atkinson 2005, Silverman et al. 2007). Coccolithophores, foraminifera, and euthecosome pterapods produce nearly all of the $CaCO_3$ in the upper ocean (Fabry et al. 2008), thus perturbations of their biological processes induced by ocean acidification may have profound effects to marine ecosystems and the ocean carbon cycle. When grown in high CO_2 some studies found the coccolithophorid *Emiliania huxleyi* to display reduced calcification rates (Riebesell et al. 2000, Delille et al. 2005, Engel et al. 2005), while other studies found no calcification abnormalities in *Coccolithus pelagicus* or reduced calcification rates and increased growth abnormalities in *Calcidiscus leptoporus* (Langer et al. 2006). Shell mass declined in two foraminifera species, *Orbulina universa* and *Globigerinoides sacculifer*, by 8 and 14% when exposed to high CO_2 (Spero et al. 1997, Bijma et al. 2002). The pteropod *Cila pyramidata* suffered net shell dissolution when exposed to seawater undersaturated with respect to aragonite ($\Omega < 1.0$) (Orr et al. 2005). Some benthic calcifiers also appear vulnerable to high CO_2 and low [CO_3^{2-}]. Reductions to fertilization success, cleavage and growth rates of juvenile urchins *Hemicentrotus pulcherrimus* and *Echinometra mathaei* were observed after long-term exposures to extreme CO_2 levels (Kurihara & Shirayama 2004, Shirayama & Thornton 2005).

Elevated CO_2 adversely affected the growth of the benthic gastropod *Strombus luhuanus* (strawberry conch) (Shirayama & Thornton 2005). Calcification rates of *Mytilus edulis* (blue mussel) and *Crassostrea gigas* (Pacific oyster) declined linearly with increasing CO_2 (Gazeau et al. 2007). Delayed development, morphological abnormalities, and reduced growth were observed in larvae of *Mytilus galloprovincialis* (Mediterranean mussel) after a 144h exposure to high CO_2 (Kurihara et al. 2009). Talmage at al. (2009) showed reduced survivorship, size, and delayed metamorphosis in response to high CO2 levels in larvae of three benthic invertebrates, *Mercenaria mercenaria* (hard clam), *Argopecten irradians* (Atlantic bay scallop) and *Crassostrea virginica* (eastern oyster).

Most effects of elevated CO_2 on marine organisms appear to be negative, but there's evidence for non-linear (Ries et al. 2009), neutral (Aberle et al. 2013, McConville et al. 2013) and even positive effects (Gooding et al. 2009, Ries et al. 2009, Lohbeck et al. 2012), suggesting variable biological responses (Kroeker et al. 2010). For example, larvae of *C. virginica* suffered reduced growth and calcification under high CO_2, but larvae of the closely related *Crassostrea ariakensis* (Suminoe oyster) showed no such response (Miller et al. 2009). A heavily calcified morphotype of *Emiliania huxleyi* found in current day low pH environments suggests that adaptation to environmental hypercapnia is possible and potentially common (Beaufort et al. 2011). The vulnerability of marine organisms to elevated CO_2 is likely to be highly complex and species-specific (Miller et al. 2009, Ries et al. 2009, Kroeker et al. 2010).

Past experiments have demonstrated adult fish can tolerant CO_2 levels up to 16,000 ppm (Ishimatsu et al. 2008), highlighting their advanced acid-base regulatory capacity (Melzner et al. 2009b). Thus, marine fish have long been thought to be largely resistant to rising oceanic pCO_2 levels, particularly for levels predicted to occur over the next few centuries (Ishimatsu et al. 2008,

Melzner et al. 2009b). To date, however, most research evaluating the effects of CO_2 on fish have focused on adult and juvenile stages of predominantly freshwater species, using short-term exposures of CO_2 levels far beyond what is predicated for the open ocean in the coming centuries (Ishimatsu et al. 2008). A meta analysis published in 2010 analyzing all studies on the effects of predicted CO_2 increases on marine organisms included only two studies that focused on marine fish (Kroeker et al. 2010), highlighting a clear knowledge gap. A recent upsurge in publications has produced a broad spectrum of responses, changing the traditional assumption that marine fish are robust to ocean acidification, but it remains unknown how realistic future acidification or even modern CO_2 variability affects the development or timing of proficient acid-base regulatory mechanisms in early fish life stages. Nevertheless, observations from these experiments have provided valuable insights into teleost osmoregulation.

In their aqueous medium, fish regulate their acid-base equilibrium predominately through exchanges of acid-base relevant molecules passing between their gills and the environment, primarily through adjustments of blood bicarbonate (Evans et al. 2005). Secretion of such molecules can also occur through the skin, rectal gland and kidneys, but gill epithelia can account for over 90% of acid-base compensation (Evans et al. 2005). Gills of most fish are densely populated with ionocytes (also known as mitochondria-rich cells or MRCs) containing high concentrations of basolateral Na^+/K^+ ATPase, an enzyme considered to be the "motor" of the gill ion-regulatory machinery. It provides an electro-chemical gradient for other ion transporters involved in acid-base compensation (Claiborne et al. 2002, Colina et al. 2007, Hwang et al. 2011). The Na^+/K^+ exchanger operates by pumping two K^+ ions into the cell while removing three Na^+ ions, thus creating low intracellular [Na^+]. As excess CO_2 diffuses into MRCs, it is hydrated by cytosolic carbonic anhydrase producing H^+ and HCO_3^-. The resulting proton is exported from the

cell via a Na+/H+ exchanger powered by low intracellular [Na^+], while bicarbonate is transported into the plasma by either a Cl^-/HCO_3^- exchanger of Na^+/HCO_3^- transporter (Melzner et al. 2009b). Once in the plasma, HCO_3^- may undergo another protonation/dehydration/hydration cycle and further H^+ extrusion from the gills (Deigweiher et al. 2008).

The cellular and molecular mechanisms involved in teleost osmoregulation are far more complex than described here. Existing literature has produced a myriad of possible models, often with conflicting or unresolved issues (Evans et al. 2005). For example, HCO_3^- uptake from the environment was recently found to drive intracellular pH compensation in *Opsanus beta* (Gulf toadfish) (Esbaugh et al. 2012*)*, a previously unaccounted factor in acid-base regulation. Recent advancements in molecular techniques have refined these models, but the lack of sufficient model species still hampers progress (Hwang et al. 2011). These mechanisms likely allow fish to compensate extra and intracellular pH during periods of extreme metabolic activity, and are thought to have enough excess capacity to cope with low level hypercapnia associated with future acidification in the adult stage (Melzner et al. 2009b). However, early fish life stages rely on epidermal ionocytes for ion transport as they are still developing adult-like acid-base regulatory structures in gills (Hwang et al. 2011, Tseng et al. 2011). The efficacy of these early structures in acid-base regulation prior to gill development is currently unknown (Baumann et al. 2012, Tseng et al. 2013), despite decades of research devoted to understanding the selective pressures controlling gill development in teleost fish. Several studies have indicated the development of gills in early embryos and larvae is not driven by O_2 limitations, but by the need for advanced ion exchange mechanisms (Li et al. 1995, Rombough 2002, Rombough 2004). A review by Rombough (2007) found that branchial MRCs (cells critical for gas exchange) appear on the gill well before secondary lamellae in every species examined to date, suggesting the

rapid maturity of ion regulation is critical in developing fish. As the larvae grow their ability to satisfy ion regulation through cutaneous exchange is reduced with a decreasing surface-to-volume ratio, and while this is also true of gas exchange, O_2 can be absorbed across most of the fish's surface area, while ion regulation is limited to specialized cells (Rombough 2007). In addition, increasing skin thickness reduces likelihood that epidermal ionocytes remain situated between their environment and a blood vessel (Varsamos et al. 2005), a requirement for ion exchange. Thus, ion exchange quickly becomes critical during larval development. Although the precise timing varies among species, epidermal ionocyte densities generally reach a maximum early in larval development before declining significantly (Rombough 2007). For example, body surface density of MRCs was reduced from 28.9% to 2.2% during the development of larval *Dicentrarchus labrax* (European sea bass) (Varsamos et al. 2002). When epidermal ionocyte activity is at its maximum, branchial ionocytes begin to emerge on the gills of the developing embryos or early embryos, resulting in a rapid increase of ion exchange capacity. The number MRCs on the gills of developing *Oncorhynchus mykiss* (rainbow trout) increased from zero at five days pre-hatch to ~50,000 8 days post-hatch (dph), corresponding to a 5-fold increase in Na^+/K^+ ATPase activity (Brauner & Wood 2002). In the gills of larval tilapia *Oreochromis mossambicus* (Mozambique tilapia) the mass specific Na^+/K^+ ATPase activity associated with the proliferation of MRCs was 400 times greater than that of an adult gill (Li et al. 1995). Thus, it would appear that ion exchange, including mechanisms for acid-base regulation (e.g. Na^+/K^+ ATPase activity), are the first critical functions of gills. In freshwater fish, the development of advanced ion exchange mechanisms around the time of hatch most likely reflects the need to accumulate body cations and anions to support growth and combat diffusive ion loss (Rombough 2007). For marine taxa, which tend to gain ions through diffusive processes, early development

of ion exchange may require an alternative explanation. The proliferation of branchial ionocytes prior to the development of secondary lamellae may instead reflect the need for advanced acid-base regulation early in development; however, associations between acid-base regulation, ammonia excretion, and other ion exchange processes make it difficult to assess which process first become limiting (Rombough 2007).

While acid-base regulation is clearly possible in fish early life stages, its efficacy remains poorly understood. Little information exists on how elevated CO_2 affects the development and proton excretion mechanisms (Tseng et al. 2013). The lack of developed adult-like acid-base regulatory structures may be a reason for increased susceptibility to high CO_2 in the earliest life stages (Ishimatsu et al. 2008, Hu et al. 2010, Ishimatsu & Dissanayake 2010, Hu et al. 2011). In fact, a growing number of studies have demonstrated early life sensitivity to high CO_2. For example, many coral reef species were found to exhibit detrimental behavioral abnormalities when reared under high CO_2. Juvenile *Amphiprion percula* (orange clownfish) exposed to ambient CO_2 within an auditory choice chamber consistently avoided daytime reef noise, where predation would be high, but fish exposed high CO_2 lacked this ability (Simpson et al. 2011). Under high CO_2, *A. percula* larvae failed to distinguish critical olfactory cues, such as appropriate settling habitat (Munday et al. 2009a) and predator from non-predator cues (Dixson et al. 2010). Settlement-stage damselfish larvae *Pomacentrus wardi* (Ward's damsel) acclimated to high CO_2 exhibited more risky behavior when transplanted to a series of patch reefs, resulting in increased mortality compared to ambient larvae (Munday et al. 2010). In the wild, such behavior abnormalities may result in substantial increases in mortality as young reef fish rely on environmental cues to locate suitable settling habitat (Atema et al. 2002, Heenan et al. 2008) or locate predators (Kelley & Magurran 2003).

The mechanism behind behavioral abnormalities seems to be an impairment of the GABA-A receptor. GABA-A is a major inhibitory neurotransmitter receptor in vertebrate central nervous systems (Fatima-Shad & Barry 1993). When reared in high CO_2 abnormal olfactory preferences and loss of behavior lateralization in *A. percula* larvae and settlement-stage *P. wardi* were reversed when treated with the GABA-A receptor antagonist gabazine, implicating the GABA-A receptor in the loss of behavioral function (Nilsson et al. 2012). Under normal CO_2 conditions, an open GABA-A receptor will influx Cl^- and HCO_3^- over the neuronal membrane resulting in a hyperpolarized neuron. When larvae are exposed to high CO_2, acid-base regulation alters Cl^- and HCO_3^- gradients over the neuronal membrane causing a net outflux of anions, depolarizing the neuron. This process forces the GABA-A receptors to remain in an excited state, altering behavior and sensory (auditory and olfactory) preferences (Nilsson et al. 2012). Given the rapid pace of anthropogenic acidification and the ubiquity of GABA-A receptors in marine fish, this previously unknown consequence of ocean acidification may affect population replenishment and ecosystem functions (Munday et al. 2010, Nilsson et al. 2012). In addition, fish use otoliths (ear bones) made of aragonite embedded in a protein matrix, to aid in stato-acoustic perception (Popper & Lu 2000). Hence, a reduction in $\Omega_{aragonite}$ associated with ocean acidification may substantially affect otolith growth (Fabry et al. 2008). Larval *Atractoscion nobilis* (white sea bass) reared in 993 µatm and 2558 µatm of CO_2 grew otoliths 10-14% and 24-26% larger, respectively, than larvae reared under ambient conditions (Checkley et al. 2009). Larval *A. percula* showed similar otolith overcalcification under high CO_2 (Munday et al. 2011b). Increased otolith size may be counterintuitive in low $\Omega_{aragonite}$ conditions where one would expect reductions in calcification rates. However, acid-base regulation of intercellular pH results in increased $[HCO_3^-]$ in extracellular fluids, including around the endolymph (fluid

around the otolith) (Esbaugh et al. 2012). High carbonate ion concentrations within the endolymph would increase $\Omega_{aragonite}$, accelerating the calcification of otoliths (Checkley et al. 2009). It remains unknown whether larger otolith will negatively affect early life fish, but larvae with asymmetrical otoliths have been shown to exhibit significantly higher rates of mortality (Gagliano et al. 2008). Furthermore, models have shown that heavier otoliths may not be displaced as readily as 'normal' otoliths, hence stato-acoustic perception would be impaired (Bignami et al. 2013), but this has yet to be observed.

Low level hypercapnia can reduce fitness in early life fish. Delayed development in *Oryzias latipes* (Japanese rice fish) embryos when reared at high CO_2 was correlated to a strong down-regulation of genes associated with major metabolic pathways; including glycolysis, Krebs cycle, and the electron transport chain (Tseng et al. 2013). Delayed growth was observed 3-5 days post fertilization (dpf), a period when embryos begin to develop epidermal ionocytes and may still lack efficient, adult-like acid-base regulatory systems. Metabolic depression is a common strategy employed by animals under environmental stress (Guppy 2004) suggesting reduced development rates in *O. latipes* embryos may be a coping mechanism against high CO_2. Interestingly, *O. latipes* embryos reared in high CO_2 waters reached full development by time of hatch, possibly through an activation of genes associated with metabolizing amino acids to fuel growth (Tseng et al. 2013). *Gadus morhua* (Atlantic cod) larvae also exhibited major histological damage to the liver, pancreas, kidney, eye and gut when reared under high CO_2, with implied but not demonstrated negative consequences for survival (Frommel et al. 2012b). Direct CO_2-related reductions in fish early life survival and growth have also been documented. Survival of *Paralichthys dentatus* (summer flounder) larvae was reduced by 50% and 75% relative to controls when reared at elevated (1,860 µatm) and high (4,717 µatm) CO_2 levels, respectively (Chambers et al. 2013).

Early larvae of *Menidia beryllina* (inland silverside) reared at 1,000 µatm exhibited significantly reduced survival (74%) and growth (22%) compared to ambient treatments (Baumann et al. 2012). The authors observed that the embryonic stage of *M. beryllina* was most susceptible to high CO_2. In a separate experiment, Baumann and colleagues reared embryos at low CO_2 then switched to a high CO_2 treatment post hatch (5 dpf). At 5 dph survival of larvae in the "switch" group was only marginally lower than survival in the constant low CO_2 group, but significantly higher than the constant high CO_2 group. Thus, high CO_2 seems to be most damaging during the embryonic stage but manifests as mortality in the first few hours to days post hatch (Baumann et al. 2012). Thus, studies testing the effects of ocean acidification on fish without including the egg stage may not observe the full impact of adverse CO_2 effects.

Further complicating this spectrum of responses, many species or populations appear unaffected by CO_2. No evidence for increased mortality, reduced growth, or otolith malformation were found in *A. percula* (Munday et al. 2009b, Simpson et al. 2011), *Acanthochromis polyacanthus* (spiny chromis damselfish) (Munday et al. 2011a), or *Theragra chalcogramma* (walleye pollock) (Hurst et al. 2012, Hurst et al. 2013). Experiments on Atlantic larval *G. morhua* and *Clupea harengus* (Atlantic herring) suggested some adverse CO_2 related effects (Frommel et al. 2012b), while experiments on Baltic *G. morhua* and *C. harengus* did not (Franke & Clemmesen 2011, Frommel et al. 2012a). The broad range of responses among early fish life stages mirrors the complexity observed in other marine taxa (Ries et al. 2009, Kroeker et al. 2010). Such complexity suggests highly species-specific reactions, but it may indicate the existence of so far overlooked or insufficiently controlled factors during laboratory experiments. One such factor is the naturally occurring spatio-temporal CO_2/pH variability typical of many marine habitats (Hendriks et al. 2010, Hofmann & Todgham 2010, Hofmann et al. 2011, Kelly et al. 2013,

McElhany & Busch 2013). Coastal environments, where most ecologically and economically important species spend all or parts of their life cycle are influenced by seasonal fluctuations in ecosystem metabolism (Wootton et al. 2008), river discharge (Salisbury et al. 2008), upwelling (Feely et al. 2008) or anthropogenic influences (Cai et al. 2011). Thus, species that inhabit these dynamic habitats already experience CO_2 levels up to 4,500 µatm and thus not predicted to occur in the open ocean for several hundred years (Cai et al. 2011, Melzner et al. 2012, Duarte et al. 2013) and have likely evolved mechanisms to tolerate extended periods of environmental hypercapnia (Melzner et al. 2009b, Frommel et al. 2012a).

One such mechanism, which organisms may employ to cope with rapidly changing CO_2/pH conditions is transgenerational epigenetic inheritance or transgenerational plasticity (TGP). TGP describes the ability of parents to enhance offspring fitness through the inheritance of beneficial epigenetic or transcriptomic states, determined by the parental environment, without requiring changes in DNA sequences (Salinas & Munch 2012). The ability of parental environments to alter offspring reaction norms appears ubiquitous across eukaryotes and prokaryotes, with prominent examples from plants (Molinier et al. 2006, Galloway & Etterson 2007, Champagne 2008), as well as bacteria, protists, fungi, insects, marine invertebrates and vertebrates (Jablonka & Raz 2009, Salinas & Munch 2012, Salinas et al. 2013). Epigenetic inheritance is achieved through alterations that occur within the chromatin of a cell. DNA is wrapped in a complex of proteins known as histones; clusters of DNA/histone complexes form the cellular chromatin (Champagne 2008). For a gene to be expressed, DNA sequences must first come in contact with RNA polymerase and other transcription factors, but expression can only occur when DNA is in an 'open' state, unwrapped from histone proteins (Robertson 2005). Several types of histone modifications or DNA methylation can either tightly bind DNA to repress expression,

or unwind sequences to promote transcription (Chinnusamy & Zhu 2009). Epigenetic modifications are often a result of environmental stress (Molinier et al. 2006, Champagne 2008, Jablonka & Raz 2009, Fedoroff 2012) and are inheritable through mitotic or meiotic cell division (Chinnusamy & Zhu 2009). Thus offspring can inherit modifications to increase fitness during periods of stressful environmental conditions (Galloway & Etterson 2007, Salinas & Munch 2012), an intriguing pathway for short-term adaption to anthropogenic climate change.

Given the prospect of unprecedented ocean warming (Parry 2007) and the central role of temperature in determining the metabolic scope of fish (Roessig et al. 2004), a species' short-term capacity to acclimate and adapt to elevated temperatures may dictate their future persistence in current habitats (Pörtner & Knust 2007, Nilsson et al. 2009). TGP presents a potential pathway for thermal acclimation, where parental environment can shape thermal reaction norms in offspring. When reared at warmer than optimal temperatures, offspring of *Cyprinodon variegatus* (sheepshead minnow) displayed reduced growth (Salinas & Munch 2012) while *A. polyacanthus* demonstrated a reduced aerobic scope (Donelson et al. 2012). In both species, the effects of thermal stress were fully compensated when parents were acclimated to higher temperatures for several weeks prior to fertilization (Donelson et al. 2012, Salinas & Munch 2012). Parental acclimation seemingly shifted offspring maximum temperature-dependent fitness to a higher temperature. Laboratory experiments have also shown TGP in CO_2 resistance as a potential adaptive mechanism for coping with large CO_2 fluctuations. *Saccostrea glomerata* (Sydney rock oyster) larvae displayed reduced development, growth and survival when reared at high CO_2, but these effects were mitigated in offspring from high CO_2 acclimated adults *S. glomerata* (Parker et al. 2012). TGP in CO_2 sensitivity may also be common in marine fish. Juvenile *Amphiprion melanopus* (cinnamon clownfish) suffered reductions to length, weight,

condition and survival when reared at 1,000 ppm CO_2 compared to an ambient treatment, but these effects were absent in larvae spawned from adults conditioned to high CO_2 prior to fertilization (Miller et al. 2012). Parental CO_2 acclimation reversed poor locomotor performance in high CO_2 reared juvenile *A. melanopus* (Allan et al. 2014). Adult CO_2 exposure may have led to epigenetic alterations that readied their offspring to develop more efficient physiology mechanisms to cope with high CO_2 environments, possibly through changes in gene expressions associated with acid-base regulation or mitochondrial metabolism (Miller et al. 2012). TGP in high CO_2 acclimation may be widespread among marine organisms, particularly in species that reside or spawn in highly variable CO_2 environments, such as coastal or upwelling systems (Salinas & Munch 2012, Dupont et al. 2013, Shaw et al. 2013). However, evidence for TGP mediated acclimation in wild populations has yet to be shown, a clear knowledge gap in the understanding of how species will adapt to rapid anthropogenic climate change.

The purpose of this study was to test the effects of high CO_2 on the survival and growth of early offspring from coastal marine fish. Specifically, we tested the hypothesis that offspring from wild *Menidia menidia* (Atlantic silverside) collected and spawned throughout their spawning season will show consistent negative effects when reared under high CO_2, as observed in the closely related silverside *M. beryllina (*Baumann et al. 2012*).* In addition to well-developed captive rearing methodologies (Conover & Present 1990, Billerbeck et al. 2000, Munch & Conover 2002), *M. menidia* exhibit ideal life history characteristics for studying the possibility of short-term adaptive mechanisms organisms may employ to inhabit highly variable environments. *M. menidia* is an ecological important coastal forage fish native to North America's east coast, ranging from Nova Scotia to northern Florida (Conover & Ross 1982). Adults overwinter offshore and return to coastal habitats with warming spring temperatures to

spawn from April – July following a semi-lunar spawning periodicity (Conover 1985, Middaugh et al. 1987). The intertidal zones of estuaries and salt marshes are their preferred spawning habitat, i.e., environments that are extremely productive and thus exhibit large diel to seasonal fluctuations in CO_2 and pH conditions (Wang & Cai 2004, Baumann et al. 2014). *M. menidia* appear to thrive in these dynamic environments and thereby may utilize short-term CO_2/pH adaptive mechanisms to produce more robust offspring during periods of natural acidification.

In my thesis, I conducted a series of fully replicated and standardized experiments (N_{2012} = 5, N_{2013} = 5) to test the null hypothesis that offspring from wild adult *M. menidia* sampled bi-weekly throughout the spawning season would show comparable survival and growth reaction norms when reared under ambient (~600 μatm, pHNBS = 8.07), elevated 130 (~1,100 μatm, pHNBS = 7.77), and high CO_2 levels (~2,300 μatm, pHNBS = 7.47) from fertilization to 10 dph. To quantify seasonal patterns of pH variability typical *M. menidia*'s spawning habitat we analyzed long-term (2008-2012) high frequency pH monitoring data from the Flax Pond tidal salt marsh (Long Island, NY). The novel approach of combining wild adult field sampling and offspring CO_2 exposure experiments with *in situ* pH data from a spawning habitat allowed us to compare offspring CO_2 reaction norms in the lab with the concurrent pH conditions experienced by the wild population.

Methods

Monitoring of Flax Pond salt marsh conditions

Flax Pond is a tidal salt marsh (40°57.78'N, 73°8.22'W) situated on the north shore of Long Island (Figure 1). A single inlet connects the 0.59 km^2 marsh to the Long Island Sound allowing a mean tidal range of 1.8 meters (Richard 1978). The marsh is characterized by open water banked by a

mosaic of *Spartina alterniflora* stands, bare mud and upland islands (Richard 1978). The geography of Flax Pond is not conducive for beach seining, thus adult *M. menidia* were collected at a structurally similar site 2 km away at Poquot Beach (40° 57.78′ N, 73° 8.22′ W, Fig. 1). Without specifically monitoring the Poquot site we cannot know the exact environmental conditions experienced by the adults collected there, but we assumed that the seasonal changes of temperature and CO_2/pH conditions at Flax Pond are representative of tidal salt marshes on the north shore of Long Island. The specific timing and magnitude of seasonal change may vary between sites but the general seasonal patterns are likely to be very similar. The system has been monitored by the US Geological Survey (USGS, site # 01304057, http://ny.water.usgs.gov/rt/pub/01304057.html) since April 2008, collecting high frequency data (six minute intervals) of temperature (C°), pH (National Bureau of Standards, NBS), dissolved oxygen (DO, mg 1^{-1}), and tidal elevation (m), among other parameters. PH was measured using a Fast-Response pH sensor (precision: 0.1 pH units, accuracy: 0.2 pH units). Sensors were deployed in the main channel of the marsh, mounted 0.5 m above a sandy bottom on a YSI 6600 multi-parameter sonde. The probes were cleaned, checked for biofouling and electronic drift, and recalibrated (NBS) according to USGS guideline every month in summer, every six weeks in spring and fall, and bimonthly in winter (Wagner et al. 2000). Funding constrains resulted in the discontinuation of pH monitoring; hence the time series analyzed here are for periods January 2009 - November 2012. Minima, maxima, means and amplitudes (max – min) of temperature and pH were calculated for each day, month, and year of the time series. To describe the seasonal variability of pH and temperature we estimated monthly averages (± 1 s.d) from daily minima, means and maxima from all years of data. Annual amplitudes were estimated from each year's monthly minimum and maximum averages. Linear regressions were used to estimate general

seasonal trends in pH minima, maxima, ranges, and means during the first half of *M. menidia's* spawning season (1 April to 30 May) or during the seasonal acidification of the marsh (15 February to 15 August). In addition, we calculated the seasonal change in hours per day (hpd) an organism in Flax Pond would spend in waters at or below the three pH_{NBS} levels (8.10, 7.80 and 7.50) used in our laboratory experiments.

CO_2 treatments and measurements

Experiments were conducted by strictly following the guidelines for best practices of ocean acidification research outlined by the European Project on Ocean Acidification (Riebesell et al. 2010). Gas proportioners (Cole Parmer® Flowmeter system, multi-tube frame) delivered a precise mix of 5% CO_2 gas and pressurized air to all replicate containers (19L white buckets) via air stones. Manual adjustments of CO_2 flow rates using high precision valves maintained precise pCO_2 levels in experimental containers (Table 1). This method of acidification best mimics the natural acidification process of seawater and ensured that treatments were saturated with respect to oxygen (> ~8 mg L^{-1}). Experimental containers were filled with aged and aerated saline well water from Flax Pond, NY wet lab, a facility that has been used for decades to rear experimental fish like Atlantic Silversides (Conover & Present 1990, Schultz & Conover 1997, Billerbeck et al. 2000). Well water was aerated for two weeks to ensure ambient CO_2 levels. Untreated well water contains high levels of Manganese but thorough aeration in settling tanks fully precipitates the metal. Each replicate container was routinely monitored for pH (calibrated bi-weekly with three-point NIST traceable pH_{NBS} references, Orion ROSS Ultra pH/ATC Triode and Orion Star A121 pH Portable Meter; Thermo Scientific), which indicated consistent levels throughout all experiments (Table1). During each experiment, seawater was sampled from all treatments for total dissolved inorganic carbon (DIC) and alkalinity analysis, using protocols established in Dickson et al. (2007).

Container water was siphoned into borosilicate bottles and immediately preserved using 200µL of a saturated $HgCl_2$ solution (10g $HgCl_2$ per 100ml H_20). A thin line of Apiezon L grease was used to seal bottle stoppers and prevent gas exchange and DIC alteration before analysis. Bottles were kept refrigerated until analyzed. An EGM-4 Environmental Gas Analyzer measured DIC after separating the gas phase from seawater using a Liqui-Cel Membrane (Membrana; Talmage and Gobler 2010). Dissolved CO_2 levels (µatm) were obtained using the program CO2SYS that calculates dissolved CO_2 based on levels of DIC, pH (NBS), temperature, salinity, and the first and second dissociation constants of carbonic acid in seawater (Roy et al. 1993). A complete overview of carbonate chemistry measurements from all experiments can be found in Table 1. Three CO_2 levels were tested during this project: ambient (air only, 2012: 670 µatm, pHNBS = 8.04; 2013: 529 µatm, pHNBS = 8.10), elevated (air:CO_2, 2012 & 2013: 1,100 µatm, pHNBS = 7.77), and high CO_2 conditions (air:CO_2, 159 2012 & 2013: 2,300 µatm, pHNBS = 7.47). These levels represent projected average open ocean CO_2 levels over the next 100-300 years (Alley et al. 2007) and cover the range of CO_2 levels recommended by EPOCA for experimental design of ocean acidification experiments (Riebesell et al. 2010). In addition, these levels are already common in coastal ecosystems during the spawning season of our model species *M. menidia* (Baumann et al. 2014).

Experimental design

Wild mature adults were collected bi-weekly from Poquot, Long Island (40°56’51”N, 73°6’10”W, Figure 1), four days prior to full or new moons, coinciding with the species’ semilunar spawning activity between late March and early July (Middaugh et al. 1987). Collections were made from shore using a large beach seine (30 x 2 m, 5 mm mesh size). To ensure sufficient genetic variability, at least 20 males and 20 females were collected and transported back to our laboratory

facility at Flax Pond, NY. However, this target number was compromised during the end of the spawning season in both years, because of the disproportionate disappearance of mature males from the population (Figure 2). A complete overview of the 2012 and 2013 seining effort can be found in Table 8. Collected fish were separated by sex and held for ~24h at 24°C and ambient CO_2. Adults were strip-spawned onto window screen segments (~1 mm mesh) submerged in seawater. Fertilized eggs attach to the screen via chorionic filaments and are easily quantified under low magnification. Strip-spawned adults were measured for length (SL, lower 0.5 cm). For each experiment one temperature controlled bath (700L, 24°C) housed up to 18 experimental containers (19 L white buckets), i.e., six replicates per CO_2 level (~600, ~1,100, ~2,300 µatm). Experiments were initiated within two hours of fertilization, and each replicate received a random 100 fertilized eggs by hanging embryo screens in the water column of the container. A summary of fertilization dates can be found in Table 2.

At 24°C and 25 psu embryos hatched 5-6 dpf. Larvae were provided with newly hatched brine shrimp *Artemia salina* nauplii (San Francisco strain, Brine Shrimp Direct, Inc.) immediately post hatch. For the first three dph larvae were fed commercial larval powder food (Otohime Marine Weaning Diet, size A, Reed Mariculture®) to reduce mortality related to the onset of first feeding. Dead nauplii and excess food were siphoned daily from containers. At 1 dph surviving larvae were counted by gently scooping them in groups of five to eight into a replacement rearing container. Dead larvae and unhatched eggs were also quantified. A subsample (n = 10) of surviving larvae were preserved in 10% formaldehyde/seawater solution for initial SL measurements (nearest 0.01mm). Due to our focus on in the earliest life stages, all experiments were terminated 10 dph and surviving larvae were counted. Survivors were preserved for SL measurements. All SL

measurements were made from calibrated digital pictures by using image analysis software (ImagePro® 6.0).

Statistical analysis

Non-normally distributed data were log transformed, and percent-survival data were arcsin transformed to fulfill normal distribution assumptions of parametric statistical tests (McDonald 2009). Data were also tested for homogeneity of variance using Levin's test (McDonald 2009). All statistical analyses were done using IBM SPSS Statistics v. 20. A Pearson correlation was used to test the hypothesis that larger females produce offspring with higher rates of survival (fertilization (f) – 10 dph) and 1 dph length (2-tailed, $p < 0.05$) throughout the spawning season. For each experiment, separate one-way ANOVAs were used to test for the effect of CO_2 level ($p < 0.05$) on survival (1 dph, f – 10 dph), 1 dph length, and growth rate (GR = $(SL_{10dph}-SL_{1dph})/9$). Least-significant-difference (LSD) or Dunnet-T3 post-hoc test were used in case of homogeneous or heterogeneous variances between groups, respectively.

The seasonal offspring survival patterns suggested two main periods; "early offspring" that were fertilized and reared during first half of the spawning season (April and early May; experiments 1, 2 and 3 from 2012 and experiments 1 and 2 from 2013) and "late offspring" reared during the second half of the season (late May, June and July; experiments 4 and 5 from 2012 and experiments 3, 4 and 5 from 2013).Hence, the effects ($p < 0.05$) of fertilization date (FD) on CO_2-dependent survival (f – 10dph) and GR were tested for each period using a General Linear Model.

Survival (arcsin(%)) or GR (mm d^{-1}) = CO_2 + FD + CO_2*FD + ε (error)

FD was chosen as a fixed factor over other possible factors, including mean Flax Pond pH at time of adult collection, or the cumulative time adults spent below some pH threshold prior to spawning, for two main reasons. Funding constraints beyond our control resulted in no monitoring data from Flax Pond during the 2012 spawning season. Consequently, using pH data in the model would include a multi-year average and not a direct measurement of conditions at the time of adult collection. In addition, spawning adults were not collected in Flax Pond but at the adjacent estuary in Poquot. To two estuaries are likely to exhibit different diel pH variability, but are also likely to exhibit very similar seasonal trends. That is, early in the spawning season both estuaries have high, more ambient, pH conditions with little diel variability; later in the spawning season as biological activity intensifies the estuaries become more acidic with greater diel variability. Thus, we believe the best proxy for the seasonal acidification experienced by adults collected from Poquot is FD. We do acknowledge that without directly testing adult CO_2 exposure we cannot robustly infer its role in determining offspring CO_2 tolerance. Alternative possibilities are explored in the Discussion. Linear regressions were used to quantify the relationship between survival (f – 10 dph) or GR and FD within a CO_2 treatment during each spawning interval. All means are given ± 1 s.e., unless noted otherwise. P-values below 0.05 were considered statistically significant.

Results

A total of 12 collections were made during two years of experimentation, seven in 2012 and five in 2013, resulting in a total of 10 experiments. Two collections made in 2012 (4 May and 2 June) resulted in failed experiments. Larvae fertilized from the 4 May collection were killed by an invasive polyp that infested our experimental containers. The 2 June collection resulted in a failed fertilization attempt. The first collection of spawning adults in 2013 was made ~ 1 month later than in 2012 in spite of an intensive seining effort starting March 2013. A

summary of fertilization dates, CO_2 treatments administered, replication, and results can be found in Table 3. Measurements of strip-spawned adults showed that seasonal variability in female spawner SL was not correlated to offspring survival or 1dph length (Pearson correlation, $P > 0.05$, Figure 2).

Flax Pond environmental data

Flax Pond is a dynamic tidal salt marsh system characterized by a high degree of seasonal and diel variability in temperature and pH. On average, January exhibited the coldest mean water temperatures (0.98±1.73°C) and August was the warmest (24.21±1.10°C). Mean diel temperature fluctuations were smallest in January (2.97±1.09°C) and most variable in May (6.61±2.38°C). Mean pH was highest in February (8.19±0.08) and progressively decreased with an increase in variance throughout the spring and summer, until reaching a seasonal low in August (7.57±0.14). Daily fluctuations of pH were smallest between January - and March (0.22±0.08), but steadily increased throughout the spring and summer reaching a maximum in September (0.75±0.15), before gradually returning to their initial state. Interannual pH variability was substantial, though limited by the brevity of the time series, with absolute monthly anomalies ranging from 0.09 pH units in February and November to 0.31 units in June. A complete summary of temperature and pH data can be found in Table 3 and Figure 3.

From a maximum in February, marsh pH fell at an average rate of -0.003 units day^{-1} through August, but the period of most rapid pH change consistently occurred from early April through end of May. Daily pH_{mean} declined by 0.31 units (pH_{mean} 8.06 → 7.65, -0.005 units d^{-1}), while daily pH_{min} declined by 0.70 units during this period (pH_{min} 7.80 → 7.10, -0.01 units d^{-1}) (Figure 3). This period also coincides with first half of *M. menidia*'s spawning season, hence

offspring fertilized early in the season experienced more basic and less variable pH conditions than conspecifics spawned at later dates. In an effort to relate these seasonal changes in pH to our experimental treatment levels we calculated the hours per day (hpd) a wild fish would spend in pH conditions below one of our CO_2 treatment levels (ambient, pH 8.10; elevated, pH 7.80; high, pH 7.50). In April, pH levels were below ambient levels an average of 20.3 hpd, but only 4.5 and 0.1 hpd under elevated and high levels, respectively (Figure 3). In May, wild fish experienced pH levels below the ambient level on average 23.1 hpd, with more than half the day spent under elevated (13.9 hpd) and 3.9 hpd under high levels (Figure 3). By late June, pH in the marsh was almost exclusively below ambient levels at 23.9 hpd. Average pH under elevated and high levels had increased to 17.1 hpd and 5.9 hpd, respectively (Figure 4).

Survival

2012: Offspring fertilized from the first collection of wild adults (4 April) displayed comparable 1 dph survival across CO_2 treatments ($P = 0.328$, Table 2, Figure 5); however, at 10 dph a strong CO_2 effect emerged. Then, embryos and early larvae exposed to high CO_2 exhibited significantly lower average survival (39±6%) compared to elevated (71±4%, $P = 0.006$) and ambient (83±5%, $P = 0.001$) treatments ($P = 0.002$; Figure 6). Offspring tested during experiment 2 (fertilized 19 April) were similarly vulnerable to high CO_2. Average 1 dph survival was slightly, but not significantly reduced at high CO_2, 18% and 14% lower than elevated and ambient treatments, respectively ($P = 0.428$, Table 2, Figure 5). A larger effect was observed at 10 dph when only 48±3.0% of larvae reared at high CO_2 remained, significantly fewer than in elevated CO_2 (86±1.0%, $P = 0.038$), but not statistically significant than ambient (77±7.0%, $P = 0.089$) treatments ($P = 0.085$, Figure 6). Mean survival in the elevated treatment was not significantly different than ambient survival ($P = 0.561$, Table 2). Larvae reared during a third

experiment (fertilized 18 May) showed significantly lower 1 dph survival in elevated CO_2 compared to ambient (P = 0.001) and high (P = 0.004) treatments (P = 0.002, Table 2, Figure 5). However, no substantial differences in survival were observed 10 dph when high, elevated and ambient average survivals were 79±3%, 68±4% and 75±5%, respectively (P = 0.192, Figure 6). During experiment 4 (fertilized 15 June) 1 dph mean survival was significantly higher in ambient CO_2 (92±3.0%) compared to high CO_2 (55±11%) replicates (P = 0.006, Figure 5). However, only a marginal difference in survival between treatments was observed 10 dph, a result of high 1-10 dph mortality in ambient replicates (Table 2, Figure 6). During the final experiment in 2012 (fertilized 3 July) embryos exposed to high CO_2 had slightly but not significantly lower 1 dph survival than those reared under ambient conditions (Table 2, Figure 5). Survival at 10 dph was significantly lower in high CO_2 (17±4.0%) compared to the low CO_2 (32±3.0%) (P = 0.016, Figure 6). Ambient f – 10dph survival in experiment 4 and 5 was significantly lower than ambient survival in experiments 1, 2 and 3 (P = <0.001, Table2, Figure 6) and significantly lower than high CO_2 survival in experiment 3 (P = <0.001, Table 2, Figure 6).

2013: Embryos fertilized for experiment 1 (26 April) exhibited significantly lower 1dph survival when reared under high CO_2 (78±3.0%) compared to elevated (97±3.0%, P = 0.002) and ambient (92±3.0%, P = 0.004) treatments (P = 0.001, Figure 7). This reduction in survival continued at 10 dph when 46±4.0% of larvae remained in high CO_2, significantly fewer then ambient (69±7.0%, P = 0.012) treatments (Figure 8). Mean 10 dph survival in elevated CO_2 (65±7.0%) was not significantly different from ambient (P = 0.561) or high (P = 0.123) treatments (Figure 8). Mean 1 dph survival during experiment 2 (fertilized 7 May) was not significantly different between treatments (P = 0.529, Table 2, Figure 7); however, high 1-10 dph mortality inflicted on high CO_2 larvae produced near significant differences between CO_2

treatments 10 dph (P = 0.067, Table 2, Figure 8). A third experiment (fertilized 21 May) revealed no differences in 1 dph survival between CO_2 treatments (P = 0.193, Table 2, Figure 7). This trend continued 10 dph when no significant differences were observed between high (64±6.0%), elevated (62±7.0%) and ambient (55±7.0%) replicates (P = 0.594, Figure 8). Experiment 4 (fertilized 6 June) was characterized by near complete 1 dph survival in all treatments (Table 2, Figure 7). Comparable 1-10 dph mortality resulted in similar 10 dph survival across high (48±6.0%), elevated (55±6.0%) and ambient (49±8.0%) treatments (P = 0.760, Figure 8). A final experiment was initiated on 22 June that exhibited no significant differences in mean 1 dph survival between treatments (P = 0.599, Table 2, Figure 7). High levels of mortality inflicted 1-10 dph resulted in very low but similar mean 10 dph survival at high (25±7.0%), elevated (31±4.0%) and ambient (23±5.0%) CO_2 treatments (P = 0.555, Table 2, Figure 8). The low overall survival during experiment 5 was consistent with results from 2012's final experiment (Table 2).

Growth

2012: Mean 1 dph lengths for larvae reared during experiment 1 were not significantly different between CO_2 treatments (P = 0.157, Table 2, Figure 9); however, after 10 dph significantly faster growth rates were recorded in larvae reared at ambient (0.80±0.03 mm d^{-1}) compared to elevated (0.59±.05 mm d^{-1}, P = 0.007) and high (0.57±.04 mm d^{-1}, P = 0.005) treatments (P = 0.008, Figure 10). Experiment 2 exhibited no significant differences between treatments for mean 1 dph length (P = 0.446, Table 2, Figure 9) or growth (P = 0.403, Table 2, Figure 10). During experiment 3, 1 dph larvae were significantly longer from high CO_2 (5.45±0.06 mm) compared to elevated (5.14±0.06 mm, P = <0.001) and ambient (5.08±0.06 mm, P = <0.001) treatments (P = <0.001, Figure 9). Growth rates were also significantly different

between treatments, with slowest rates in ambient CO_2 (0.67±0.02 mm d^{-1}), significantly slower than elevated (0.72±0.02 mm d^{-1}, P = 0.032) and high (0.78±0.02 mm d^{-1}, P = 0.001) treatments (P = 0.003, Figure 10). Larvae reared during experiment 4 exhibited similar 1 dph length (P = 0.748, Table 2, Figure 9) and growth rates (P = 0.464, Table 2, Figure 10) in high and ambient CO_2. During experiment 5 mean 1 dph lengths were nearly identical from high (4.74±0.05 mm) and ambient (4.74±0.04 mm) treatments (Figure 9); however, 1-10 dph growth was significantly faster in larvae reared at ambient CO_2 (0.52±0.02 mm d^{-1}) compared to those reared under high CO_2 (0.39±0.03 mm d^{-1}) (P = 0.013, Figure 10).

2013: Mean 1 dph lengths (P = 0.888, Table 2, Figure 11) and 1–10 dph growth rates (P = 0.183, Table 2, Figure 12) during experiment 1 were not significantly different between CO_2 treatments. During experiment 2, 1 dph larvae reared in elevated CO_2 (5.67±0.04 mm) were significantly longer than those reared in ambient CO_2 (5.47±0.05 mm, P = 0.017), and nearly significantly longer than high CO_2 (5.54±0.05 mm, P = 0.055) larvae (P = 0.042, Figure 11). Mean 1 dph lengths from high and ambient treatments did not vary significantly (P = 0.360, Table 2, Figure 11). Mean 1-10 dph growth was similar across all CO_2 treatments (P = 0.215, Table 2, Figure 12). Mean 1 dph lengths of larvae reared during experiment 3 were marginally but significantly larger in elevated CO_2 (5.69±0.04 mm) compared to high (5.54±0.04 mm, P = 0.027) and ambient treatments (5.55±0.05 mm, P = 0.038) (P = 0.045, Figure 11). Significant differences in 1-10 dph growth were observed, because larvae from high CO_2 (0.71±0.02 mm d^{-1}) grew significantly faster than elevated (0.64±0.02 mm d^{-1}, P = 0.050) and ambient (0.62±0.02 mm d^{-1}, P = 0.013) treatments (P = 0.033, Table 2, Figure 12). Growth rates in elevated and ambient treatments were not significantly different (P = 0.511, Table 2, Figure 12). Experiment 4 exhibited similar 1 dph lengths (P = 0.989, Table 2, Figure 11) and 1-10 dph growth rates

(P=0.589, Table 2, Figure 12) across all CO_2 treatments. A similar results was found during experiment 5 when 1dph length (P = 0.534, Table 2, Figure 11) and 1-10 dph growth (P = 0.865, Table 2, Figure 12) varied little between CO_2 treatments.

Seasonal Response

When combined, results from both years indicated that *M. menidia* embryos and early larvae display a high degree of seasonal variation in response to high CO_2. Offspring fertilized from adults collected at the onset of the spawning season (4 April 2012, 26 April 2013) were negatively affected when exposed to high levels of CO_2; however, offspring tested at and after the height of the spawning season (18 May 2012, 21 May 2013) had similar survival and growth reaction norms in response to CO2 (Figure 13B, Figure 14B). A General Linear Model was constructed to test for the effects of CO_2, fertilization date (FD) and their interaction on larval survival (f - 10 dph) and growth (1 – 10 dph). The experiments were grouped into two halves; "early offspring" that were fertilized and reared during first half of the spawning season (April and early May; experiments 1, 2 and 3 from 2012 and experiments 1 and 2 from 2013) and "late offspring" reared during the second half of the season (late May, June and July; experiments 4 and 5 from 2012 and experiments 3, 4 and 5 from 2013). Experiments fertilized during the first half of the season used wild adults collected during the seasonal acidification of their spawning habitat but prior to the minimum Flax Pond pH dropping below our high CO_2 level, while adults used for experiments during the second half of the season regularly experience pH levels below our high CO_2 treatment (Figure 13A). The model indicated survival of early offspring was significantly affected by CO_2 level (P = <0.001), FD (P = 0.019) and a CO_2*FD interaction (P = 0.009) (Table 4, Figure 13B). A regression analysis found larval survival at high CO_2 levels increased significantly during the first half of the spawning season (P = 0.002, Table 5, Figure

13b), while reaching a plateau in elevated and ambient treatments (Table 5, Figure 13B). The model found CO_2 level (P = 0.041), FD (P = <0.001) and a CO_2*FD interaction (P = <0.001) were significant sources of variation influencing larval growth rates (Table 6, Figure 14B). However, regression analyses found that CO_2 treatment did not significantly predict growth rate (Table 7, Figure 14B).

During the second half of the spawning season, however, the model characterized survival of "late offspring" differently. Now, FD (P = <0.001) was the only significant source of variation in larval survival, while CO_2 (P = 0.367) and CO_2*FD interaction (P = 0.494) had no significant effect (Table 4). Regression analysis found that larval survival decreased significantly in all CO_2 treatments (high; P = <0.001, elevated; P = 0.002, ambient; P = 0.002, Table 5, Figure 13B). CO_2 level had no effect on the growth of late offspring (Table 6); however, FD (P = <0.001) and a CO_2*FD interaction (P = 0.012) were significant sources of variation (Table 6). Regression analyses found a significant decrease in growth at high CO_2 (P = 0.007) over the second spawning interval, but no significant correlations between growth and FD in elevated or ambient treatments (Table 7, Figure 14B).

Discussion

Embryos and early larvae of *M. menidia* were found to display a considerable degree of seasonal plasticity in response to high CO_2. Offspring fertilized from adults collected at the onset of species' spawning season (early to mid-April) and reared under high CO_2 suffered significant reductions in survival and growth (only in 2012) relative to ambient conditions. However, offspring reared from adults collected at the height of the spawning season (late May) appeared no longer sensitive to high CO_2, as similar survival and growth rates were observed across all

CO_2 treatments. During this interval, survival under high CO_2 increased significantly, while remaining stable or decreasing slightly in elevated and ambient treatments. The rapid shift from CO_2-sensitive to CO_2-tolerant offspring coincided temporally with the seasonal acidification of the species' spawning habitat.

Estuary pH was consistently most basic and least variable in February, but levels decreased steadily throughout the spring and summer becoming most acidic and most variable in August. Fastest rates of acidification were consistently observed from early April to late May, which encompasses the first half of *M. menidia*'s spawning season. The benefit of producing CO_2 resistant offspring during periods of seasonal acidification suggests the species is employing an adaptive strategy, by which adult silversides modify offspring CO_2 tolerance and/or alter metabolic provisioning based on their own environmental experience. This interpretation is supported by laboratory experiments, which have shown that parental conditioning to high CO_2 prior to fertilization can mitigate effects of high CO_2 in offspring (Miller et al. 2012, Parker et al. 2012, Allan et al. 2014). In fact, this parent-offspring link is consistent with a growing understanding of transgenerational effects found in many other traits and taxonomic groups (Jablonka & Raz 2009), especially traits influenced by rapid anthropogenic environmental change (Salinas et al. 2013). In the case of *M. menidia,* transgenerational plasticity (TGP) may allow offspring from a single population to rapidly adapt to the continuum of CO_2 and pH conditions experienced during a spawning season. While laboratory experiments and field observations support TGP as a plausible explanation for seasonal variability in CO_2 tolerance, several questions remain. The mechanisms of direct CO_2 related mortality in fish early life stages is still poorly understood; it is unclear why exactly low level hypercapnia kills young fish. The mechanisms of TGP in altering offspring CO_2 reaction norms are unknown, too, although

epigenetic inheritance and maternal provisioning are alternative possibilities. The results of this study suggest this species is robust to rapid acidification, but it is unknown how early life stages will tolerate the combined effects of other anthropogenic stressors such as warming sea temperatures and increased occurrences of coastal hypoxia and anoxia. In future studies, investigators must fine-tune their methodology to consider the parental environment when testing early life stages. The existence of seasonally changing CO_2 reaction norms will potentially influence the direction of future ocean acidification research.

What are the mechanisms of CO_2 related mortality?

While exposure to extreme CO_2 levels (>50,000 µatm) can kill fish (Ishimatsu et al. 2008) recent work has shown exposure to even low-level hypercapnia (<1,900 µatm) can induce respiratory acidosis (Esbaugh et al. 2012). Generally, adult and juvenile fish have enough acid-base regulatory capacity to fully compensate respiratory acidosis (Melzner et al. 2009b). Fish early life stages, however, may not yet possess this ability, given their still large surface-to-volume ratio (Mangor-Jensen 1987) and developing ion exchange and acid-base regulatory mechanisms (Perry & Gilmour 2006, Tseng et al. 2013). The exact mechanisms of how acidosis kills fish early life stages is presently not well understood; however, reductions in extracellular pH induced by environmental hypercapnia has been shown to cause changes in physiological functions at the molecular, cellular, tissue, and systematic levels (Pörtner et al. 2011). For example, cardiac failure in adult *Seriola quinqueradiata* (Japanese amberjack) exposed to extreme hypercapnia (50,000 µatm CO_2) led to the complete mortality of test subjects (Lee et al. 2003). Exposure to CO_2 can lower intracellular pH of the myocardium which depresses cardiac contractility (Gesser & Poupa 1983). A precipitous drop in cardiac output through a reduction in stroke volume severely limited oxygen delivery to the tissues of *S. quinqueradiata*. Although

CO_2 levels administered here were substantially lower, chronic exposure to low level hypercapnia may have similar effects on the sensitive early developmental stages of early season *M. menidia* offspring. The effects of realistic CO_2 levels in the context of ocean acidification on cardiac performance, as well as other critical metabolic functions in fish early life stages, warrant further investigation.

Alternatively, larval or early life stages of fish may be capable of advanced acid-base and ion regulation at the cost of increased metabolic demands (Perry & Gilmour 2006, Rombough 2007). Enhanced rates of ion exchange may significantly increase the standard metabolic rate of water breathing organisms when exposed to high CO_2 (Pörtner et al. 2011). For example, under long-term acclimation to high CO_2, adult *G. morhua* and *Zoarces viviparous* (viviparous eelpout) demonstrated a persistent shift in energy budget towards acid-base regulation, possibly at the expense of other factors, such as growth (Deigweiher et al. 2008, Melzner et al. 2009a). Furthermore, increased energy demands for acid-base regulation may be compensated through decreased rates in protein turnover, anabolism, or activity levels (Deigweiher et al. 2008), further compromising normal metabolic function. For example, early offspring of *O. latipes* increased amino acid catabolism as an energy support when reared under high CO_2, which suggests that early offspring have increased energy requirements when subjected to hypercapnia (Tseng et al. 2013). Early season *M. menidia* offspring may suffer a higher risk of starvation as they deplete their energy reserves countering high CO_2 during the critical transition from yolk- to first-feeding larvae. This is consistent with the findings from Baumann et al. (2012), where survival reductions to early *M. beryllina* were found to be primarily a consequence of embryonic exposure, even though the most significant mortality was observed post hatch. Presently, few

studies have explored changes in the metabolic budget of fish early life stages exposed to high CO_2, yet this should remain a focus of future ocean acidification research.

Mortality could also be a result of detrimental behavioral abnormalities. Increased CO_2 has been shown to influence a number of behavioral attributes in marine fish; including anti-predator response (Ferrari et al. 2011, Allan et al. 2014), sensory performance (Munday et al. 2009a, Simpson et al. 2011) and lateralization (Domenici et al. 2012, Jutfelt et al. 2013). Nilsson et al. (2012) suggested high CO_2 interferes with the major vertebrate inhibitory neurotransmitter receptor GABA-A, leading to most or all observed behavioral effects. When larvae are exposed to high CO_2 ion-regulatory adjustments alter Cl^- and HCO_3^- gradients over neuronal membranes, causing membrane hyperpolarization and depolarization of post-synaptic neurons, resulting in a reversal of normal GABA-A receptor function. GABA-A receptors are ubiquitous in the central nervous system of vertebrates, thus, complex behaviors like mating rituals, predator avoidance and prey capture may be compromised in a high CO_2 world (Jutfelt et al. 2013). For example, juvenile *A. polyacanthus* exhibited significantly slower retinal responses under high CO_2 compared to a control group (Chung et al. 2014). Such a decrease in visual speed might result in reduced reaction times to identify and capture evasive copepod nauplii or lead to higher predation mortality in the wild. Larval fish are primarily visual hunters (Houde & Schekter 1980), thus visual impairment may substantially increase mortality around the vulnerable period of transition from endogenous to exogenous feeding. Although no observations were made in prey capture success between ambient and high CO_2 treated early season *M. menidia* larvae, reduced capture success is an intriguing possibility contributing to mortality under high CO_2.

What are the mechanisms that seasonally modify offspring CO_2 reaction norms?

There are several alternative explanations why CO_2 tolerance varies seasonally in *M. menidia* offspring. Theoretically, discrete populations of *M. menidia* spawning at different periods of the spawning season could produce genetically distinct offspring with varying levels of CO_2 tolerance. However, the species exhibits a semelparous, batch-spawning life history (Conover & Kynard 1984), where adults overwinter in coastal shelf waters and move as single population into spawning habitats, where they remain until death (Conover & Ross 1982). Therefore, we assume that we sampled adults from a single population throughout the spawning season. Instead, seasonal plasticity in offspring CO_2 tolerance may be triggered by non-genetic gamete modifications. Adult *M. menidia* sensing a more variable and acidic environment while preparing to spawn in a semi-lunar rhythm may modify offspring CO_2 sensitivity via two potential mechanisms: transgenerational epigenetic inheritance and/or maternal provisioning.

Transgenerational plasticity (TGP) enhances offspring fitness through the inheritance of epigenetic states, from parents of both sexes, which are determined by parental environmental experience (Jablonka & Raz 2009, Salinas et al. 2013). Epigenetic mechanisms control gene expression via several pathways (e.g., DNA methylation, repressive histone modification: Chinnusamy & Zhu 2009) without requiring changes to genetic sequences. Inheritance of specific epigenetic configurations (e.g., corresponding to high or low CO_2 levels) could potentially enhance offspring physiological performance under environmental hypercapnia. Adult *M. menidia* experiencing progressively more variable and acidic environments throughout their spawning season may condition their offspring with beneficial epigenetic configurations resulting in the increased offspring survival we observed in lab. One possible pathway to increase offspring CO_2 tolerance is altering the expression of key enzymes involved ion-regulation or mitochondrial metabolism (Esbaugh et al. 2012, Tseng et al. 2013). Many studies

have shown transcriptomic regulated changes in gene expression was exposed to acidified waters. *Z. viviparus* exposed to high CO_2 initially showed a downregulation, but then an upregulation of several key ion transporters, including Na^+/ HCO_3^- and Cl^-/ HCO_3^- exchangers (Deigweiher et al. 2008). Na^+/K^+ ATPase activity increased steadily throughout the exposure, mRNA and protein levels increased to 200 and 140% of control levels. *G. morhua* showed a similar increase in Na^+/K^+ ATPase activity under a long term acclimation (Melzner et al. 2009a). Such transcriptomic plasticity has been detected in fish larvae as well. The zebrafish *Danio rerio* embryos increased the quantity and size of epidermal H^+-ATPase-rich (HR) cells when exposed to low pH water (Horng et al. 2009). A detailed molecular approach by Tseng et al. (2013) found *O. latipes* larvae increased expression of several genes involved in acid secretion, HCO_3^- regulation, and Na^+/K^+ ATPase activity. Given the critical role of these enzymes in ion and acid-base regulation under environmental hypercapnia (Deigweiher et al. 2008), inheritance of an epigenetic configuration which upregulates their expression may comprise a potential pathway for increased offspring CO_2 tolerance. Given that early life stage vulnerability to hypercapnia has been linked to inefficient or under-developed acid-base regulatory mechanisms, either the timing of the development of adult-like structures (e.g. brachial ionocytes) or the enhancement of early structures (e.g. epidermal ionocytes) may play significant roles in determining species or population specific vulnerabilities.

Maternal effects comprise an alternative mechanism by which adult female *M. menidia* could alter offspring CO_2 reaction norms. Maternal effects describe non-genetic phenotypic variations in offspring as a consequence of maternal provisioning (Mousseau & Fox 1998). Theoretically, female *M. menidia* sensing the acidification of their spawning habitat could provide their eggs with more yolk lipids, thereby increasing embryonic energy reserves to meet

enhanced metabolic demands of acid-base regulation. Alternatively, seasonal increases in egg quality may reflect an improvement in maternal fitness. Adult *M. menidia* that survive the winter are typically in poor condition, but body condition improves drastically during their first weeks in spawning estuaries as temperatures rise and feeding increases (Conover & Ross 1982). Maternal effects are generally thought to manifest via larger eggs that produce larger larvae, which in turn have greater chances for survival (Green 2008). Seasonal increases in offspring CO_2 tolerance could therefore be unrelated to seasonal estuarine acidification and instead reflect seasonal increases in maternal condition; however, our length-at-hatch data does not support this argument. Across all CO_2 treatments, length of 1 dph larvae was largest in April and declined steadily throughout the season showing no correlation between hatch length and CO_2 tolerance. However, seasonal changes in maternal condition or egg quality may explain the steady, CO_2-unrelated, decline in survival and growth during the second half of the spawning season. Such a rapid decline in offspring fitness may be linked to deteriorating adult condition, and with it the quality of eggs, as *M. menidia* conclude their annual lifecycle (McEvoy & McEvoy 1992, Trippel 1998). Assuming this to be true, and given that the offspring fitness declined similarly across all CO_2 treatments, offspring CO_2 reaction norms may not be affected by maternal condition, bolstering the argument for the existence of TGP in CO_2 tolerance. Targeted microscopic or energetic measurements maternal condition and of egg quality, color, and size are needed to support or discount any such maternal effects.

What factors contributed to interannual variability?

The general pattern of seasonal increases in offspring tolerance to CO_2 was consistent during both years of experimentation, however, some interannual variability was observed. During the first experiment of 2012, both survival and growth rates were significantly lower for

larvae reared under high CO_2 compared to ambient levels, however, subsequent experiments in 2012 and those in 2013 did not observe a significant growth effect. Metabolic depression may be a typical response of an organism suffering from uncompensated acidosis (Melzner et al. 2009b), thus reduced growth under chronic high CO_2 is expected in sensitive early life stages. However, our approach of feeding larvae *ad libitum* food rations may have masked any CO_2 related growth reductions. Those larvae exposed to high CO_2 that survived the initial wave of CO2 related mortality may have increased their food consumption to meet the increased metabolic demands associated with acid-base regulation, thus maintaining similar growth as larvae from ambient treatments. Increased feeding rates may explain why most studies testing the effects of high CO_2 on fish early life stages have found neutral or positive growth effects (Munday et al. 2009b, Frommel et al. 2012b, Bignami et al. 2013, Chambers et al. 2013, Hurst et al. 2013).

In addition, our seasonal seining effort observed substantial differences in the onset and duration of the spawning season between the two years; ripe adult *M. menidia* appeared three weeks earlier and disappeared two weeks later in 2012 than in 2013. Warm late winter temperatures in 2012 may have prompted adults to ripen gametes and return to coastal estuaries sooner than after typical winters, given that *M. menidia* are likely cued by warming temperatures to make the seasonal spawning ingress into shallow coastal habitats (Conover & Ross 1982). Temperatures in our Flax Pond study estuary were roughly 5°C warmer during much of March 2012 than March 2013 (Figure 15). Unfortunately, how thermal differences translated to seasonal pH dynamics in the estuary is unknown due to the lack of pH data during the 2012 season. However, combining temperature data with the observed two week lag of increased offspring CO_2 tolerance (Figure 13 B) suggested that lower 2013 temperatures translated to a two week

delay in environmental acidification. Further investigation is needed to determine how interannual variability of seasonal warming and acidification may affect offspring CO_2 tolerance.

What are the broader implications for ocean acidification research?

The discovery of seasonally shifting CO_2 sensitivities in the early life stages of a widely distributed coastal marine fish may influence the direction and methodology of future ocean acidification research. The primary motivation for most OA experimentation has been, and remains to be, an assessment of species-specific vulnerabilities to elevated CO_2 (Denman et al. 2011, Branch et al. 2013). This task is complicated by the existence of seasonal plasticity in offspring CO_2 tolerance influenced by parental environments. For example, many studies, but particularly those targeting early life stages, often choose species based on feasibility and access to sufficient and reliable quantities of embryos. This is often achieved by relying on adult broodstocks in aquaculture or research labs, where culturing conditions (light, temperature, pH, food) are unlikely to resemble conditions experienced by wild populations, particularly for species which inhabit highly variable habitats. A case in point, Baumann et al. (2012) observed that *M. beryllina* offspring reared under elevated (~1,000 µatm) CO_2 displayed reductions to growth (18%) and survival (74%) compared to ambient (~400 µatm) levels. In this study, *M. menidia* offspring were largely tolerant of 1,000 µatm, only showing significant effects at ~2,300 µatm early in the spawning season. *M. beryllina* embryos were obtained from a large, genetically diverse commercial broodstock that experiences stable not highly variable CO_2 conditions unlike wild *M. menidia* populations. The varying level of CO_2 tolerance between these closely related species may reflect strict species-specific CO_2 reaction norms, however, it may be that wild populations that experience substantial CO_2 variability are more robust to high CO_2. Triggering changes in offspring CO_2 reaction norms through parental acclimation is clearly possible in the

lab (Miller et al. 2012, Parker et al. 2012, Allan et al. 2014) but without experiencing such variability broodstocks may consistently produce CO_2-sensitive, or under high CO_2 conditions, CO_2-tolerant offspring. The use of commercial or lab-maintained broodstocks in ocean acidification research will continue to produce valuable mechanistic data, but such experiments are unlikely to accurately reflect a species vulnerability rising oceanic pCO_2.

The seasonal acidification experienced by *M. menidia* is consistent with a growing number of studies arguing that most ecologically and economically important species inhabit dynamic coastal habitats exhibiting large diel and seasonal CO_2 fluctuations (Hendriks et al. 2010, Hofmann et al. 2011, Duarte et al. 2013, Kelly et al. 2013, McElhany & Busch 2013). The extent of spatio-temporal CO_2 variability typical of a species' habitat may influence their CO_2 tolerance and thus vulnerability to future ocean acidification. In late summer, *M. menidia* juveniles inhabiting Flax Pond experience diel CO_2 fluctuations of 400 – 4,000 µatm (Baumann et al. 2014), i.e., CO_2 levels about twice the magnitude of the predicted maximum acidification in the open ocean over the next 300 years (Caldeira & Wickett 2003). Thus, the evolution of transgenerational acclimation, or other genetic or phenotypic CO_2 adaptions, to cope with rapid and large natural CO_2 variability may have 'pre-adapted' *M. menidia*, and potentially many species that inhabit dynamic coastal environments, to tolerate future anthropogenic acidification (Melzner et al. 2009b, Kelly et al. 2013). Ocean acidification may indeed be an 'open-ocean syndrome'; while coastal species may tolerate acidification, pelagic species that have evolved in the stable CO_2/pH conditions of the open ocean may have a reduced capacity to tolerate the anthropogenic reductions in pH (Duarte et al. 2013).

However, CO_2/pH conditions in coastal habitats are also strongly correlated to levels of dissolved oxygen (Cai et al. 2011, Waldbusser et al. 2011, Melzner et al. 2012, Baumann et al.

2014). The combined effects of other anthropogenic influences, including coastal eutrophication and global oceanic warming, could synergistically exacerbate CO_2/pH, temperature and O_2 variability in coastal systems (Hofmann & Todgham 2010, Shaw et al. 2013, Gobler et al. 2014) and challenge even species like *M. menidia* to persist in afflicted coastal habitats.

Future work

The effects of ocean acidification on the early life stages of marine fish continue to be an important field rich of future research needs. The existence of seasonally shifting CO_2 reaction norms suggests *M. menidia* are capable to adapt to rapid changes in environmental CO_2/pH. To substantiate the existence of TGP in offspring CO_2 tolerance, however, experimental manipulations of parental spawning conditions are required. This was attempted during spring 2013, but our approach of holding adults in acclimation tanks for six weeks after collection (following protocols described in Miller et al. 2012) did not yield sufficient egg quantities. However, given the rapid increase in offspring CO_2 tolerance (two weeks in 2013, Figure 8) the mechanisms that alter offspring reaction norms must be occurring over a similar period. Thus, future attempts to manipulate CO_2 spawning conditions should use shorter acclimation periods (e.g., 7 – 14 days)

A detailed expression profile of genes involved in acid-base regulation and metabolism would also help understanding the mechanisms of TGP in offspring CO_2 tolerance. Of particular interest are genes involved in proton secretion, HCO_3^- regulation, NH_4^+ transport and Na^+/K^+ ATPase regulation, whose expressions are strongly influenced by CO_2/pH conditions (Tseng et al. 2013). Profiling the gene expression adults and their offspring acclimated to high CO_2 may reveal genes involved in the epigenetic control of inherited CO_2 reaction norms. Expression of

genes from major metabolic pathways are also influenced by CO_2 exposure (Tseng et al. 2013), but little is known of the metabolic costs imposed on early larvae exposed to environmental hypercapnia. Expression profiles of metabolic pathways would reveal any CO_2-induced metabolic or developmental depression. In this study, CO_2-related metabolic effects may have been masked by our *ad libitum* feeding methodology. *Ad libitum* rations were used to maximize offspring survival, potentially resulting in artificially high survival and growth within the high CO_2 treatments. Carefully designed experiments under different levels of food limitation may elucidate the metabolic costs to larvae developing under high CO_2. Theoretically, larvae under high CO_2 would drain energy reserved from their egg yolk at a more rapid pace, thus starvation first appear in high compared to ambient CO_2.

There's an increasing awareness that treating organisms with constant CO_2/pH levels is not a realistic approach when determining vulnerabilities to coastal acidification. For example, during late summer Flax Pond salt marsh exhibits extreme diel CO_2 variability, ranging from 400 to 4,000 µatm within 12 hours (Baumann et al. 2014). In coastal estuaries, tidal effects can dominate diel pH variability (Baumann et al. 2014), creating predictably rapid fluctuations that can be simulated in the lab. Additionally, coastal acidification is necessarily coupled to temperature and O_2 variability. Ocean warming and eutrophication will increase community respiration in coastal habitats, exacerbating levels of acidification and hypoxia (Pörtner et al. 2004, Melzner et al. 2012, Duarte et al. 2013) that can have additive and synergistic negative effects on fitness (Gobler et al. 2014). An automated system that varies CO_2, O_2 and temperature simultaneously could administer treatments that replicate current or predicted environmental conditions, generating results more useful in determining species vulnerability to anthropogenic impacts. Testing pelagic species, on the other hand, poses additional challenges. Over the last 2

million years open ocean pH has been incredibly stable (Hönisch et al. 2009), thus sensitive early life stages of pelagic species may lack the acid-base regulatory capacity exhibited by coastal species and therefore be more vulnerable to small decreases in seawater pH. However, few studies have attempted to use pelagic species in CO_2 exposure experiments given the difficulties involved in obtaining viable embryos. Adaption to local conditions may dictate a species capacity to tolerate ocean acidification (Kelly et al. 2013), thus only a comprehensive effort to test different species, and even different population within a species, from different habitat regimes will yield a better understanding of how ocean acidification will affect fish populations.

Summary

This study suggested that transgenerational acclimation to increasing CO_2 levels is not just a laboratory phenomenon, but may be a common adaptive strategy employed by marine fish and other organisms experiencing natural pH and CO_2 variability in their coastal habitats. It entails that *M. menidia*, and maybe many coastal species, have the capacity to cope with predicted CO_2 over the next several centuries. How these species will cope with the synergistic effects of several stressors (CO_2, O_2, temperature) remains unknown. In addition, our results strongly suggest that realistic, experimental assessments of species vulnerabilities to ocean acidification (and other stressors) will require more comprehensive approaches that controls for parental environments (Miller et al. 2012, Parker et al. 2012, Reusch 2014).

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APPENDIX

Figures

Figure 1. Satellite image of the tidal marsh lagoons on the north shore of Long Island, NY, USA (inlet: Long Island, red dot: study area), with pins depicting the exact position of spawner collection (Poquot) and long-term, high-frequency pH monitoring sites (Flax Pond Bridge). Google earth, Image © TerraMetrics.

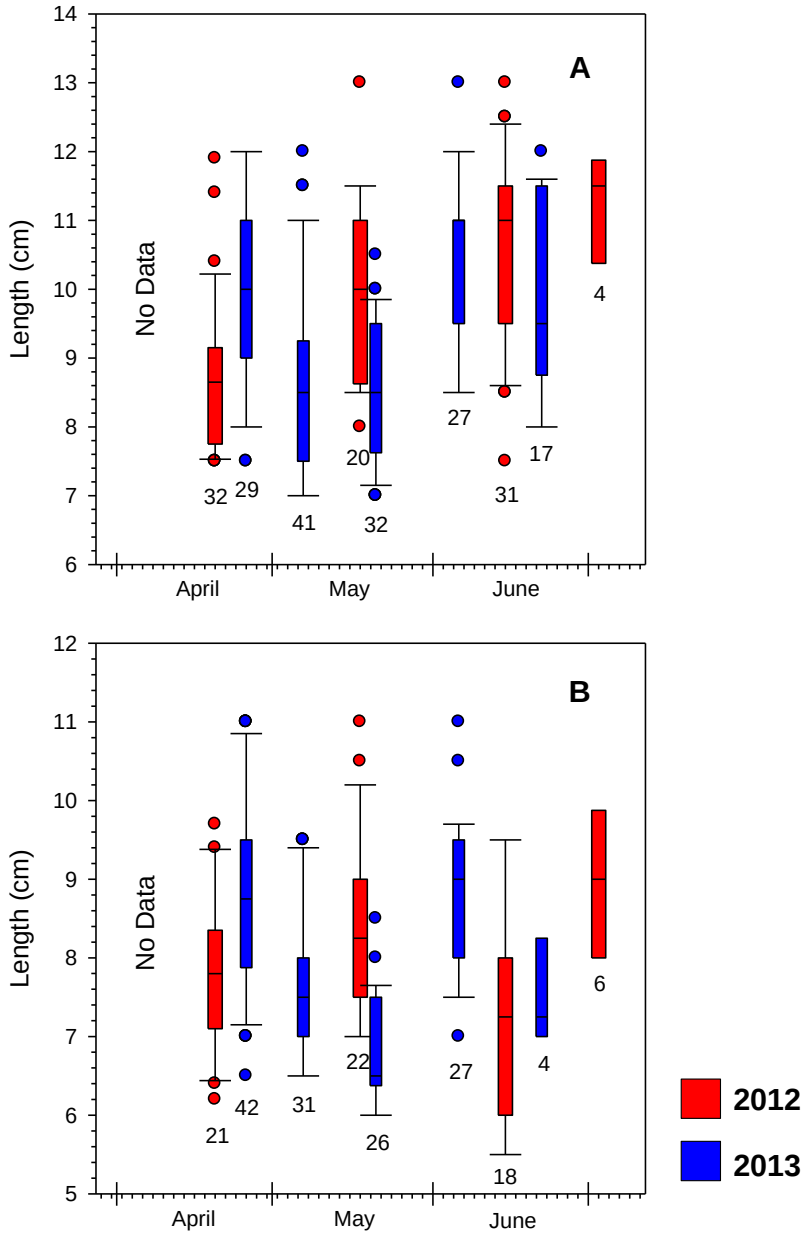

Figure 2. *M. menidia*. Box-Whisker Plots showing 10th, 25th, 50th, 75th, 90th percentiles and outliers of standard lengths of adults (A) females and (B) males used to fertilize offspring tested in CO_2 exposure experiments in 2012 (red) and 2013 (blue). Numbers below each box denote the number of individuals used. Adults used during the first experiment in 2012 were accidentally lost.

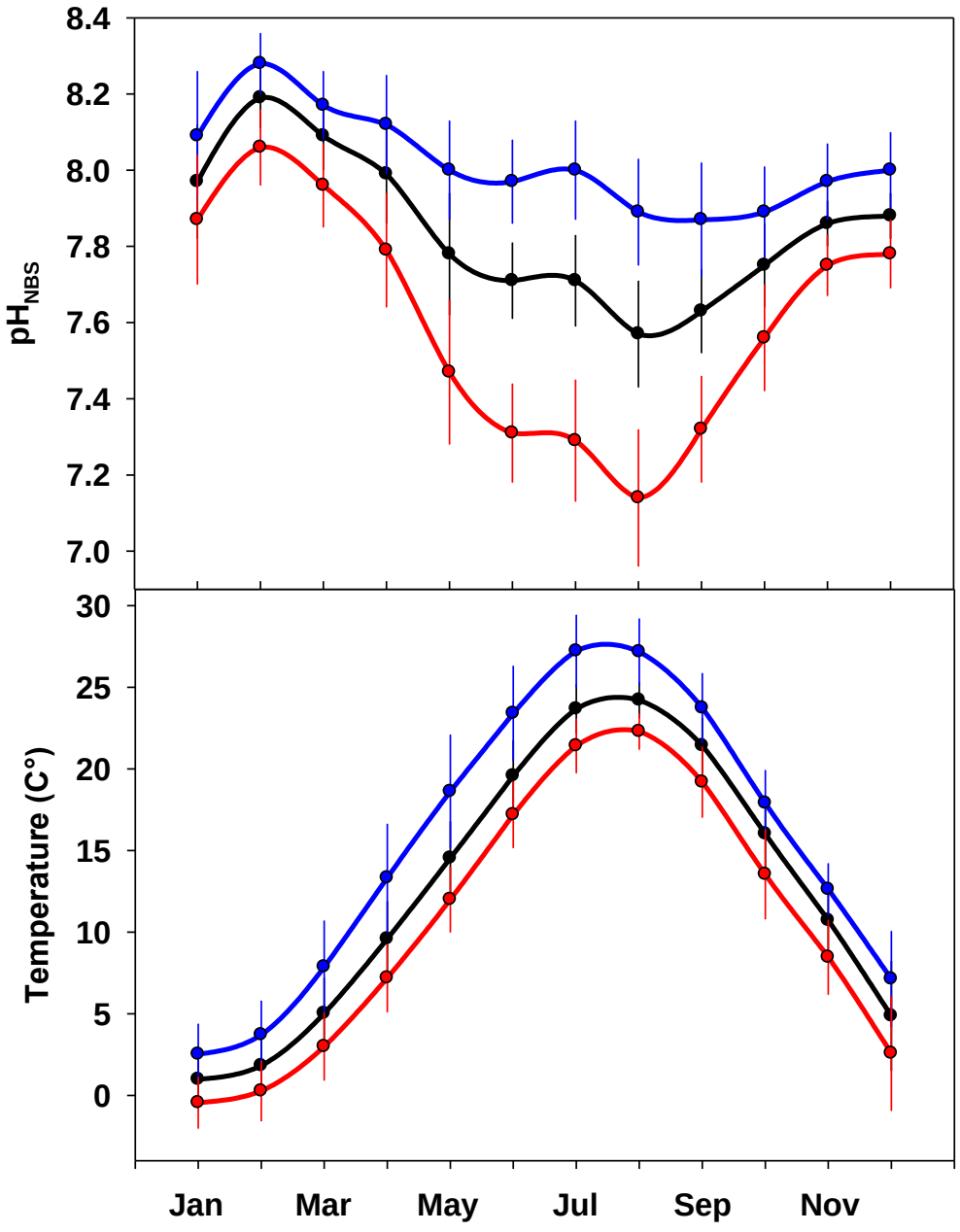

Figure 3. Seasonal variability of monthly mean (black lines, dots) maximum (blue lines, dots) and minimum (red lines, dots) (A) pHNBS and (B) temperature (C°) at Flax Pond salt marsh (2009 – 2012). Error bars represent ± 1 s.d

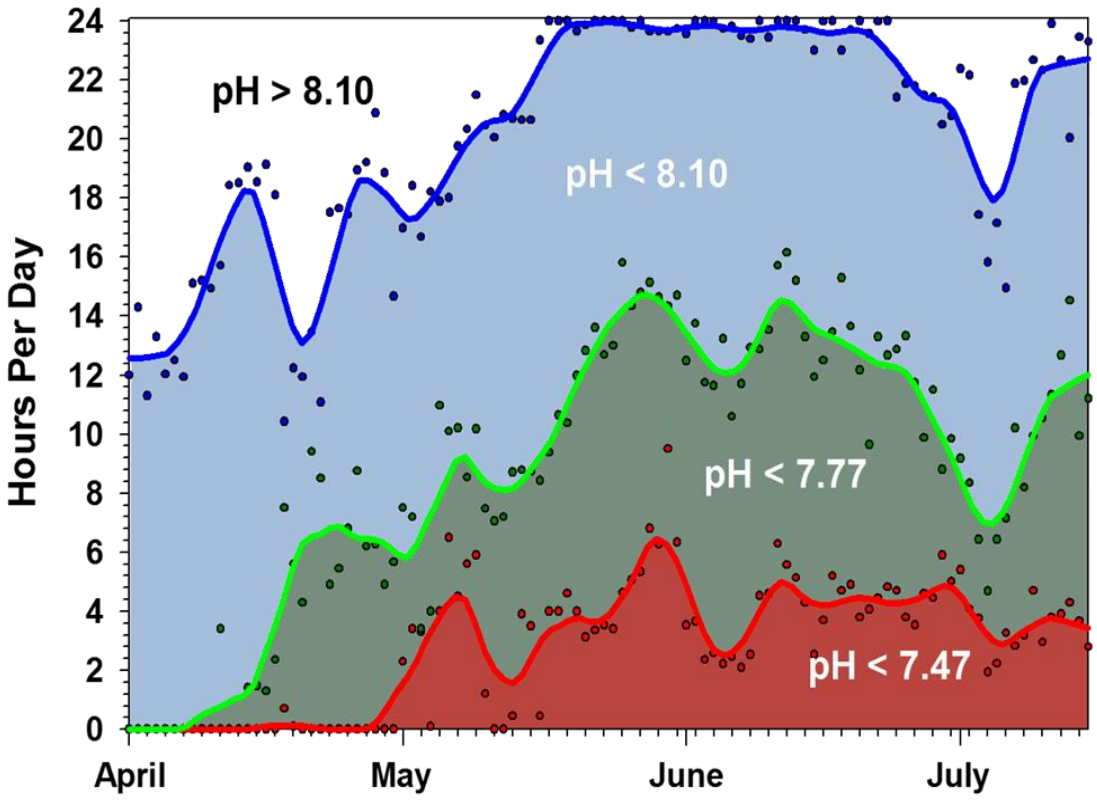

Figure 4. Seasonal changes in daily pH exposure of a hypothetical organism in Flax Pond. Dots represent the average time per day (h) when aquatic organisms would experience conditions below pH 8.1 (blue), pH 7.8 (green) and below pH 7.5 (red). Lines represent nonparametric local fits (Loess, bandwidth = 11 days).

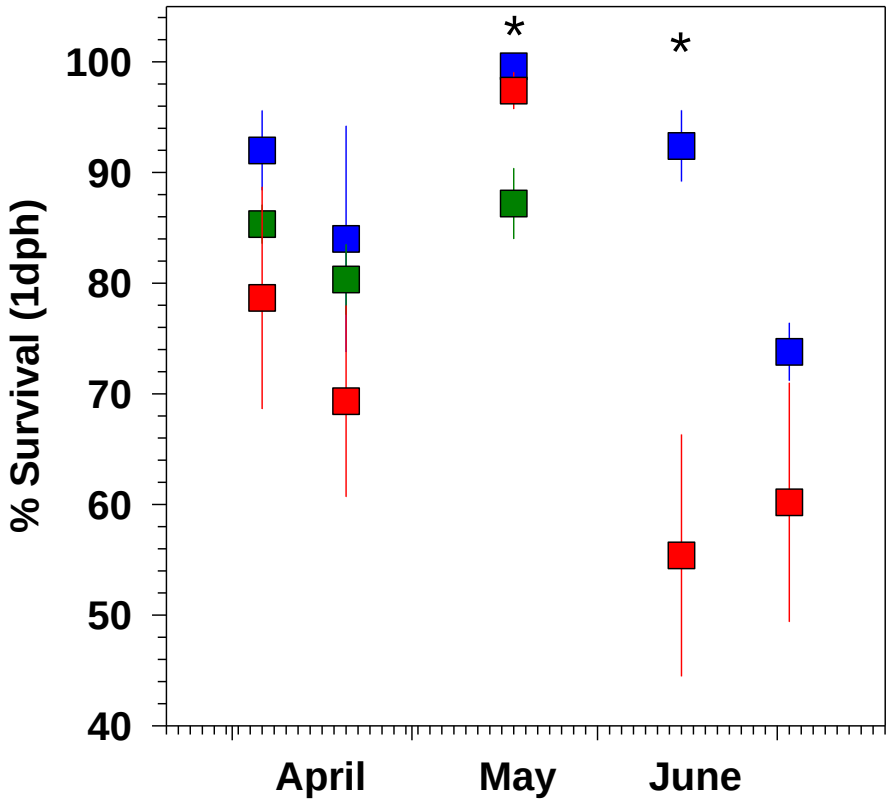

Figure 5. *M. menidia*. Offspring survival (%, 1 dph) when reared at three CO_2 levels; ambient (670 µatm, pHNBS = 8.04, blue squares), elevated (~1,100 µatm, pHNBS = 7.77, green squares), and high CO_2 levels (~2,300 µatm, pHNBS = 7.47, red squares). Symbols represent treatment averages ± 1 s.e. of 3-6 replicates over 5 experiment during 2012. Dates correspond to the day of fertilization. Significant differences between CO_2 treatments are denoted by (*).

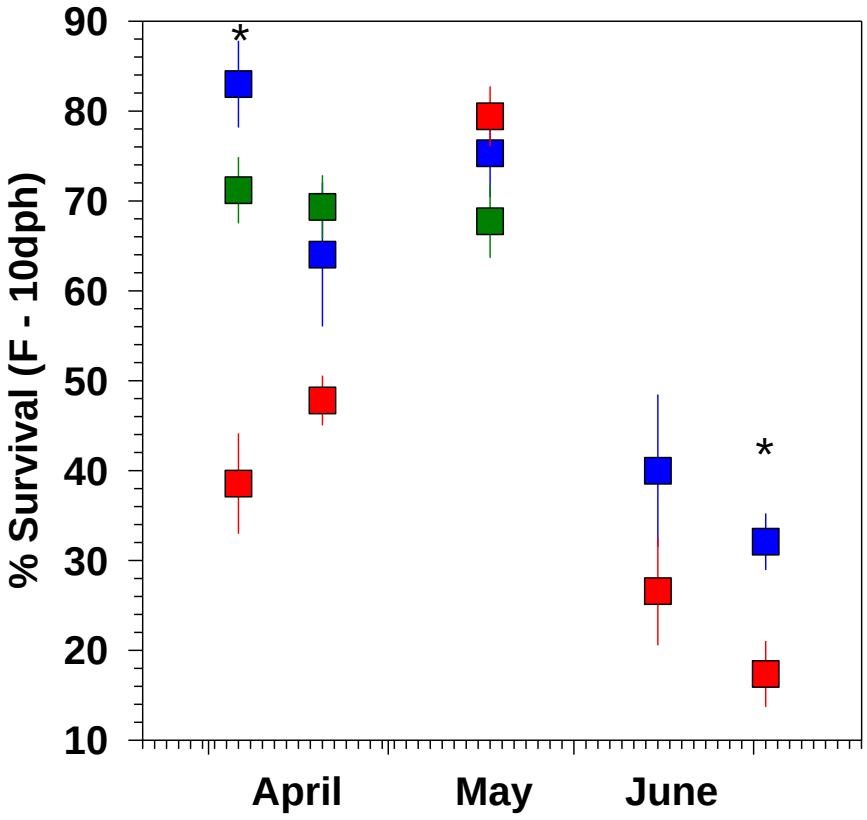

Figure 6. *M. menidia*. Offspring survival (%, f - 10dph) when reared at three CO_2 levels; ambient (670 µatm, pHNBS = 8.04, blue squares), elevated (~1,100 µatm, pHNBS = 7.77, green squares), and high CO_2 levels (~2,300 µatm, pHNBS = 7.47, red squares). Symbols represent treatment averages ± 1 s.e. of 3-6 replicates over 5 experiment during 2012. Dates correspond to the day of fertilization. Significant differences between CO_2 treatments are denoted by (*).

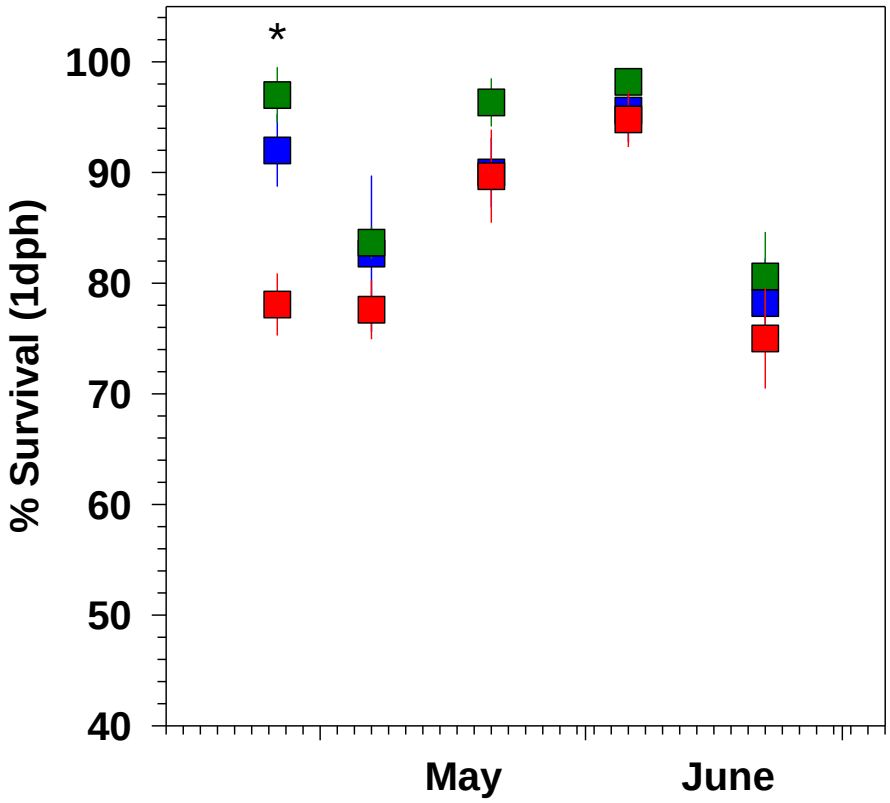

Figure 7. *M. menidia*. Offspring survival (%, 1 dph) when reared at three CO_2 levels; ambient (530 µatm, pHNBS = 8.10, blue squares), elevated (~1,100 µatm, pHNBS = 7.77, green squares), and high CO_2 levels (~2,300 µatm, pHNBS = 7.47, red squares). Symbols represent treatment averages ± 1 s.e. of 3-6 replicates over 5 experiment during 2013. Dates correspond to the day of fertilization. Significant differences between CO_2 treatments are denoted by (*).

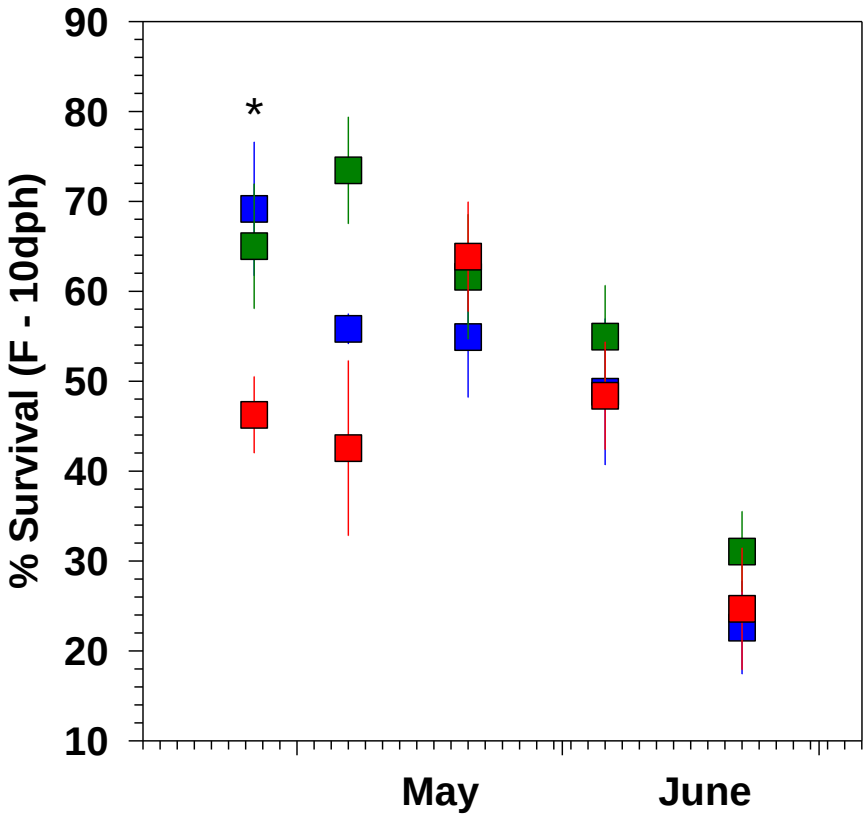

Figure 8. *M. menidia*. Offspring survival (%, f - 10dph) when reared at three CO_2 levels; ambient (530 µatm, pHNBS = 8.10, blue squares), elevated (~1,100 µatm, pHNBS = 7.77, green squares), and high CO_2 levels (~2,300 µatm, pHNBS = 7.47, red squares). Symbols represent treatment averages ± 1 s.e. of 3-6 replicates over 5 experiment during 2013. Dates correspond to the day of fertilization. Significant differences between CO_2 treatments are denoted by (*).

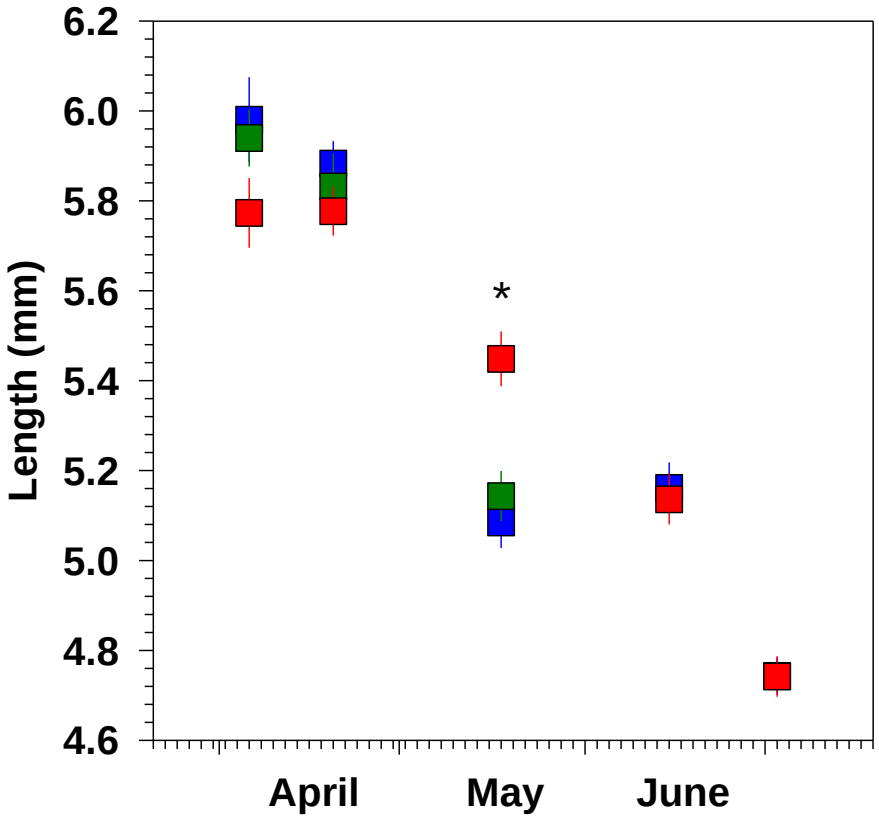

Figure 9. *M. menidia.* Offspring length (mm, 1 dph) when reared at three CO_2 levels; ambient (670 µatm, pHNBS = 8.04, blue squares), elevated (~1,100 µatm, pHNBS = 7.77, green squares), and high CO_2 levels (~2,300 µatm, pHNBS = 7.47, red squares). Symbols represent treatment averages ± 1 s.e. of 3-6 replicates over 5 experiment during 2012. Dates correspond to the day of fertilization. Significant differences between CO_2 treatments are denoted by (*).

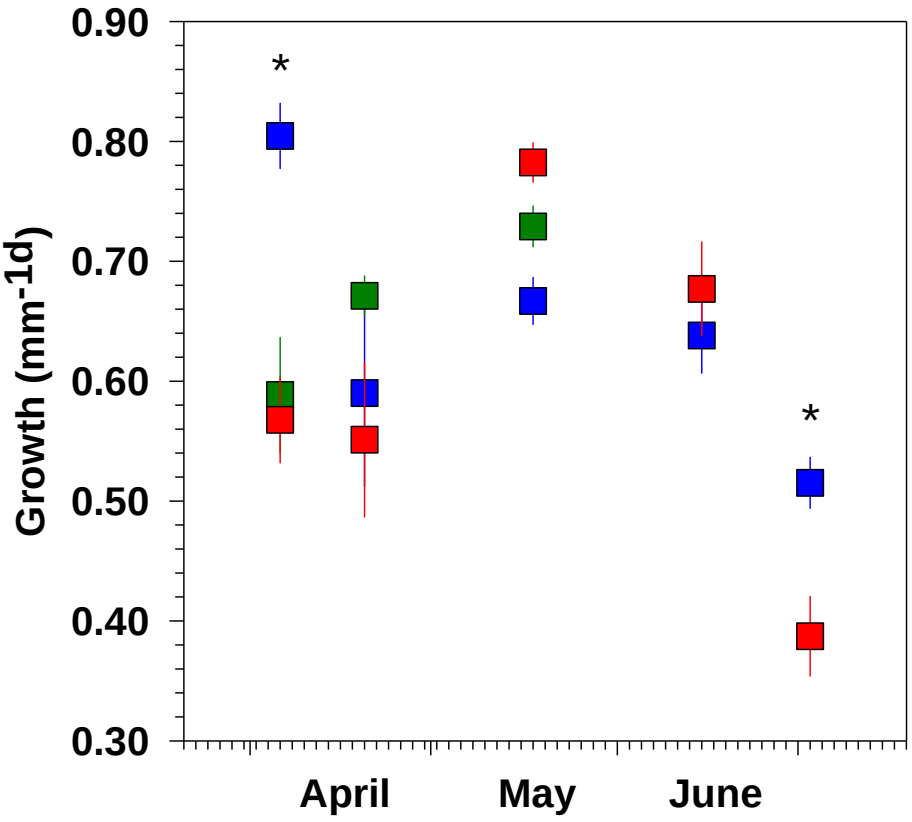

Figure 10. *M. menidia.* Offspring growth (mm d^{-1}, 1 – 10dph) when reared at three CO_2 levels; ambient (670 µatm, pHNBS = 8.04, blue squares), elevated (~1,100 µatm, pHNBS = 7.77, green squares), and high CO_2 levels (~2,300 µatm, pHNBS = 7.47, red squares). Symbols represent treatment averages ± 1 s.e. of 3-6 replicates over 5 experiment during 2012. Dates correspond to the day of fertilization. Significant differences between CO_2 treatments are denoted by (*).

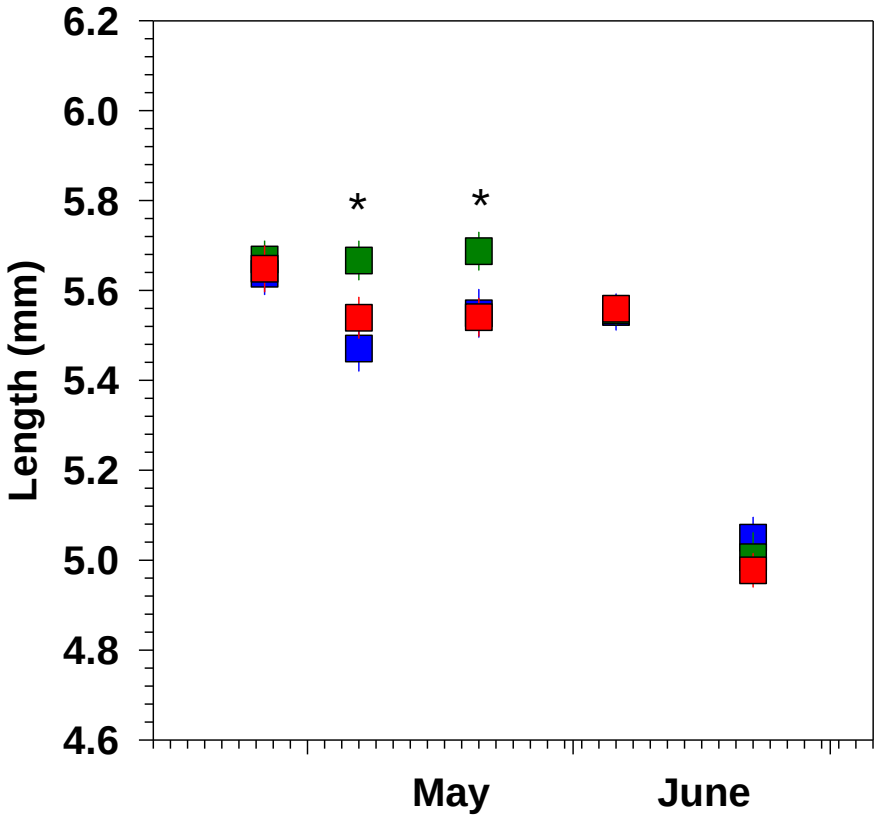

Figure 11. *M. menidia.* Offspring length (mm, 1 dph) when reared at three CO_2 levels; ambient (530 µatm, pHNBS = 8.10, blue squares), elevated (~1,100 µatm, pHNBS = 7.77, green squares), and high CO_2 levels (~2,300 µatm, pHNBS = 7.47, red squares). Symbols represent treatment averages ± 1 s.e. of 3-6 replicates over 5 experiment during 2013. Dates correspond to the day of fertilization. Significant differences between CO_2 treatments are denoted by (*).

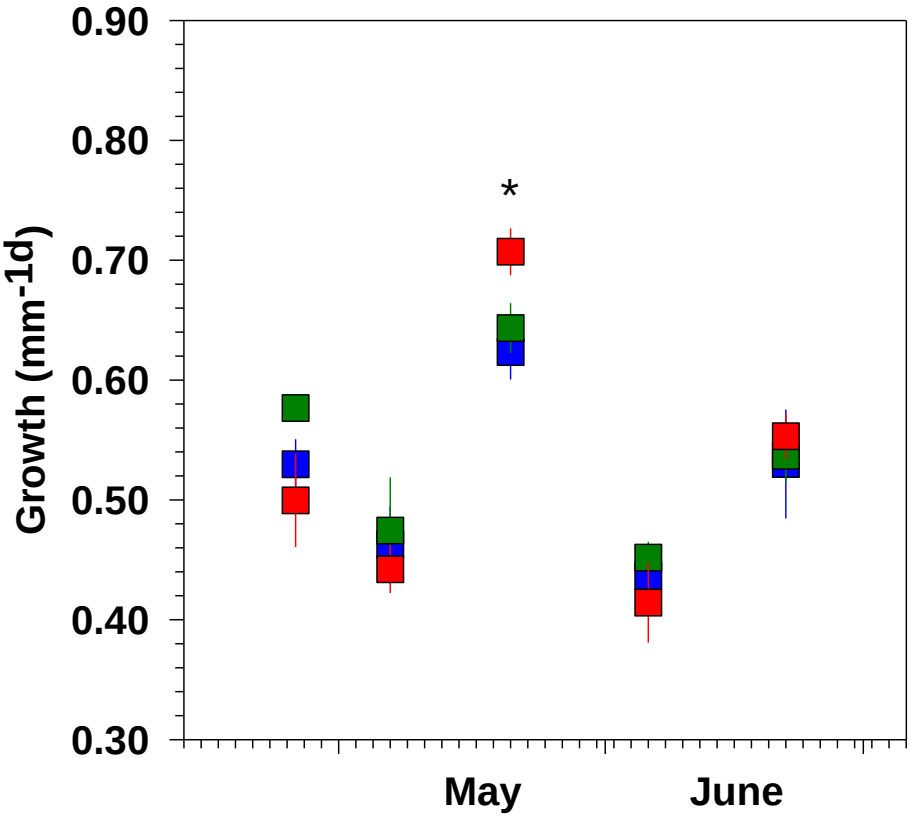

Figure 12. *M. menidia* Offspring growth (mm d^{-1}, 1 – 10dph) when reared at three CO_2 levels; ambient (530 µatm, pHNBS = 8.10, blue squares), elevated (~1,100 µatm, pHNBS = 7.77, green squares), and high CO_2 levels (~2,300 µatm, pHNBS = 7.47, red squares). Symbols represent treatment averages ± 1 s.e. of 3-6 replicates over 5 experiment during 2013. Dates correspond to the day of fertilization. Significant differences between CO_2 treatments are denoted by (*).

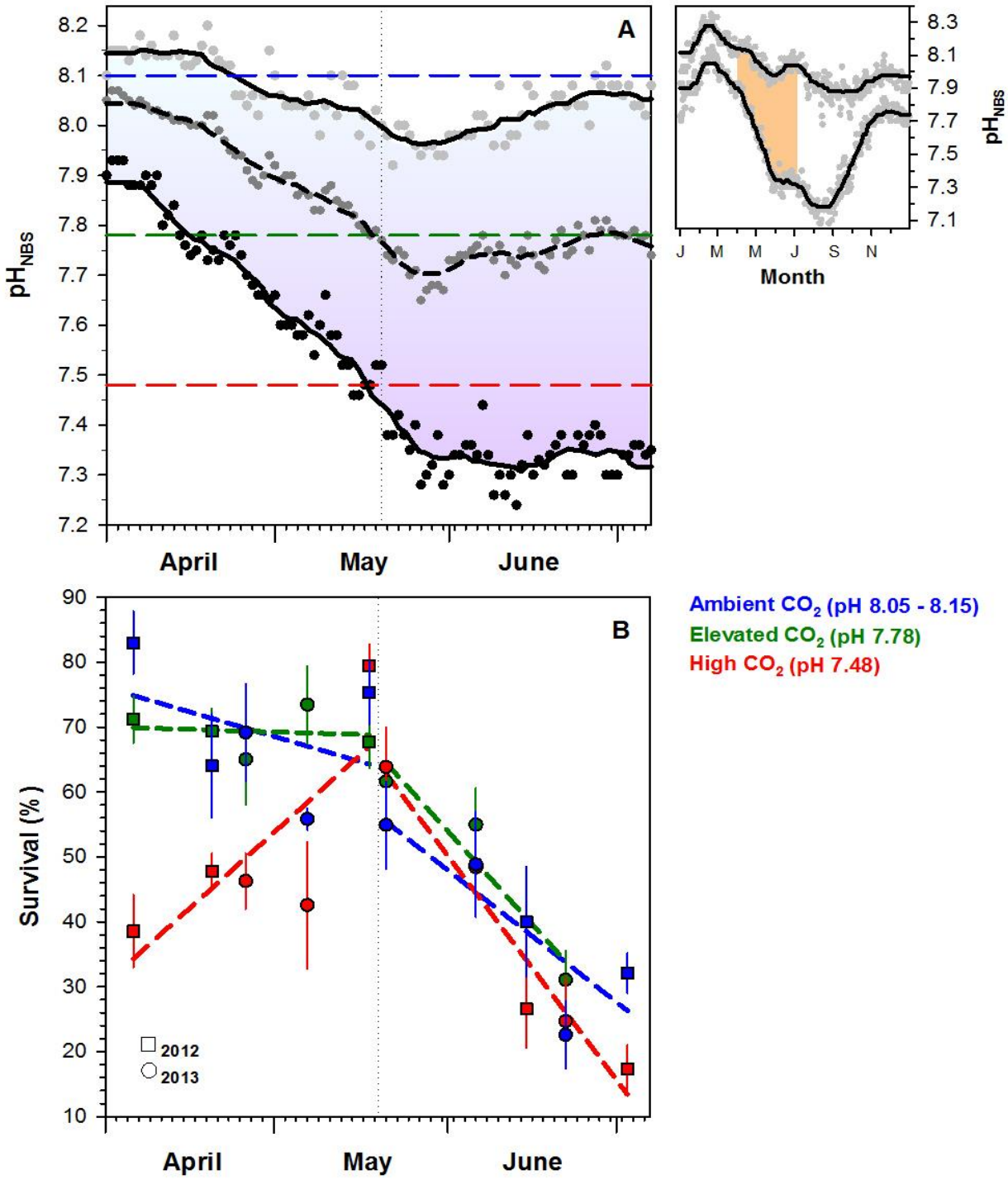

Figure 13. Seasonal acidification in the spawning habitat of *M. menidia* and its correspondence to changes in offspring survival under different CO_2 levels. A: Five year (2008-2012) average daily pH minima, means, and maxima (black, dark grey, grey circles, respectively) in April, May, and June (A1: January – December, shading = M. menidia spawning season) at Flax Pond

salt marsh, fitted with a moving median local smoother (bandwidth = 10% of data). Dashed lines depict experimental pHNBS levels. B: *M. menidia*. Mean (± 1 s.e.) survival of offspring reared from fertilization to 10 dph at ambient (2012: 670 µatm, pHNBS = 8.04; 2013: 530 µatm, pHNBS = 8.10, blue circles), elevated (~1,100 µatm, pHNBS = 7.77, green diamonds), and high CO_2 levels (~2,300 µatm, pHNBS = 7.47, red squares) throughout the 2012 (see Figure 6) and 2013 (see Figure 8) spawning seasons. Dates correspond to the day of fertilization. Dashed lines represent linear fits to the combined data during the first and second half of the spawning season.

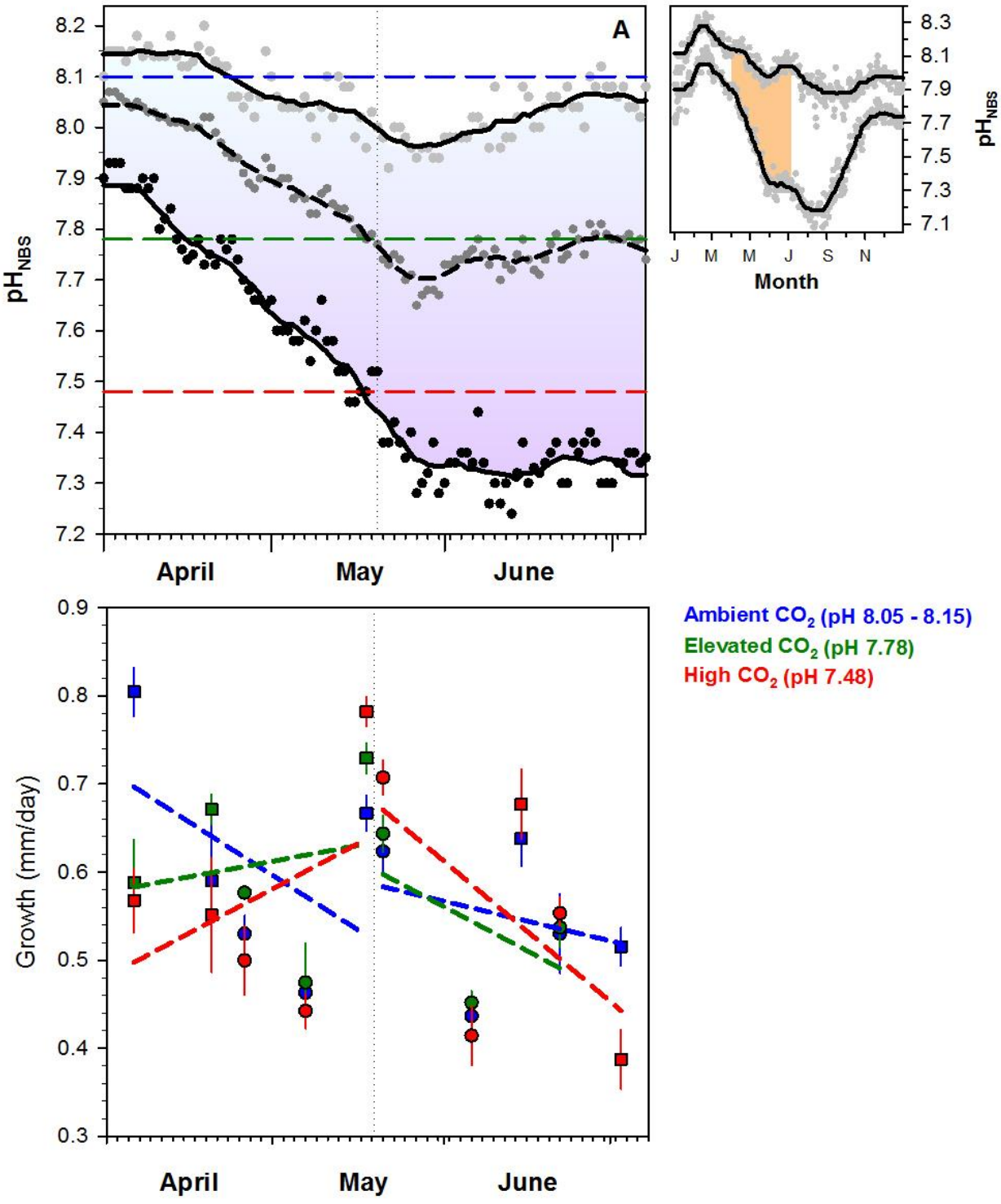

Figure 14. Seasonal acidification in the spawning habitat of *M. menidia* and its correspondence to changes in offspring growth under different CO_2 levels. A: Five year (2008-2012) average

daily pH minima, means, and maxima (black, dark grey, grey circles, respectively) in April, May, and June (A1: January – December, shading = M. menidia spawning season) at Flax Pond salt marsh, fitted with a moving median local smoother (bandwidth = 10% of data). Dashed lines depict experimental pHNBS levels. B: *M. menidia*. Mean (± 1 s.e.) growth (mm d^{-1}) of offspring reared from 1 -10 dph at ambient (2012: 670 µatm, pHNBS = 8.04; 2013: 530 µatm, pHNBS = 8.10, blue circles), elevated (~1,100 µatm, pHNBS = 7.77, green diamonds), and high CO_2 levels (~2,300 µatm, pHNBS = 7.47, red squares) throughout the 2012 (see Figure 10) and 2013 (see Figure 12) spawning seasons. Dates correspond to the day of fertilization. Dashed lines represent linear fits to the combined data during the first and second half of the spawning season.

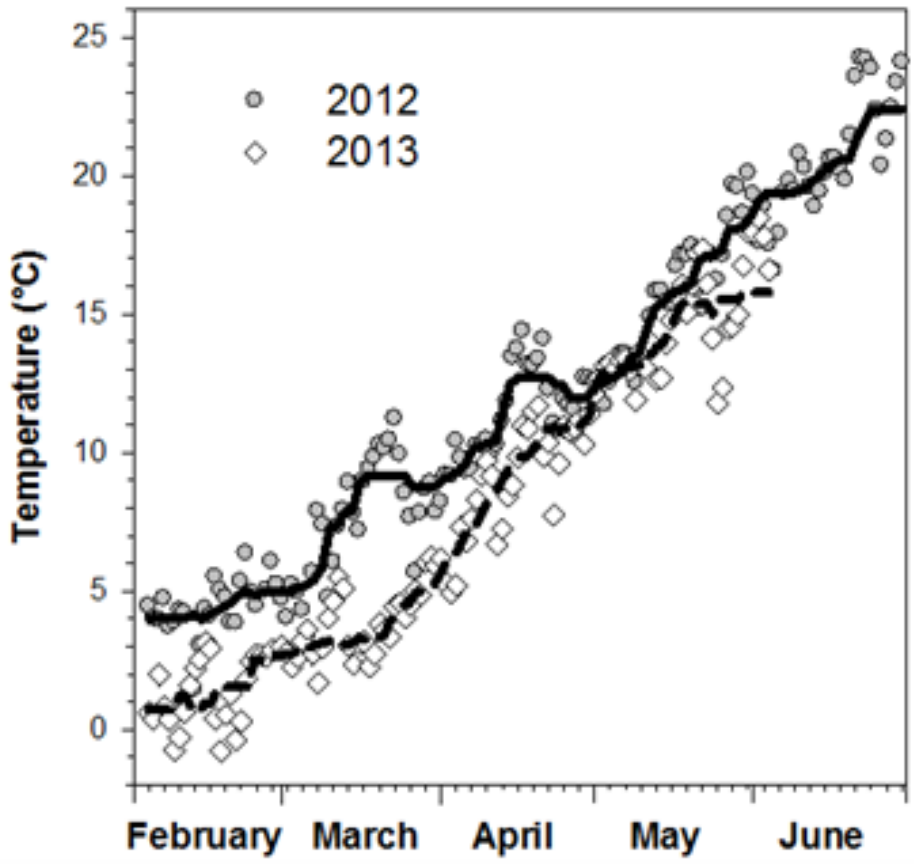

Figure 15. Mean daily water temperature at the Flax Pond salt marsh in spring 2012 (grey circles) versus 2013 (white diamonds), measured in the main channel ~0.5 above the bottom (US Geological Survey monitoring site #01304057, http://ny.water.usgs.gov/rt/pub/01304057.html). Black and dashed lines represent non-parametric, running median local fits (band width = 10% of the data).

TABLES

Year	Exp	CO_2 level	Temp	Salinity	pH	pCO_2	Total DIC	Total alkalinity
2012	1	A	24	25	8.03 ± 0.02	660	2226	2281
		E	24	25	7.79 ± 0.04	1042	2218	2219
		H	24	25	7.48 ± 0.05	2153	2338	2249
	2	A	24	25	8.02 ± 0.03	653	2197	2251
		E	24	25	7.75 ± 0.06	1031	2198	2201
		H	24	25	7.47 ± 0.03	2289	2487	2393
	3	A	24	25	8.03 ± 0.03	624	2250	2303
		E	24	25	7.78 ± 0.03	1117	2359	2346
		H	24	25	7.48 ± 0.03	2190	2315	2212
	4	A	24	25	8.09 ± 0.04	758	2628	2698
		H	24	25	7.45 ± 0.06	2250	2471	2372
	5	A	24	25	8.05 ± 0.01	648	2425	2503
		H	24	25	7.47 ± 0.04	2380	2541	2446
2013	1	A	24	25	8.11 ± 0.07	459	2175	2262
		E	24	25	7.75 ± 0.03	1020	2153	2147
		H	24	25	7.45 ± 0.04	2294	2324	2235
	2	A	24	25	8.13 ± 0.07	544	2379	2546
		E	24	25	7.76 ± 0.02	1243	2517	2590
		H	24	25	7.46 ± 0.03	2145	2254	2157
	3	A	24	25	8.09 ± 0.05	539	2379	2514
		E	24	25	7.76 ± 0.04	1071	2517	2219
		H	24	25	7.47 ± 0.04	2540	2254	2475
	4	A	24	25	8.09 ± 0.04	560	2146	2326
		E	24	25	7.76 ± 0.04	1142	2265	2374
		H	24	25	7.47 ± 0.05	2401	2380	2396
	5	A	24	25	8.10 ± 0.06	545	2317	2491
		E	24	25	7.77 ± 0.04	1220	2336	2443
		H	24	25	7.49 ± 0.03	2146	2173	2192

Table 1. Temperature (C°), salinity (psu), pH (NBS), pCO_2 (µatm), dissolved inorganic carbon (DIC), and total alkalinity during 10 exposure experiments on *M. menidia* offspring in 2012 and 2013, testing ambient (A), elevated (E) and high CO_2 levels (H) on embryos and early larvae. Results are given as treatment means (± 1 s.d. when applicable).

Year	Exp	FD	CO_2 Level	# Replicates	F-1 dph survival	1-10 dph survival	F-10 dph survival	1 dph length	10 dph length	Growth
2012	1	6 April	A	3	92 ± 4	90 ± 3	83 ± 5	5.98 ± 0.09	13.22 ± 0.11	0.80 ± 0.03
			E	3	85 ± 2	84 ± 7	71 ± 4	5.94 ± 0.30	11.23 ± 0.16	0.59 ± 0.05
			H	3	79 ± 10	49 ± 2	39 ± 6	5.77 ± 0.08	10.88 ± 0.18	0.57 ± 0.04
	2	20 April	A	3	84 ± 10	77 ± 7	64 ± 8	5.88 ± 0.05	11.19 ± 0.25	0.59 ± 0.08
			E	3	80 ± 3	86 ± 1	69 ± 4	5.83 ± 0.07	11.87 ± 0.19	0.67 ± 0.02
			H	3	69 ± 9	70 ± 6	48 ± 3	5.78 ± 0.05	10.74 ± 0.25	0.55 ± 0.06
	3	18 May	A	5	100 ± 1	76 ± 5	75 ± 5	5.08 ± 0.06	11.08 ± 0.11	0.67 ± 0.02
			E	5	87 ± 3	78 ± 3	68 ± 4	5.14 ± 0.06	11.70 ± 0.11	0.73 ± 0.02
			H	5	97 ± 2	82 ± 5	79 ± 3	5.45 ± 0.06	12.47 ± 0.10	0.78 ± 0.02
	4	15 June	A	5	92 ± 3	43 ± 9	40 ± 8	5.16 ± 0.06	10.94 ± 0.18	0.64 ± 0.03
			H	5	55 ± 11	48 ± 6	27 ± 6	5.14 ± 0.06	11.31 ± 0.18	0.68 ± 0.04
	5	3 July	A	5	74 ± 3	44 ± 4	32 ± 3	4.74 ± 0.04	9.38 ± 0.14	0.52 ± 0.02
			H	5	60 ± 11	28 ± 2	17 ± 4	4.74 ± 0.05	8.44 ± 0.16	0.39 ± 0.03
2013	1	26 April	A	5	92 ± 3	75 ± 6	69 ± 7	5.64 ± 0.05	10.40 ± 0.16	0.53 ± 0.02
			E	3	97 ± 3	67 ± 6	65 ± 7	5.67 ± 0.04	10.86 ± 0.17	0.58 ± 0.01
			H	13	78 ± 3	59 ± 4	46 ± 4	5.65 ± 0.05	10.18 ± 0.18	0.50 ± 0.04
	2	7 May	A	5	83 ± 7	68 ± 4	56 ± 2	5.47 ± 0.05	10.38 ± 0.10	0.49 ± 0.03
			E	3	84 ± 1	88 ± 6	73 ± 6	5.67 ± 0.04	10.47 ± 0.13	0.47 ± 0.04
			H	5	78 ± 3	54 ± 12	43 ± 10	5.54 ± 0.05	9.99 ± 0.08	0.44 ± 0.02
	3	21 May	A	6	90 ± 3	60 ± 6	55 ± 7	5.55 ± 0.05	11.16 ± 0.12	0.62 ± 0.02
			E	6	96 ± 2	64 ± 7	62 ± 7	5.69 ± 0.04	11.48 ± 0.09	0.64 ± 0.02
			H	6	90 ± 4	72 ± 7	64 ± 6	5.54 ± 0.04	11.90 ± 0.09	0.71 ± 0.02
	4	6 June	A	5	96 ± 3	52 ± 10	49 ± 8	5.55 ± 0.04	9.47 ± 0.12	0.44 ± 0.03
			E	5	98 ± 1	56 ± 6	55 ± 6	5.56 ± 0.03	9.62 ± 0.10	0.45 ± 0.01
			H	5	95 ± 2	51 ± 6	48 ± 6	5.56 ± 0.03	9.29 ± 0.13	0.41 ± 0.03
	5	22 June	A	5	78 ± 4	29 ± 6	23 ± 5	5.05 ± 0.05	10.02 ± 0.12	0.53 ± 0.05
			E	5	81 ± 4	39 ± 5	31 ± 4	5.01 ± 0.06	9.86 ± 0.11	0.54 ± 0.02
			H	5	75 ± 5	33 ± 9	25 ± 7	4.98 ± 0.04	9.99 ± 0.13	0.55 ± 0.02

Table 2. *M. menidia.* Results overview of 10 experiments (Exp) by fertilization date (FD) testing the effects of ambient (A), elevated (E), and high (H) CO_2 levels on the survival (%, F-1 day post hatch (dph), 1 – 10 dph, F – 10 dph), standard length (mm, 1 dph, 10 dph) and growth rate (mm d-1, 1 – 10 dph) of offspring during the 2012 and 2013 spawning seasons. Results are given as treatment means ± 1 s.e.

A

Month	Max	Mean	Min	Amp
1	8.09 ± 0.17	7.97 ± 0.15	7.87 ± 0.17	0.22 ± 0.08
2	8.28 ± 0.08	8.19 ± 0.08	8.06 ± 0.10	0.22 ± 0.08
3	8.17 ± 0.09	8.09 ± 0.09	7.96 ± 0.11	0.22 ± 0.08
4	8.12 ± 0.13	7.99 ± 0.13	7.79 ± 0.15	0.33 ± 0.13
5	8.00 ± 0.13	7.78 ± 0.16	7.47 ± 0.19	0.52 ± 0.14
6	7.97 ± 0.11	7.71 ± 0.10	7.31 ± 0.13	0.66 ± 0.12
7	8.00 ± 0.13	7.71 ± 0.12	7.29 ± 0.16	0.71 ± 0.16
8	7.89 ± 0.14	7.57 ± 0.14	7.14 ± 0.18	0.75 ± 0.15
9	7.87 ± 0.15	7.63 ± 0.11	7.32 ± 0.14	0.55 ± 0.18
10	7.89 ± 7.89	7.75 ± 0.11	7.56 ± 0.14	0.33 ± 0.11
11	7.97 ± 0.10	7.86 ± 0.06	7.75 ± 0.08	0.22 ± 0.09
12	8.00 ± 0.10	7.88 ± 0.06	7.78 ± 0.09	0.23±0.10

B

Month	Max	Mean	Min	Amp
1	2.52 ± 1.87	0.98 ± 1.73	-0.45 ± 1.58	2.97 ± 1.09
2	3.71 ± 2.09	1.82 ± 1.79	0.27 ± 1.85	3.44 ± 1.28
3	7.87 ± 2.84	5.02 ± 2.18	2.99 ± 2.08	4.88 ± 2.03
4	13.32 ± 3.32	9.58 ± 2.32	7.19 ± 2.10	6.13 ± 2.37
5	18.61 ± 3.49	14.53 ± 2.25	12.00 ± 2.02	6.61 ± 2.38
6	23.40 ± 2.92	19.57 ± 2.18	17.19 ± 2.04	6.20 ± 2.07
7	27.22 ± 2.22	23.67 ± 1.54	21.41 ± 1.67	5.82 ± 1.79
8	27.17 ± 2.04	24.21 ± 1.10	22.29 ± 1.11	4.88 ± 1.58
9	23.73 ± 2.14	21.42 ± 1.70	19.19 ± 2.18	4.53 ± 1.63
10	17.90 ± 2.03	16.02 ± 2.29	13.54 ± 2.75	4.37 ± 1.55
11	12.62 ± 1.60	10.72 ± 1.86	8.46 ± 2.29	4.16 ± 1.52
12	7.13 ± 2.94	4.87 ± 3.37	2.58 ± 3.53	4.55 ± 1.50

Table 3. Seasonal variability of monthly mean, maximum and minimum (A) pHNBS and (B) temperature (C°) at Flax Pond salt marsh (2009 – 2012). Error represents ± 1 s.d.

Spawning Period	Source of Variation	DF	F	*P*
April to mid-May	***Model***	***14***	***4.8***	***<0.001***
	Intercept	1	879.0	<0.001
	CO_2	2	11.4	<0.001
	FD	4	3.3	0.019
	CO_2*FD	8	2.9	0.009
Mid-May to early July	***Model***	***12***	***6.4***	***<0.001***
	Intercept	1	420.9	<0.001
	CO_2	2	1.0	0.367
	FD	4	16.0	<0.001
	CO_2*FD	6	0.9	0.494

Table 4. *M. menidia.* Effect of CO_2 level (ambient, elevated, high), fertilization date (FD, both years combined) and their interaction on the survival (%, fertilization – 10 dph, arcsine transformed) of offspring during the first and second half of the spawning season (Figure 13), using a General Linear Model.

Spawning Period	CO_2 level	DF	*P*	Slope
April to mid-May	Ambient	1	0.570	-
	Elevated	1	0.838	-
	High	1	0.002	+
Mid-May to early July	Ambient	1	0.002	-
	Elevated	1	0.002	-
	High	1	<0.001	-

Table 5. *M. menidia.* Offspring survival (%, fertilization – 10dph) as a linear function of fertilization date (2012, 2013 combined) during the first and second half of the spawning season (Figure 13).

Spawning Period	Source of Variation	DF	F	*P*
April to mid-May	***Model***	***14***	***12.3***	***<0.001***
	Intercept	1	4724	<0.001
	CO_2	2	3.5	0.041
	FD	4	3.3	<0.001
	CO_2*FD	8	2.9	<0.001
Mid-May to early July	***Model***	***12***	***14.6***	***<0.001***
	Intercept	1	4748	<0.001
	CO_2	2	0.01	0.999
	FD	4	39.0	<0.001
	CO_2*FD	6	3.02	0.012

Table 6. *M. menidia.* Effect of CO_2 level (ambient, elevated, high), fertilization date (FD, both years combined) and their interaction on the growth (mm d^{-1}, 1 – 10 dph) of offspring during the first and second half of the spawning season (Figure 14), using a General Linear Model.

Source of Variation	DF	F	*P*
Model	***10***	***4.4***	***<0.001***
Intercept	1	2383.4	<0.001
CO_2	2	1.7	0.190
Month	3	11.5	<0.001
CO_2*Month	5	1.8	0.120

Table 7. *M. menidia.* Offspring survival (mm d^{-1}, 1 – 10 dph) as a linear function of fertilization date (2012, 2013 combined) during the first and second half of the spawning season (Figure 14).

Date	Location	Time	Temp	pH	Salinity	Number of seines	Adults collected	CPUE
4/5/2012	Poquot		10.2		24.7	3	20	7
4/19/2012	Poquot		13.5	7.9	23.5	3	56	19
5/3/2012	Poquot							
5/17/2012	Poquot	10:00	15.7	7.75	22.5	3	78	26
6/2/2012	Poquot		17.9	7.8	22.34	3		0
6/14/2012	Poquot		19.5	7.72	21.5	3	48	16
7/3/2012	Poquot/					4	10	3
3/15/2013	Poquot	13:30	5	8.07	22.25	3	0	0
3/29/2013	Poquot	11:15	5.2	8.02	24.5	3	0	0
4/3/2013	Poquot	17:00	10.1	8.18	27.55	3	0	0
4/7/2013	Poquot	8:30	7.2	8.05	27.1	3	0	0
4/8/2013	Poquot	9:15	7.9	8.01	26.9	3	0	0
4/9/2013	Poquot	10:00	7.6	8.04	27.12	3	0	0
4/10/2013	Poquot	11:00	10.9	7.83	24	3	67	22
4/11/2013	Poquot	10:30	10.3	8.01	24.27	3	0	0
4/12/2013	Poquot	11:45	10.3	8.06	24.33	3	0	0
4/13/2013	Poquot	13:00	10	8.06	22.54	3	0	0
4/14/2013	Poquot	13:30	9.7	7.85	23.5	3	0	0
4/15/2013	Poquot	14:35	10.5	8.03	23.88	3	0	0
4/16/2013	Poquot	15:00	11.7	8.02	23.25	3	0	0
4/17/2013	Poquot	15:50	15.3	8.04	21.6	3	0	0
4/18/2013	Poquot	16:30	15.3	8.02	22.34	3	0	0
4/19/2013	Poquot	17:15	12.5	8.09	24.5	3	50	17
4/20/2013	Poquot	18:00	11.1	7.9	23.5	3	52	17
4/22/2013	Poquot	8:00	10.6	8.09	23.4	1	193	193
4/25/2013	Poquot	10:45	11.7	7.97	24.33	1	167	167
5/6/2013	Poquot	14:30	13.4	8.09	22.43	2	124	62
5/20/2013	Poquot	18:30	16.2	8.06	23.75	2	82	41
5/22/2013	Poquot	9:00	15.9	8.01	23.88	2	69	35
6/5/2013	Poquot	9:00	16.2	7.83	23.9	4	6	2
6/5/2013	Poquot	19:00	18.8	7.85	23.8	6	160	27
6/19/2013	Poquot	8:00	18.1	7.88	23.79	4	50	13
6/20/2013	Poquot	9:00	18.3	7.94	24.2	3	6	2
6/20/2013	Stony Brook	12:30				1	12	12
6/21/2013	Stony Brook	1:45	19.1	7.85	23.21	1	6	6
6/21/2013	Thatch Meadow	3:30				3	30	10

Table 8. *M. menidia.* An overview of wild adults collected by beach seine from Poquot Beach or surrounding estuaries during the 2012 and 2013 spawning seasons. Missing values indicate lost or unrecorded data.