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Using quantitative genetics methods to estimate heritability of larval CO_2-resistance in a coastal marine fish

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Abstract of the Thesis

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Marine organisms are under pressure to adapt to anthropogenic changes that are rapidly altering ocean water chemistry. Since the Industrial Revolution, human activities have caused an increase in atmospheric CO_2 which is partly absorbed by the world's oceans, thereby reducing ocean pH, aragonite and calcite saturation states in a process known as ocean acidification (OA). Concern over these changes has caused a surge in scientific interest to better understand and predict how OA affects marine organisms from single celled algae to marine calcifying invertebrates and vertebrates such as fish. Since most studies thus far have focused on short-term responses of marine organisms exposed to future CO_2 levels, the long-time, i.e., evolutionary potential for adaptation to high CO_2 environments remains poorly understood. This thesis investigated the potential for evolutionary responses in the Atlantic Silverside, *Menidia menidia*, an abundant and ecologically important coastal forage fish with a large spatial distribution from Florida, USA to Nova Scotia, CA. Laboratory experiments have demonstrated that when reared under conditions of elevated CO_2 (~2,300 µatm) larval *M. menidia* show increased, but not complete mortality when compared to control larvae reared at ambient CO_2 (~600 µatm). This suggests that larvae from some parental lines may be resistant to these conditions. If this tolerance to high CO_2 is genetically determined, at least in part, fish may adapt to OA through natural selection in the long-term. Using quantitative genetic methods (the 'animal model'), I simultaneously estimated the heritability and maternal effects of larval resistance to elevated CO_2. Mean days survived post-hatch (MDS) ('the trait value') was quantified on a daily basis for 1,000 fertilized eggs reared from crossing 71 parents (42

males, 29 females) caught from an undisturbed estuary on the north shore of Long Island (Poquott, 40º 57.78'N, 73º 8.22'W) at the beginning of the spawning season. This approach quantitatively assessed the species potential of responding to the selection introduced by a high CO_2 environment. Heritability and maternal effects of post-hatch survival at high CO_2 for 772 larvae were estimated to be 0.11±0.07 and 0.03±0.03, respectively. Therefore the potential for an evolutionary response to ocean acidification in *M.menidia* appears to be low, perhaps because this species already utilizes alternative adaptive mechanisms to cope with rapid environmental change in the short-term. The ability to assess the microsatellite genotypes of all individuals within the ten replicates (n =100) used in this experiment revealed that survival increased with increasing heterozygosity, allelic richness and number of parents, perhaps suggesting that larval survival is dependent on sufficient genetic variability among populations.

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Chapter 1

Introduction

Current levels of atmospheric CO_2 concentrations have exceeded 395 ppm and are now expected to rise to 750-1000 ppm by the end of this century. These changes represent a projected reduction in pH of 0.3-0.4 units by 2100 (Metz et al. 2005, Alley et al. 2007, Change 2007, Rost et al. 2008a) and may have dramatic impacts on many ecologically and commercially important marine organisms (Hendriks et al. 2010, Kroeker et al. 2013). Atmospheric CO_2 concentrations are currently increasing at a rate 100 times faster than during the last 650,000 years, and approximately a third of this CO_2 has already been absorbed by ocean waters (Caldeira & Wickett 2005, Orr et al. 2005, Frommel et al. 2011, Dupont & Pörtner 2013). As atmospheric CO_2 is absorbed and reacts with the surface ocean, carbonic acid is formed ($CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow 2H^+ + CO_3^{-2}$) and goes through a series of dissociations. These dissociations result in a net increase of protons [H+] measured as a reduction in pH, while decreasing the availability of free carbonate ions [CO_3^{2-}] reducing the calcium carbonate ($CaCO_3$) saturation state (Ω). The complex anthropogenic changes in ocean chemistry are often referred to simply as ocean acidification (OA) (Rost & Riebesell 2004, Hofmann et al. 2010).

Present oceanic alterations have had significant effects on important calcifying organisms, including coral reef ecosystems. The Great Barrier Reef has experienced a 20.6% drop in growth rate in the last 16 years and has struggled to maintain its structural integrity by reducing skeletal density of its coralline structures (Hoegh-Guldberg et al. 2007). Experimental research has revealed similar patterns suggesting that OA will pose a significant threat to the survival, growth and overall fitness of many marine calcifying species that may be vulnerable to dissolution, including foraminifers and coccolithophores (Doney et al. 2009), pteropods (Orr et al. 2005), bivalves, echinoderms and crustaceans (Fabry et al. 2008, Kurihara 2008). Interestingly, culture experiments examining the response of calcification of coccolithophores exposed to elevated CO_2 have produced varying results between and within species (Beaufort et al. 2011). The overall response of multiple species has been a decline in coccolith mass with decreasing concentrations of CO_3^{-2}, however some species such as the ubiquitous *Emiliania huxylei* have shown heavy calcification under low pH and low CO_3^{-2} water conditions, emphasizing the uncertainty in making large scale predictions for the future (Rost & Riebesell 2004, Rost et al. 2008b, Beaufort et al. 2011). Calcifying organisms can use multiple mineral forms of $CaCO_3$ to produce and maintain their carbonate structures and as a result, organisms utilizing more soluble forms of $CaCO_3$, such as aragonite and high-Mg calcite may be more vulnerable to OA than organisms using less

soluble mineral forms of $CaCO_3$ such as low-Mg calcite (Ries et al. 2009). Additionally, the vulnerability of organisms to OA may vary depending on life-history stage, where early life history stages may be more susceptible to the adverse effects of OA than later life stages (Kroeker et al. 2013). Sensitivity to OA may also depend on motility, organisms with the ability to move freely and regulate their extracellular pH through active ion-transport such as fish, cephalopods and crustaceans may be more tolerant to OA. Sessile organisms that are unable to internally regulate changes in extracellular pH may be more vulnerable due to depressed metabolisms leading to reductions in growth and overall fitness (Gutowska et al. 2008, Pörtner 2008, Melzner et al. 2009). The impact of OA on calcifying organisms has understandably received most attention within the scientific community because of their hypothesized vulnerability to dissolution.

Existing open ocean predictions may pose a notable threat to marine organisms; however, more importantly, ocean acidification may be more complex in that many ecologically and commercially important marine species inhabit coastal zones that experience vastly different and more variable CO_2/pH regimes than those proposed for the open ocean. Several natural and anthropogenic factors contribute to the carbon systems of coastal regions, making it much more difficult to predict the complex dynamics of these habitats from a climate change perspective (Duarte et al. 2013). Coastal acidification mechanisms include ecosystem metabolism, such that pH may fluctuate rapidly due to release of CO_2 via biological respiration and uptake of CO_2 through photosynthesis (Wootton et al. 2008). Inputs of acidic river plumes through variable temporal scales can also impact pH of coastal ecosystems (Salisbury et al. 2008). In addition, anthropogenic inputs of excess nutrients leading to eutrophication can have combined effects with ocean acidification, resulting in greater than expected drops in pH than predicted values may suggest (Cai et al. 2011, Melzner et al. 2012). These confounding contributions to coastal acidification emphasize the need for experimental evidence investigating the response of marine organisms to high CO_2 between and within species, as variable responses are becoming more evident. Recent OA research has begun to broaden its scope to include vertebrate organisms such as fish, which has resulted in increased complexity in the response to high CO_2.

Adult and juvenile fish have been documented to tolerate very high levels of CO_2 due to their well-developed acid-base regulatory mechanisms such as carbonate buffering through the gills (Ishimatsu et al. 2008, Munday et al. 2009b). Previous experimental research on hypercapnia in adult or juvenile fish was mostly aquaculture-motivated and used higher CO_2 levels (>10,000 µatm) than what is relevant for ocean acidification research (EPOCA; www.epoca-project.eu/index.php/Home/Guide-to-OA-Research; (Riebesell et al. 2010). The high tolerance of juvenile and adult fish led some researchers to believe fish may not be susceptible to OA. Ishimatsu et al. (2008) published a review of existing studies

(116 papers) that concentrated their efforts on exploring the effects of elevated CO_2 on fish. They concluded that the CO_2 sensitivity of fish eggs and larval stages comprises a knowledge gap that needs to be addressed before inferring fish vulnerability to OA.

The relatively recent focus on fish early life stages revealed that ocean acidification may impact larval fish in a number of ways, ranging from indirect effects such as impairing the ability of larvae to detect and respond to sensory cues (Munday, McCormick et al. 2012) to direct reductions in survival and growth (Baumann et al. 2011). Munday et al. (2009a) demonstrated that tropical reef fish larvae lose the ability to distinguish odors from predator and non-predator individuals, and were more attracted to the smell of predators when exposed to elevated levels of CO_2 (1,000 µatm). In addition to the impairment of olfactory cues, studies have suggested that juvenile fish exposed to elevated levels of CO_2 showed reduced visual threat perception, learning ability, behavioral lateralization and auditory preferences (Ferrari et al. 2011, Munday et al. 2012). Significant reductions in survival (74%) and growth (18%) were observed in larvae of the Inland Silverside, *Menidia beryllina* when exposed to projected end-of-century levels of CO_2 (~1,000 ppm) (Baumann et al. 2011). Larval cod, *Gadus morhua*, showed increased tissue damage in the gut, liver, pancreas and kidneys of larvae with increasing CO_2 concentrations (Frommel et al. 2011). The inability to detect predators and settle on habitats, the risk of reduced survival, length and damaged internal organs are all important factors that may act as a bottleneck to recruitment and therefore impact fish productivity.

Not all evidence shows such vulnerability of larval fish to high CO_2; in fact, some species have shown little to no effect when exposed to high levels of CO_2. Walleye Pollock, *Theragra chalcogramma*, a commercially important temperate fish, showed no significant effects on growth or condition during high CO_2 early life exposure experiments (Hurst et al. 2012). The otoliths (calcareous inner ear bones) of White Sea Bass, *Atractoscion nobilis*, reared in high CO_2 were significantly larger than those reared in low CO_2 (Checkley et al. 2009). Several tropical marine fish seem to tolerate elevated CO_2 conditions during early life development; the spiny damselfish, *Acanthochromis polyacanthus*, exhibited no change in early life growth or survival at high CO_2 (Munday et al. 2011), and cobia, *Rachycentron canadum* showed resistance to high CO_2 treatment (~2100ppm) effects on growth, development, swimming ability and swimming activity (Bignami et al. 2013). Clearly, the early life stages of some fish species are sensitive to elevated CO_2 levels while other species seem to tolerate high levels of CO_2.

Many studies have assessed the response of contemporary organisms to CO_2 conditions predicted hundreds of years in the future but to what extent any of these species will be able to adapt to these conditions in the short or long-term remains largely unknown. Survival under elevated levels of CO_2 has

been observed in several species, but whether or not this trait can evolve through natural selection in marine fish is still unclear. The lack of research quantifying the adaptive potential of marine fish faced with climate change represents a major missing link in the true evaluation of the long term impacts OA can have on marine fish.

Current evidence for adaptations to elevated CO_2 for marine organisms is scarce; however, several studies are beginning to emerge. Adaptive responses such as changes in photosynthesis and cell size under elevated CO_2 conditions have been observed in an alga species, *Chlamydomonas reinhardtii* (Collins & Bell 2005), and individually based genetic variation in fitness responses to high CO_2 was experimentally detected in a coccolithophore, *Emiliania huxleyi,* (Langer et al. 2009) and a bryozoan species, *Celleporella hyaline*, (Pistevos et al. 2011) suggesting the possibility to adapt to changing seawater conditions through non-mutational natural selection. A study investigating the potential for a coccolithophore species, *Gephyrocapsa oceanica*, to evolve in response to high CO_2 (~1,000 µatm) found that after 670 generations, selected cells showed increased photosynthetic carbon fixation, growth rate and production of cellular particulate organic carbon and particulate organic nitrogen (Jin et al. 2013). Sunday et al. (2011) measured the possibility for evolutionary adaptation to OA in larval development rate in a sea urchin species, *Strongylocentrotus franciscanus*, and a mussel species, *Mytilus trossulus*, and concluded that due to greater levels of phenotypic and genetic variation for larval size under future OA conditions, sea urchins may show relatively fast rates of adaptation. Similarly, a recent study observed sufficient genetic variation for body size under high CO_2 (~1,200 ppm) in the ecologically important purple sea urchin, *Strongylocentrotus purpuratus*, indicating the ability to evolve (Kelly et al. 2013). Albeit critical for understanding the potential for evolutionary adaptation in marine organisms, no studies to date have addressed these issues for marine fish.

Experimental evolution and artificial selection experiments comprise potential approaches to determine and observe the realized evolutionary responses of organisms facing selection pressures linked to OA or other climate stressors (Hoffmann & Sgrò 2011, Munday et al. 2013). Experimental evolution can be accomplished in a variety of ways depending on the organism of interest, the trait(s) of interest and by varying manipulations of the experimental design and treatments. Generally, experimental approaches involve altering the laboratory conditions (e.g. CO_2, temperature, etc.) of a contained population to introduce artificial selection while maintaining a control population (Garland & Rose 2009). Through time, the altered population may produce new genetic modifications and reproduction of future generations may increase the frequency of favored genotypes. After a certain amount of generations, the control population can then be compared (e.g. genetically, morphologically, or physiologically) to the altered population depending on the hypothesis of the experimenter. An advantage to this type of

approach is the ability to replicate the altered experimental conditions in order to consistently repeat any observed evolutionary change that may be occurring (Futuyama et al. 2009). These experiments have the potential to provide direct observations of evolution and adaptation; however, there are some limitations to such approaches. The generation time of the study organism is crucial as these experiments require observations of evolutionary change across multiple generations. Many studies thus far investigating the adaptive processes of organisms faced with OA using experimental evolution or artificial selection have been limited to short-lived, large-populations of fast growing marine organisms (e.g. phytoplankton) that can be easily maintained and tracked in an experimental setting.

Estimating heritability of fitness related traits is another way to evaluate a species' potential to adapt to selective forces introduced by changing environmental conditions. Experimentally, this requires a pedigree that includes all individuals in the experiment and their respective trait values. This can be achieved by crossing individual parental parents and keeping their larvae in separate experimental vessels to keep track of their parentage and trait values (i.e., without doing genetic analysis). In order to avoid this type of unwieldy design and rear all parental crosses within each replicate, quantitative genetics methods can be used to calculate the possibility of larval survival at elevated levels of CO_2 as a heritable trait that may evolve in fish. Quantitative genetics and comparisons of genetic markers provides a means to measure and resolve the underlying genetic variation within a population using a single generation (Wilson et al. 2009b). Survival under conditions of elevated CO_2 is a quantitative trait that can be measured on a scale and is likely influenced by multiple genes (Klerks et al. 2011). If elevated CO_2 decreases survival of some larval fish and survival is partially genetically determined, selection will favor less susceptible genotypes (Klerks et al. 2011). The principles of quantitative genetics lie in the understanding of relationships among individuals within a certain population and quantifiable data on phenotypic traits. With this information conclusions can be made about the selection, inheritance and evolution of particular traits within only one generation without clearly knowing what genes are involved (Wilson et al. 2010, Munday et al. 2013). The goal of quantitative genetic approaches is to estimate the proportion of phenotypic variance (V_P) that is made up of genetic variance (V_G) among individuals (i.e. heritability), and thus use that estimate of heritability to predict a populations response to selection (Kruuk 2004).

Implementing quantitative genetic approaches in the laboratory has some methodological constraints. First, the species of interest must have the ability to be bred in the laboratory under controlled conditions. In order to understand the relationships among individuals within a population, a pedigree must be constructed using a sufficient amount of parental crosses and molecular markers to accurately assign parentage to each individual (Conner & Hartl 2004). Each individual must be tracked

throughout the experiment and assigned a trait value in order to distinguish phenotypic variation among individuals. Once the trait values are assigned to individuals, genetic information is obtained and the pedigree is constructed, the phenotypic variation can be statistically partitioned into several quantitative genetic variance components that are used to calculate heritability (h^2) and maternal effects (m^2).

Estimating variance components can be done using a form of mixed model known as the 'animal model' (Lynch & Walsh 1998) which can estimate an individual's breeding value, or the additive genetic effect of an individual's genotype on a trait relative to the populations mean phenotype (Kruuk 2004, Wilson et al. 2010). The development of mixed models used in quantitative genetics has improved the efficiency of computing complex data sets and has provided more powerful statistical analyses. In the past, methods to estimate heritability were restricted to correlations of one level of relatedness, such as parent-offspring regressions (Boag & Grant 1978). The 'animal model,' on the other hand, uses restricted maximum likelihood (REML) to evaluate quantitative genetic parameters by simultaneously considering the resemblance among all individuals in any given pedigree, regardless of their level of relatedness (Åkesson et al. 2008, Wilson et al. 2009a). Comparative values of heritability calculated using both methods have resulted in lower heritability estimates accompanied by smaller standard errors when using the 'animal model' technique (Åkesson et al. 2008). The 'animal model' has been successfully implemented in the plant and animal breeding industries for decades and only recently have biologists began to use it to address evolutionary questions (Kruuk 2004).

Heritability and maternal effects are critical parameters used to describe and predict evolutionary responses to selection. A trait must be heritable in order to evolve; therefore quantifying the heritability of traits directly related to fitness or survival is crucial for making predictions of whether or not, and if so how quickly natural selection will generate lasting evolutionary change (Kruuk 2004). Within a certain population, there can be considerable diversity of individual phenotypes; the most likely reasons for explaining this diversity is either through differences in genotypes or differences influenced by environment (Barton & Keightley 2002). Heritability is an estimate of the importance of the genetic influences that may explain these phenotypic differences between individuals (Hoffmann & Merilä 1999, Visscher et al. 2008). Heritability is measured on a scale from 0 to 1, (no genetic influence to total genetic determination of a trait). High heritability of a particular trait is likely a good indication that an individual's phenotype can be used to describe their genotype. For example, in artificial selection breeding designs, if traits tend to have high heritability, they can be selected for to choose the best individuals to further breed based on their phenotypes (Visscher et al. 2008). In natural populations, this can be more complex as environmental conditions can alter individual phenotypes; therefore, individual

phenotypic diversity in a given population may be the product of genetics, the environment or a combination of the two.

The existence of maternal effects may be an additional explanation for the phenotypic differences observed among individuals within a given population. The phenotype of a mother and the environment she experiences may directly influence the phenotype of her offspring, in ways not necessarily dependent on the influence of the genes she passes on (Kruuk 2004). A mother's environmental experience can alter her physiological state and may impact her growth, weight or overall condition; these changes can be transmitted to her offspring in a variety of ways (Mousseau & Fox 1998). Mechanisms for maternal influence of offspring development may include internal cytoplasmic changes such as manipulation of yolk quantity, hormone concentrations, mRNA in the egg (Mousseau & Fox 1998, Green 2008) or maternal decisions such as where to deposit her eggs which may lead to differences in temperature, moisture, food quality and levels of predation encountered by her progeny (Bernardo 1996) . Measuring maternal effects has become increasingly important to evolutionary biologists because they can impact the rate of evolution and have been shown to cause traits to evolve in opposite directions than what is favored by selection (Bernardo 1996, Kruuk 2004, Green 2008).

This study will provide the first estimates of both maternal effects and heritability of larval survival under elevated CO_2 conditions for a marine fish. If maternal effects exist, that would suggest that certain females are able to provision their offspring so that they are better able to survive under OA conditions than offspring from other females. My estimate of heritability of larval survival under OA conditions will allow predictions of how quickly marine fish may be able to evolve in response to the unfolding acidification in order to best manage vulnerable populations and species. This study provides a novel design to quantify the adaptive potential of fish through the use of quantitative genetics methods and a model forage fish species.

Species Justification:

The study species for my research is an ecologically important forage fish, the Atlantic Silverside, *Menidia menidia.* These fish are abundant along the Northeast American coast inhabiting near-shore waters from Florida to Nova Scotia and they have a long-standing history of being a model species for laboratory experiments throughout the last 35 years (Conover & Munch 2002, Conover et al. 2005). *M. menidia* are easily sampled and bred in captivity and have a short generation time (~one year), which makes them an ideal research organism in common garden experiments. *M. menidia* spawn in the spring (roughly April to July) as they enter coastal waters where females adhere masses of eggs onto patches of algae and saltmarsh grass, *Spartina alternaflora*, in synchrony with full and new moons (Conover et al.

2005). Larval and juvenile stages are known to disperse throughout inshore waters, reaching sexual maturity during winter months when populations mix among deeper shelf waters promoting sufficient gene flow among populations (Conover 1998). *M. menidia* has long played an important role as a model species to advance the understanding of fish biology, ecology and evolution through experimental and field based observations. Ocean acidification is a relatively new field of research with most studies being published in the last decade. *M. menidia* provide a means to address many of the uncertainties regarding the short and long-term effects that OA may have on marine fish through the use of experimental methods and quantitative genetics approaches addressed in this thesis.

Research Objectives:

The main research objective for this thesis was to simultaneously estimate the heritability and maternal effects of larval CO_2-resistance in the Atlantic Silverside, *Menidia menidia*, using a quantitative genetics approach. Accomplishing this objective allowed me to assess whether genetic diversity within each experimental replicate influenced larval survival at high CO_2.

Materials and Methods:

For this experiment, two levels of CO_2 were administered: ambient (**~600 µatm)**, and high (**~2,300 µatm),** these treatment levels represent current day CO_2 levels at our study site and open ocean CO_2 levels projected for the year 2300 according to IPCC 2013 predictions.

CO_2 treatments and measurements:

CO_2 gas was delivered to seawater replicates via a gas proportionator system (Cole Parmer® Flowmeter system, multitube frame). 5% CO_2 gas and pressurized air were precisely mixed by the gas proportionator to produce desired CO_2 and pH_{NBS} treatment levels. The gas mix was delivered directly to the bottom of each replicated container (20L) via Sweetwater® air diffusers ensuring oxygen saturation (~8 mg L^{-1}). Each replicate contained aged and aerated water from a saline well (25 psu) located at Flax Pond Marine Laboratory, FPML (40º57.778'N, 73º8.216'W). OA experiments using gases mixed through these proportionator systems have produced virtually identical sea water chemistry conditions in the laboratory when confirmed by analyzing daily water samples using an EGM-4 Environmental Gas Analyzer® (PP Systems). Precise CO_2 levels (methodological precision of ±4%) and total dissolved inorganic carbon measurements (DIC, 102 ± 3% recovery) were obtained after separating the gas phase from seawater using a Liqui-Cel® Membrane (Membrana; (Talmage & Gobler 2010). Using the program CO2SYS (http://cdiac.ornl.gov/ftp/co2sys/), levels of CO_2 were calculated based on measured values of pH_{NBS}, DIC, temperature, salinity and first and second dissociation constants of carbonic acid in seawater according to Roy *et al* (1993). Daily pH measurements were taken using a Durafet® III pH Electrode that uses a non-glass, ISFET (Ion Sensitive Field Effect Transistor), Orion ROSS Ultra pH/ATC Triode and Orion Star A121 pH Portable Meter, that were regularly calibrated using three-point NIST traceable pH_{NBS} references.

Sampling and experimental design:

Routine sampling for wild, adult *M. menidia* started in early March 2012 by weekly deployment of a 30 x 2m beach seine during incoming tide at Poquot beach (40º55.48'N, 73º06.07'W), which is connected via Port Jefferson Harbor (40º58.12'N, 73º5.28'W) to the Long Island Sound. Live, adult fish

were transported to FPML, where females and males were held for 24h in separate temperature-controlled baths (200L, 24ºC) to acclimate and hydrate mature eggs. Fish were not fed during this 24h period.

After 24h, adult fish were strip-spawned into plastic dishes containing clean seawater with cut-out sections of window screen (1mm mesh size). This technique involves gently squeezing sperm from males into clean seawater dishes and allowing the water to thoroughly mix and activate the sperm. Eggs from adult females were then gently squeezed out onto the screens containing the mix of sperm and seawater. Within 15 minutes of strip-spawning, fertilized eggs attached to the window screens, while unfertilized eggs could be gently rinsed off the screens. Subsequently, 100 eggs for each replicate and treatment were randomly selected under low magnification (2x).

Screens containing the fertilized eggs (n=100) were hung in replicate containers filled with seawater within two hours of fertilization. Food in the form of newly hatched brine shrimp nauplii, *Artemia salina*, (San Francisco strain, Brine Shrimp Direct, Inc) was provided *ad libitum* to all replicates immediately before hatching (~5-6 days post fertilization) and each day post-hatch (dph). Additional food in the form of commercial larval powder (Otohime Marine Weaning Diet, Size A, Reed Mariculture®) was provided 1 to 2 days post hatch to reduce mortality normally occurring around the time of first feeding (Baumann et al. 2011). Larval survival was quantified within each replicate at 1 dph and 10dph by gently scooping small groups of live larvae into new rearing containers.

We further devised a method to quantitatively collect individual dead larvae on a twice-daily basis through a siphon from the bottom of all high CO_2 replicates starting on the day of hatch and continuing through 15dph. Survivorship was quantified as the number of days individual larvae survived after hatching ('trait value') when exposed to high CO_2 (~2,300 µatm). 10 replicates of 100 embryos ensured enough statistical power to obtain robust estimates of heritability and maternal effects using the 'animal model' (Lynch & Walsh 1998). The comparable control group from the same set of parents were raised at ambient CO_2 (~600 µatm), their survivorship was measured by counting total live larvae at 1dph and 10dph in order to verify that mortality in the experimental group was primarily due to the experimentally elevated CO_2. Each individual larva was transferred to a tissue lysis solution (100 µl tissue lysis buffer + 12µl proteinase K) for subsequent genomic DNA extraction, microsatellite amplification and sequencing. This experiment was carried out until zero mortality was recorded for three consecutive days or until 15dph, after which each surviving larvae was also placed into tissue lysis solution for genomic DNA extraction, microsatellite amplification and sequencing. A comparable control group from the same set of parents was raised at ambient CO_2, their survivorship was quantified at 1dph and 15dph in order to verify that mortality in the experimental group was primarily due to elevated CO_2.

A large temperature controlled bath (700L, 24ºC) was used to hold 10 rearing replicates, each containing 100 fertilized eggs from adult *M. menidia* caught at Poquott Estuary and held at high CO_2

(~2,300 µatm). A separate control bath (700L, 24°C) was used to hold an additional 5 rearing replicates each containing 100 fertilized eggs from the same adults and held at ambient CO_2 (~600ppm). 24°C represents the thermal growth optimum with unlimited food for *M. menidia* and is consistent with previous rearing experiments (Middaugh et al. 1987). A total of 71 parents (42 males, 29 females) were collected by beach seine from the study site and strip-spawned in the laboratory at the beginning of the 2013 spawning season (4/26/2013). Adults were strip-spawned into plastic dishes containing clean seawater with cut-out sections of 1mm window screen and measured for total length (TL) to the nearest 0.5cm. The first adult *M. menidia* spawners of the 2013 season were fertilized 20 days after those from the 2012 spawning season.

DNA extraction and genotyping:

Genomic DNA was extracted using tissue from tail clips (0.015–0.035g) of adult fish by means of an established DNA extraction protocol (Qiagen Blood and Tissue extraction kit, Valencia, CA, USA). Genomic DNA was extracted from each individual larva (1-15dph) using a 'salting out' protocol (Sunnucks et al. 1996). Nine polymorphic microsatellite loci for *M. menidia* (Sbrocco & Barber 2011) were amplified in a 10µl reaction for all parents and all offspring and sequenced at the Pritzker lab for Molecular Systematics and Evolution (Field Museum, Chicago, IL). This technique involves electrophoresing fluorescently labeled polymerase chain reaction (PCR) products on an ABI 3730 DNA analyzer along with an internal fluorescent ladder (LIZ-500, Applied Biosystems). The PCR master mix consisted of genomic DNA, 1xPCR buffer, 10x bovine serum albumin, 1.5–3.5mM $MgCl_2$, 0.12mM dNTPs, 0.16µM of the reverse primer and fluorescently labeled m13 primer, 0.04µM of the species specific forward primer and 1 unit Taq polymerase. Thermal cycling protocols consisted of 5 minutes at 94°C followed by 35 cycles of 94°C for 30 seconds, primer specific annealing temperature (Ta) for 30 seconds and 72°C for 60 seconds with a final extension at 72°C for 10 minutes. Alleles were scored by a single analyst (AJM) using the software, Peakscanner 1.0 (Applied Biosystems®) and a subset of approximately 10% of genotypes was verified by a second analyst (Kevin A. Feldheim, Lab Manager, Field Museum, Chicago, IL).

Parentage assignment/Pedigree construction:

Assignment of parentage was completed for each individual offspring after an allele frequency analysis was carried out using the maximum likelihood software, CERVUS 3.0 (www.fieldgenetics.com). The allele frequency component produced an allele frequency file needed for other analyses and calculated several summary statistics in order to gauge the fitness of loci for parentage analysis. The allele frequency analysis reads a text-based file of genotypes at all loci, and counts the number of

occurrences of each allele at each locus. Using these data calculates allows calculating loci-specific statistics such as expected heterozygosity (H_{exp}), observed heterozygosity (H_{obs}), polymorphic information content (PIC), average exclusion probabilities and Hardy-Weinberg chi-square statistics. Heterozygosity is the measure of genetic variability at each locus, if an individual has two different alleles at a certain locus, that individual is a heterozygote (Allendorf 1986). PIC is a measure of a molecular marker's usefulness in linkage analysis, or the probability of identifying which allele of a given parent was transmitted to a given offspring (Guo & Elston 1999). Hardy-Weinberg statistics allow comparisons of a population's genetic structure over time with the genetic structure expected if the population was not evolving (Guo & Thompson 1992, Mayo 2008).

CERVUS 3.0 was then used to assign individual offspring to candidate mothers and fathers using their microsatellite genotypes. For each offspring tested, a candidate parent or pair of parents was assigned based on a 95% level of confidence using the principles of exclusion probability and maximum likelihood. Candidate parents were excluded if mismatches occurred at multiple loci as CERVUS compares candidate parent's genotypes against offspring genotypes and all other parent's genotypes. There was one locus (Mm119) that was excluded from the analysis due to poor amplification. Likelihood works by statistically distinguishing parents that have not been excluded by using two pieces of information about candidate parents that exclusion does not take into account:

1) The frequency of the offspring allele or alleles that could have come from candidate parent.
2) Whether or not the candidate parent is heterozygous or homozygous

The combination of these two principles allows CERVUS to confidently assign the most probably parental pairs to each offspring.

Pedigree-Viewer:

The program PEDIGREE-VIEWER (http://wwwpersonal.une.edu.au/~bkinghor/pedigree.htm), version 6.5b was used to display a full pedigree structure of all adult (males=42, females=29) and larval fish (dead=336, survivors=436) used in this experiment using the output file of parentage assignment**.** The program allows displaying large pedigrees on one screen with the ability to select individuals based on their identity or assigned trait value while displaying assigned parents, related individuals (half-sibling, full-siblings) or individuals with similar trait values. In addition to visually displaying the connections within the population of fish used in this study, the pedigree output (Figure 2) was used for the analysis of individual fertilization patterns, number of offspring per parent, number of survivors per parent pair and number of survivors per parent pair, along with several other observations that allow for detailed analysis of the 772 fish used in this study.

ASReml: Univariate animal model:

In the final step, the program ASReml v3.0.5 (©VSN International Ltd) was used to run an 'animal model,' for a single trait (days survived post-hatch at high CO_2). The model is a form of mixed model that uses complex pedigree data to partition observed phenotypic variance into various quantitative genetic components used to estimate genetic variance and measure heritability. The explanatory terms of the model are a mixture of both 'fixed' and 'random' effects. 'Fixed' effects are constants that can be added to the model and may affect the phenotypic trait distribution (such as age, sex, etc). In this model, the fixed effect was Mean Days Survived (MDS). Random effects influence the variance of a trait, which were parameters to be estimated in order to determine heritability of our trait (Kruuk 2004, Wilson et al. 2010). The random effect used in this model is the individual's 'breeding value' or 'genetic merit' (the effect of that individual's genotype on the observed phenotypic trait). Since an individual's 'breeding value' is not known, the model uses this value as an explanatory 'random' effect.

For a single trait, or phenotype (y) of an individual (i), the simplest model can be written as:

$$y_i = \mu + \alpha_i + e_i \qquad \text{(model1)}$$

Where μ is the population mean (MDS), α_i is the additive genetic merit ('breeding value') and e_i is a random residual term. By fitting a single fixed effect and a single random effect in ASReml using model 1, the total phenotypic variance (V_P) was partitioned into two variance components, the variance of 'breeding values' (i.e., the additive genetic component V_A) and the residual component V_R. Heritability (h^2) of MDS was then calculated as:

$$h^2 = V_A/V_P = V_A/(V_A + V_R)$$

Maternal effects were calculated by adding 'maternal identity' as a random effect to model 1, such that:

$$y_i = \mu + \alpha_i + e_i + m_i \qquad \text{(model 2)}$$

Where m_i is maternal identity and its addition will lower the estimates of V_A and h^2 estimated in model 1, while obtaining an estimate of the variance caused by maternal effects (V_M).

Genetic diversity of experimental replicates:

Replicate-specific genetic diversity was assessed using five parameters (relative allelic richness (RAR), observed heterozygosity (H_{OBS}), number of dams, number of sires and total number of parents). An allele frequency analysis using the program CERVUS 3.0 was performed on all adult and larval fish for each microsatellite loci used in this study. The amount of alleles present in each replicate was expressed as a proportion of total alleles (RAR) present across all loci for all fish in this study. All five parameters were calculated for each replicate and plotted against survival (% fertilization to 15dph) in order to test the effect of genetic variability on survival.

Feasibility of using quantitative genetics methods to estimate evolutionary potential:

A preliminary experiment was conducted in 2012 using 6 replicates (n = 6*100) at the high CO_2 treatment (~2,300 µatm) and 3 replicates (n = 3*100) at the ambient CO_2 treatment (~600 µatm) in order to test the feasibility of four methodological prerequisites:

1) Test whether early-life survival was negatively impacted by high compared to ambient CO_2 conditions:

There was a significant, 53% reduction in survival (ANOVA, $F_{1,4} = 36.3$, $p = 0.004$) at high (~2,300 µatm) compared to ambient CO_2 (~600 µatm) treatment from fertilization to 10dph. Mean (±SE) survival at 1dph was 92%(±4%) and 79%(±10%) in the ambient and high CO_2 treatment, respectively. At 10dph, mean survival decreased to 83%(±5%) and 39%(±6%) in the ambient and high CO_2 treatment:

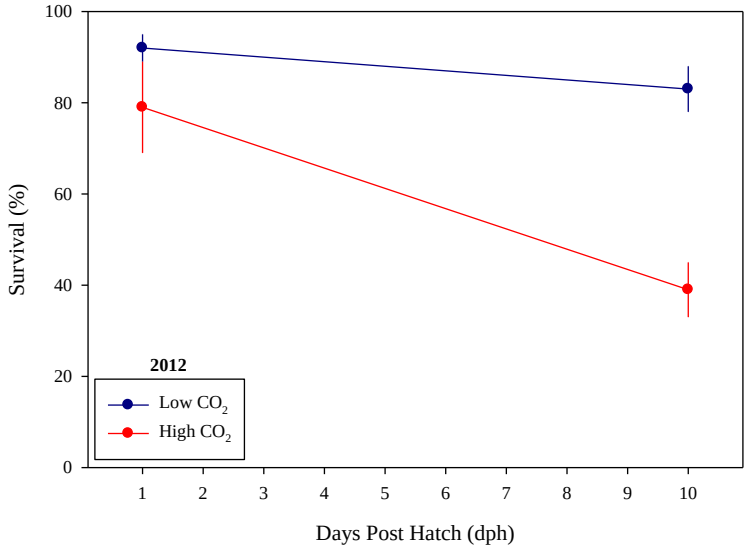

Fig 1: Survival (%) of replicates means (±SE) of ambient treatment (blue) and high CO_2 treatment (red) is plotted at 1 dph and 10dph for 2012 experiment 1 (fertilized April 6th, 2012).

2) Quantitatively estimate the 'MDS', i.e., sample every individual mortality on the day of death in sufficient condition for genetic analysis:

Siphoning six replicates twice daily for 15dph resulted in a minimum recovery of (87%) of all larvae in replicate 1 and a maximum recovery of (100%) of larvae in replicate 5. Total mean recovery of larvae was (95%) for all six replicates, verifying the success of this method:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			
Rep	Daily mortalities (recovered)															S	U	R(%)
1	33	3	-	2	-	-	-	-	1	-	-	-	-	-	-	60	2	87
2	11	1	2	3	-	4	-	-	-	-	-	-	-	-	-	77	4	98
3	20	5	2	1	1	-	-	-	-	-	-	-	-	-	-	64	5	92
4	8	1	7	5	2	-	-	-	-	-	-	-	-	-	-	60	2	92
5	5	2	3	7	-	-	-	-	-	-	-	-	-	-	-	75	4	100
6	10	-	8	1	1	2	1	-	-	-	-	-	-	-	-	58	2	99
Totals	87	12	22	19	4	6	1	-	1	-	-	-	-	-	-	394	19	95%

Table 1: Daily mortalities of experiment 2 (fertilized April 26th, 2012), **Rep** = replicate, **S** = number of survivors, **U** = unhatched eggs and **R(%)** = recovered larvae

3) Test whether useful genetic data can be obtained from larvae as young as 1 dph:

Genomic DNA was successfully extracted from individually recovered larva (1 – 15dph) using a 'salting out' protocol (Sunnucks et al. 1996) and was used for amplification at microsatellite 8 loci:

Locus	Ta(°C)	MgCl(mM)	K	N	H_{Obs}	H_{Exp}	PIC
Mm272	44	3.5	19	802	0.643	0.860	0.845
Mm248	51	1.5	10	718	0.758	0.787	0.752
Mm202	45	3.5	12	759	0.768	0.783	0.753
Mm108	47	1.5	8	800	0668	0.755	0.716
Mm251	47	3.0	21	757	0.682	0.858	0.844
Mm204	42	3.5	16	807	0.664	0.850	0.833
Mm240	42	2.0	12	789	0.791	0.811	0.787
Mm02	56	2.5	18	636	0.700	0.898	0.889

Table 2: Allele frequency for all individuals (parents and offspring) across all 8 loci, summary statistics: **K**: number of alleles, **Ta**: annealing temperature, **MgCl:** salt concentration used, **N**: number of individuals typed, **H_{Obs}**: observed heterozygosity, **H_{Exp}:** expected heterozygosity, **PIC:** polymorphic information content

4) Test the suitability of microsatellite loci to resolve relationships between individuals:

Nine microsatellite loci were amplified for all adult (42 male, 29 female) and larval fish (dead = 336, survivors = 436) and used to assign parentage to 95% of offspring. One locus (Mm119) had to be excluded from the analysis due to poor amplification; however, this did not impact the success of assigning parentage:

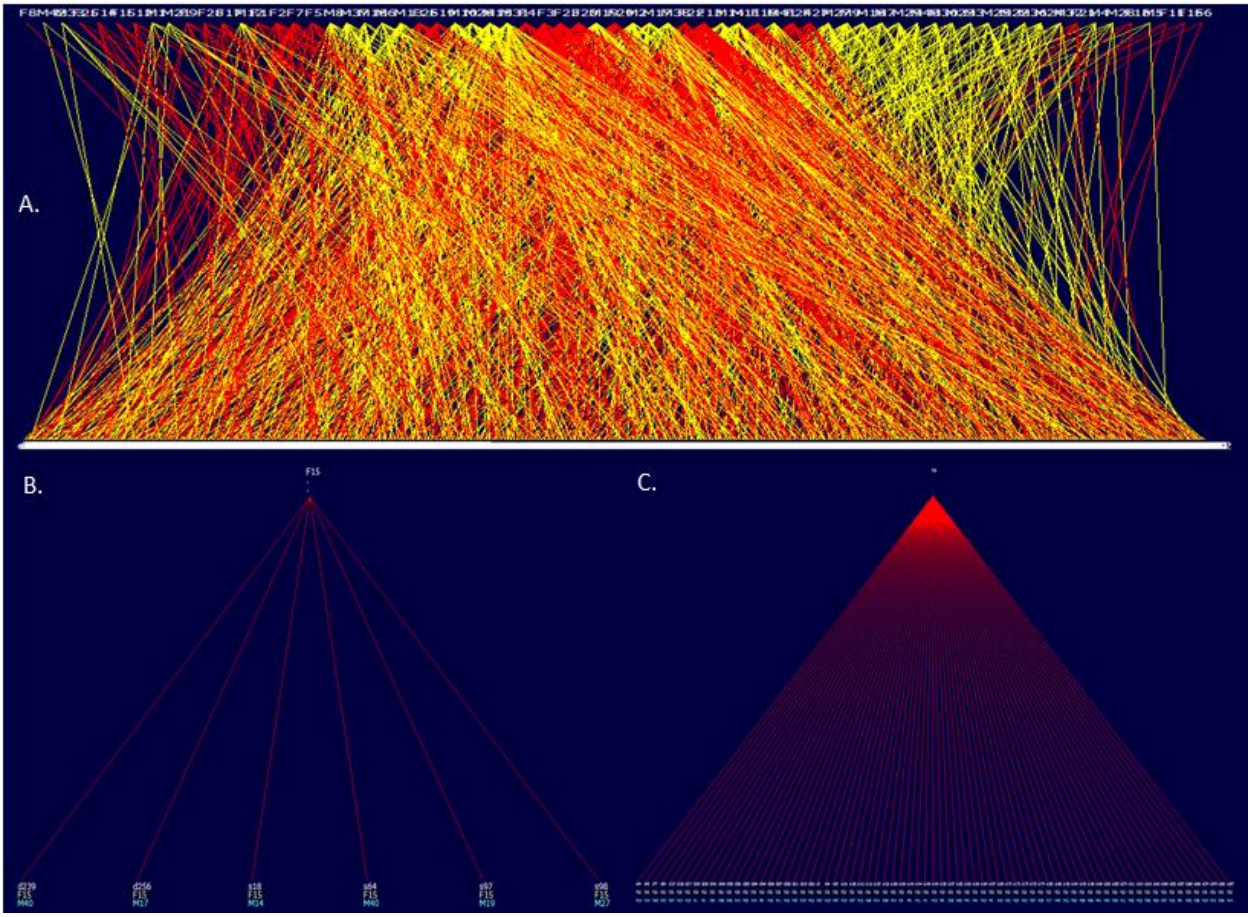

Figure 2: A) Full pedigree display of 772 larval fish from 71 parents (35 males, 29 females) is shown, females are represented by red lines and males represented by yellow lines, **B)** Example of one female (F15) that produced six offspring fertilized by five males, **C)** Example of one female (F22) that produced 70 offspring and was fertilized by 29 different males

Once it was established that we were successful in measuring all four of the above methodological components, we developed methods to conduct a full scale heredity experiment at the beginning of the 2013 *M. menidia* spawning season using 1000 fertilized eggs from 71 parents (42 males, 29 females).

Results:

Mean (±SE) survival at 1dph was 92%(±3%) and 78%(±3%) in the ambient and high CO_2 treatment, respectively. At 10dph, mean survival decreased to 69%(±7%) and 46%(±4%) in the ambient and high CO_2 treatment (Figure 3&4). At 10dph, there was a significant 33% reduction in survival (ANOVA, $F_{2,18} = 7.67$, $p = .014$) at high compared to ambient CO2 treatment.

A total of 772 (survivors=436, dead=336) offspring were tested for parentage assignment, of which 704 were successfully assigned to a parental pair, (91%). A total of 71 parents were used in the analysis, all females (29) and 35 of 42 males were assigned to offspring. The minimum number of sires per female was one, resulting in one offspring produced from that pair. The maximum number of sires to fertilize a female's eggs was 31, producing 115 offspring, 83 of which survived and 32 died. There was an exponential increase in the number of offspring produced per female as the number of sires per female increased (a=4.07, b=0.103, R^2=0.93) (Figure 5). When analyzed separately for dead and surviving larvae, we found that the exponential increase was lower for dead (a = 2.72, b = 0.08, R^2 = 0.85) than for the surviving larvae (a = 1.56, b = 0.12, R^2= 0.89) (Figure 6).

Among all high CO_2 replicates, the maximum survival was 76% and the minimum survival was 28%. By 1dph, an average of 6.7±1.8 larvae had died per replicate, by 2dph average mortality was 3.8±0.8 larvae. By 3dph, the mean number of dead larvae increased to 5±1.6 and then peaked at 4dph with 6.8±1.2 larvae per replicate with a steady decline eventually reaching 0 by 14 and 15dph (figure 7).

Relative allelic richness (RAR) was used to assess genetic diversity within each replicate. All replicates contained at least 68% of all alleles present in the entire population (Figure 8). The maximum relative allelic richness observed was 78% in replicate 10, with a survival of 52% through 15dph. The minimal proportion of total alleles was in replicates 1 and 2 where survival was 29% and 53%, respectively. Highest survival was found in replicate 9 at 76% and the relative allelic richness was 77%, the lowest survival was reported in replicate 4 at 28% with a relative allelic richness of 74%.

Each high CO_2 replicate was tested for RAR, H_{Obs}, number of dams, number of sires and total number of parents to quantify the strength of their influence on survival through 15dph using linear regression. All parameters showed a weak yet positive correlation with survival (Figure 9). Observed

heterozygosity showed the only significant (R^2 = 0.49, p = 0.03) positive increasing relationship with increasing survival (Figure 9b). The replicate that recorded the highest survival (76%) at high CO_2, also showed the highest observed heterozygosity (0.72), the highest number of dams (n=23) and the highest number of total parents (n=54)out of all 10 replicates (Figure 9b, 9c, 9e).

Heritability (h^2) and maternal effect (m^2) for larval survival at high CO_2 was calculated using quantitative genetic variance components (±SE): additive genetic variance (V_A) = 3.56±2.3, maternal variance (V_M) = 1.03±1.12, residual variance (V_R) = 27.5±2.15 and total phenotypic variance (V_P) = 32.1±1.85. The absolute heritability value was low (0.11±0.07), however; it was significantly different from zero (p<0.001), the maternal effects were also low (0.03±0.03), maternal identity explained 0-3% of total phenotypic variance in offspring survival at high CO_2 (Table 5).

Discussion:

My results suggest that mortality in larval *M. menidia* at the beginning of the spawning season is significantly increased when reared at high CO_2 conditions (~2,300 µatm) compared to ambient CO_2 conditions (~600 µatm). These findings were consistent for two years of experimentation; larval survival in April was reduced by 54% and 33% at high relative to ambient CO_2 in 2012 and 2013, respectively. These results support the notion that the early life stages of fish are sensitive to anthropogenic CO_2 increases (Munday et al. 2010, Baumann et al. 2011, Chambers et al. 2013). Importantly, even though survival was significantly lower at high CO_2 levels, a considerable fraction of larval *M.menidia* survived, which suggests that survival may partially be genetically determined. This further indicates that certain genotypes may be more tolerant to high CO_2 conditions, hence facilitating adaptation through natural selection imposed by a high CO_2 environment.

The 2013 experiment was expanded from three (n=100, 2012) to 10 (n=100, 2013) high CO_2 replicates in order to produce sufficient statistical power to provide the most robust estimates of heritability and maternal effects using the 'animal model' (Lynch & Walsh 1998). Sufficient replication is critical to the ability to discern and report valid estimates of survival from laboratory experiments. Variability in survival among our high CO_2 replicates was evident with a maximum survival of 76% and a minimum survival of 28%. 71 dead larvae were missed in the siphoning process, most likely due to timing, if some individuals died just after the second daily siphon or larvae may have already decayed by the time of siphoning. In total, there was a 23% loss of decayed dead hatchlings and eggs that failed to hatch; however, my detection probability was random, therefore all missing larvae could not belong to selected genotypes.

In this study 704 out of 772 samples were successfully assigned to a pair of parents using eight microsatellite loci. The 9% of unassigned offspring were the result of poor amplification of some loci. However, when compared to available values in the literature, our 91% success rate for parentage assignment using eight microsatellites appears to be within normal methodological constraints. Other studies assigning parentage using microsatellite markers reported a 95% success rate using eight rainbow trout, *Oncorhynchus mykiss* microsatellites (Estoup et al. 1998), a 75% success rate using nine walleye, *Sander vitreus* microsatellites (Eldridge et al. 2002) and a 95.3% success rate using eight common carp, *Cyprinus carpio* microsatellites (Vandeputte et al. 2004). All females in this study were assigned to offspring, however 7 of the 42 males were not assigned as parents. Therefore, the sperm from 7 males was either not activated, or the eggs they fertilized were not selected after strip spawning.

Analysis of the pedigree revealed that there was significant variability in the amount of offspring coming from females fertilized by a varying number of sires. The pedigree output (Figure 8) also revealed that our experimental crosses successfully produced full-siblings, half-siblings and unrelated individuals within each replicate. The most notable female was responsible for producing 15% of all the larvae in this study. The least successful female was fertilized by one male and that pair produced only 1 offspring. Females that were fertilized by 15 or more sires produced a higher proportion of survivors as compared to females with fewer sires (Figure 6). This value could be used as a key threshold when performing future survival experiments. This may be attributed to the fact that more genetic variability and a higher overall allelic richness among progeny can result in higher survival (Ruzzante et al. 1996). Interestingly, all parameters tested in each replicate (RAR, H_{obs}, number of dams, number of sires and total number of parents) showed an increasing trend with increasing survival (Figure 9). The only significant (p = 0.02) increasing linear relationship with survival was observed heterozygosity (Figure 9B); further suggesting that one of the most important genetic parameters determining larval survival may be the amount of genetic variability within a population. This is consistent with the concept that diminished genetic variability in natural populations results in increased genetic bottlenecks, inbreeding, reductions in effective population size and the potential for reduced adaptability and productivity (Hauser et al. 2002, O'Leary et al. 2013).

The estimated heritability for survival at high CO_2 levels was **0.11±0.07**. This value is low, but significantly greater than zero and must be considered as a maximum value for heritability, given that larvae from the same parents also died in the control (ambient) treatment. Since the fish in the control group were not genotyped, any estimate for heritability cannot be attributed solely to our high CO_2 treatment, but must be viewed as an estimate of the heritability of survival at high CO_2 coupled with survival in ambient conditions. Therefore, heritability of survival under high CO_2 alone will take a value

between 0 and 0.11. To disentangle normal from CO_2 inflicted mortality in the future, dead and surviving fish in the control treatment need to be collected, genotyped and sequenced in order to compare and partition estimates of heritability.

Maternal effects were simultaneously calculated by adding maternal identity as a random effect to model 1 to produce model 2. This calculated the component of variance due to differences in the observed trait value between offspring of different mothers in addition to the additive genetic effects obtained in the analysis (Kruuk 2004). Maternal effects were found to be low (m^2 = 0.03±0.03), suggesting that offspring from the same mother are no more similar in trait value (MDS) to one another than offspring from different mothers. Maternal identity in this case explained 0-3% of the phenotypic variance in offspring tolerance to high CO_2 for *M. menidia*. This result is surprising considering that maternal effects have been considered to contribute significant proportions of phenotypic variance for many marine (Green 2008) and terrestrial (Kruuk 2004) species. Heritability has been estimated for several life history traits (i.e., length, weight and survival) for multiple species of fish. Heritability estimates of 3 traits across 27 species of fish were analyzed for comparable estimates to our value of 0.11 (Figure 10). Certain morphological traits (i.e., weight and length) are consistently more heritable than others across species, however; estimates of h^2 of fitness related traits such as survival were found to typically range between (0 – 0.35).

The use of quantitative genetic methods allows for improved efficiency of the experimental design by way of a diallel breeding cross. By utilizing complex pedigree structure from a species that can be reared under controlled laboratory conditions such as *M.menidia*, quantitative genetics allows for the prediction of phenotypic evolution across a single generation in order to partition a trait's phenotypic variation into underlying additive genetic and maternal effects (Munday et al. 2013). Since *M.menidia* can be strip spawned and reared in the laboratory, we were able to cross the sperm of several males (n=42) with the eggs of many females (n=29) at the same time throughout all ten replicates, without having to fragment each cross into separate replicates. This type of design not only minimizes the unwieldy breeding crosses of old which require one replicate per cross to keep track of mating pairs, it ensures sufficient genetic variability within each replicate (Figures 8 & 9).

The value of 0.11 is, to our knowledge, the first direct estimate of the heritability and maternal effects of larval fish survival at high CO_2 conditions. When coupled with moderate selection differentials, this low h^2 value would result in slow evolutionary responses (Conover et al. 2005), particularly because 0.11 is the maximum value for heritability. This study demonstrated the feasibility

and promise of the quantitative genetic approach, which will enable future studies to predict the potential for evolutionary responses to ocean acidification and other climate stressors.

However, quantitative genetic approaches also come with considerable constraints. Such experiments require a species that can be reared in captivity, with the ability to track, measure the trait value of, and collect each individual for genetic analysis. Sufficient power to distinguish underlying genetic effects requires the use of several parents (preferably 20+ of each sex) and thousands of offspring of known parentage (Conner & Hartl 2004). This requires the construction of a complete pedigree using several genotypes across multiple loci, a design that is still relatively costly and labor-intensive. Lastly, quantitative genetic experiments do not reveal anything about specific gene expression and may overestimate heritability thus overvaluing evolutionary potential, particularly when measuring a trait such as survival that is the net result of several potential effects (Munday et al. 2013). *M.menidia* in this study were fed *ad libitum* food rations, which is highly practical but may allow survivors of high CO_2 treatments to meet potentially elevated metabolic costs due to high CO_2 exposure. Despite limitations, quantitative genetic methods offer the most direct estimates of heritable variation of traits associated with adaptation to climate change using a single generation, providing important information needed to infer the long-term impacts of climate change on marine organisms.

I hypothesize that h^2 of survival under high CO_2 is considerably lower than the estimated value of 0.11 for combined normal and CO_2-inflicted mortality. An even lower value of heritability for high CO_2 entails a lower potential for evolutionary responses to ocean acidification in *M. menidia.* In the short-term, however, species may use alternate adaptive strategies, e.g., movement or plastic responses, to cope with changing environmental conditions and water chemistry. One plausible mechanism for a short-term adaptation strategy for *M. menidia* populations faced with extreme environmental variability would be a plastic effect. Survival experiments were carried out on a biweekly basis across the entirety of the spawning season, allowing our group to observe the seasonal change in the response of offspring tolerance exposed to high CO_2. Our group had access to high-frequency long-term (2008-2012) pH monitoring data from Flax Pond tidal marsh, allowing us to observe the rapid seasonal acidification pattern typical for this habitat. Repetitive biweekly sampling of spawning adults, combined with short-term early life CO_2 exposure experiments and the corresponding environmental observational pH data allowed us to detect a shift from CO_2-sensitive to CO_2 tolerant offspring which temporally coincided with the seasonal acidification patterns of the species' habitat. These results suggest that parents may be able to condition their offspring to changing conditions within their spawning estuary, a possible case of transgenerational acclimation to increasing CO_2 levels observed for the first time in the wild. Transgenerational plasticity may help marine organisms faced with climate change to persist in the short-

term. However, in the long term, organisms may need to genetically adapt to these environmental changes. The change in phenotype that is caused by transgenerational acclimation is not permanent (i.e., epigenetic), while true adaptation involves long-term change in genetic sequence which requires sufficient genetic variability and directional selection.

Conclusions:

Understanding the long term impacts that climate change may have on marine organisms depends upon their ability to adapt to these changes. The potential for organisms to evolutionarily respond to climate change cannot be explained without a means to measure and quantify the underlying genetic variation of phenotypic traits under selection (Crozier et al. 2008, Wilson et al. 2009a). This study proposed a novel methodological approach to quantify the potential for evolution of a marine fish facing reduced survival due to anthropogenic CO_2 increases and provides the first direct estimates of the heritability and maternal effects of larval survival at high CO_2 for a marine fish, *M. menidia.* Our estimates of heritability (0.11 ± 0.07) and maternal effects (0.03 ± 0.03) for survival at high CO_2 were low, suggesting that long-term genetic change may be slow. However, our continued experiments allowed us to observe the ability for phenotypic change through a plastic (i.e. nongenetic) response which may provide a means for *M. menidia* to persist in the short-term, providing time needed for evolution to occur. Estimating the potential for adaptive responses of marine organisms in the long and short term is crucial for understanding the full biological effects of ocean acidification and far more empirical data is needed to understand varying responses across groups of species. Not only will quantifiable measurements of evolution provide estimates for the potential and speed of phenotypic change, they will be useful for making managerial decisions to protect organisms that show low adaptive potential.

CHAPTER 1 –Figures and Tables:

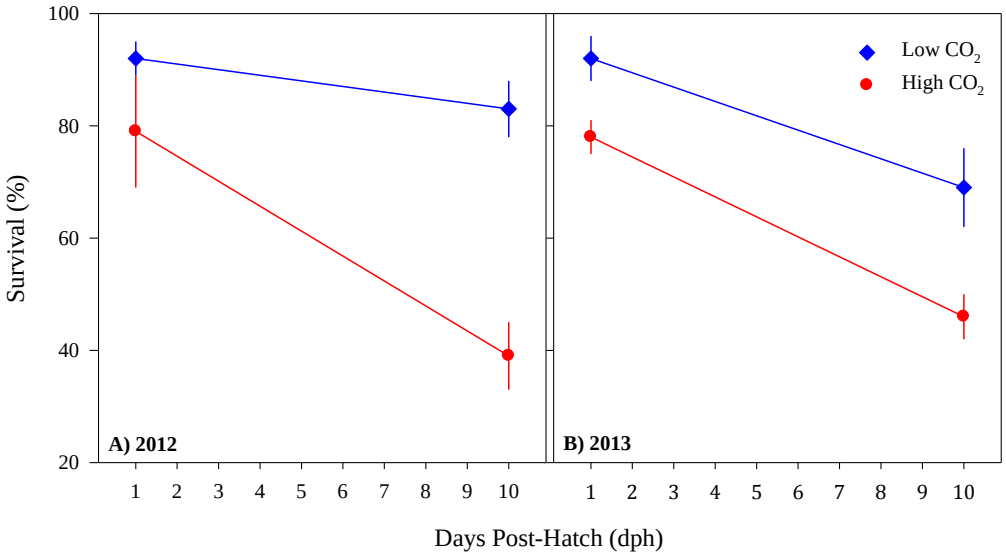

Fig 3: Survival (%) of replicates means (±SE) of ambient treatment (blue) and high CO_2 treatment (red) is plotted at 1 dph and 10dph. Figure **A** represents data from the experiment 1 (fertilized: April 6, 2012), figure **B** represents data from the main heredity experiment (fertilized: April 26, 2013).

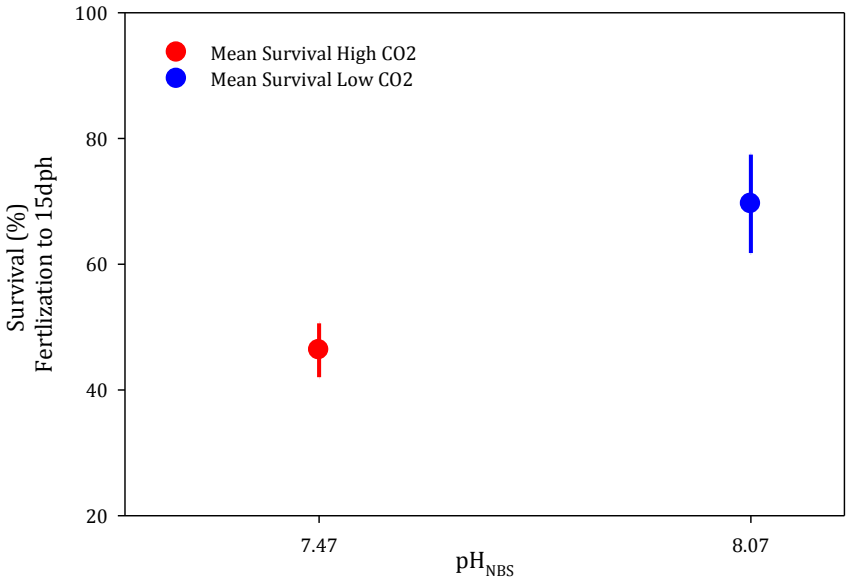

Fig 4: Mean survival (± SE) from fertilization to 15dph for the 2013 experiment is plotted for the high CO_2 treatment (red) and the low CO_2 treatment (blue).

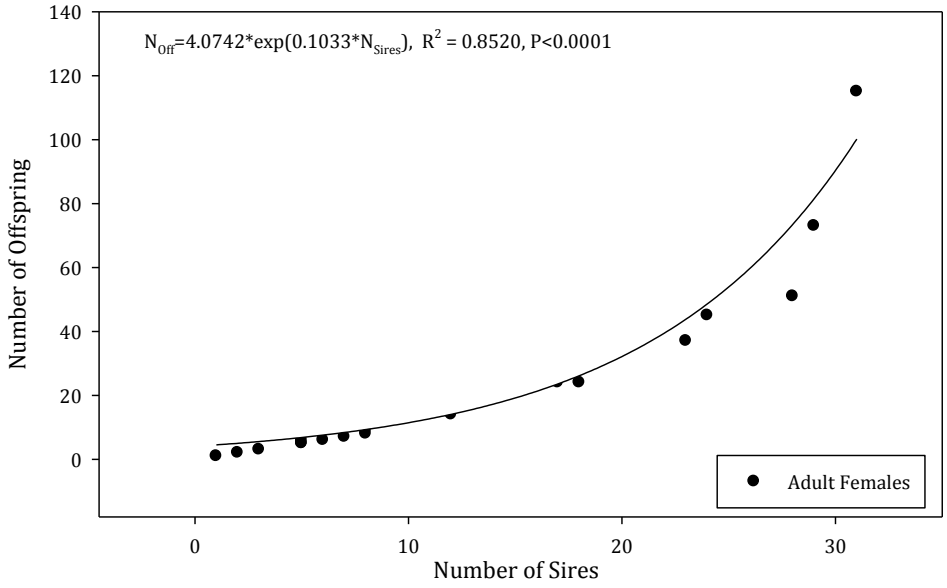

Fig 5: Number of sires per adult *M. menidia* female used in OA experiment 1 (4/26/13 - 5/17/13) is plotted with the resulting amount of offspring per adult female (circles). An exponential growth curve (a = 4.0742, b = 0.1033, R2=0.852) was fitted to the data.

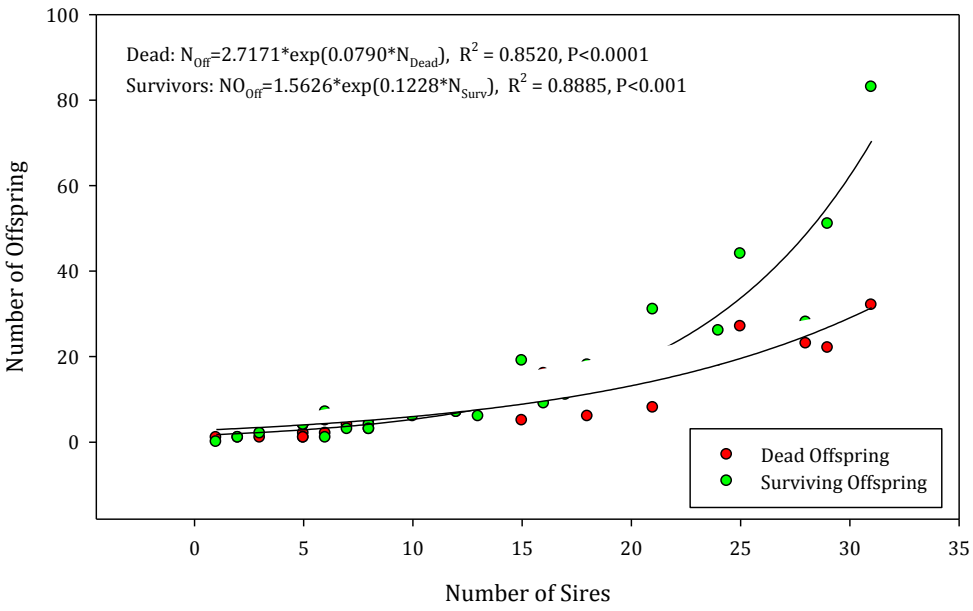

Fig 6: Number of sires per adult *M. menidia* female used in the 2013 experiment (4/26/13 - 5/17/13) is plotted with the resulting amount of dead offspring (red circles) and surviving offspring (green circles) per female. Adult females are represented by pairs of circles. An exponential growth curve for dead offspring (a = 2.7171, b = 0.0.0790, R^2 = 0.852) and surviving offpsring (a = 1.5626, b = 0.1228, R^2= 0.8885) was fitted to the data.

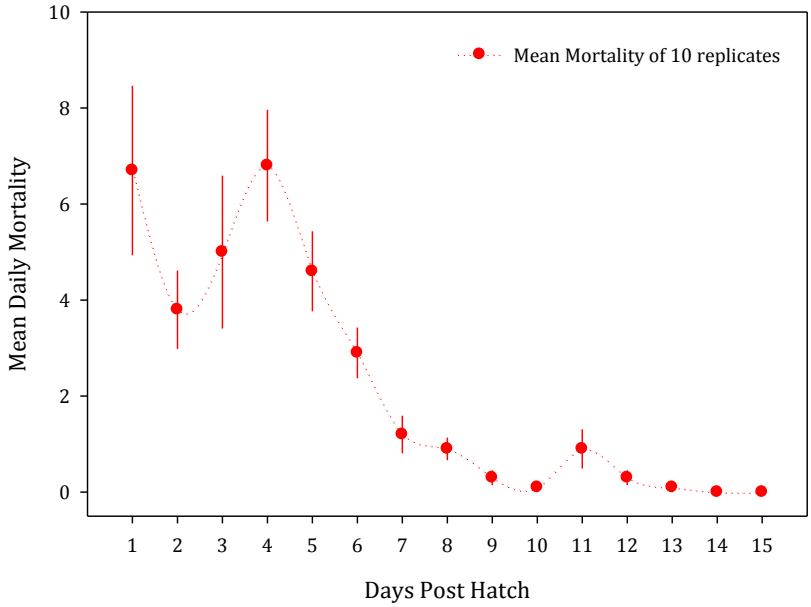

Fig 7: Mean number of daily mortalities (±1 SE) was plotted for all replicates in high CO2 (circles).

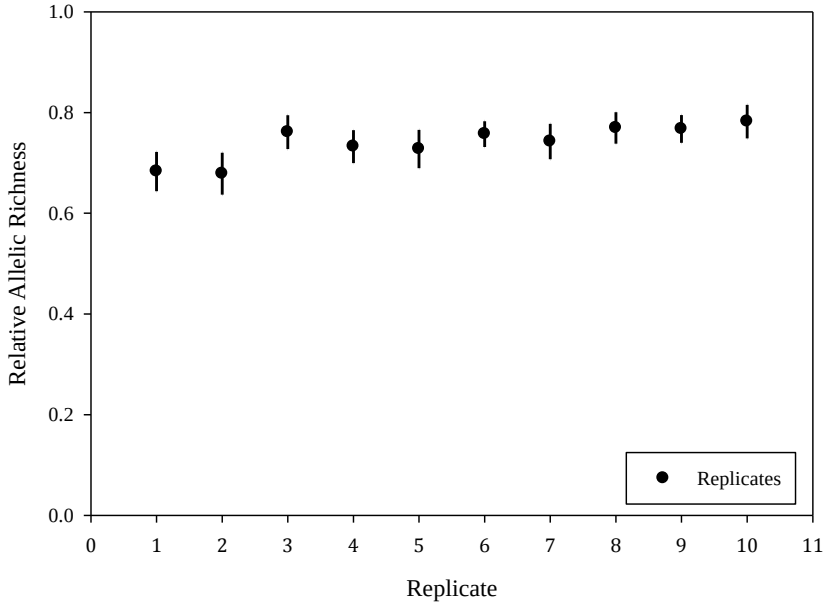

Fig 8: Relative allelic richness (±1 SE) across all microsatellite loci is plotted for each replicate (circles).

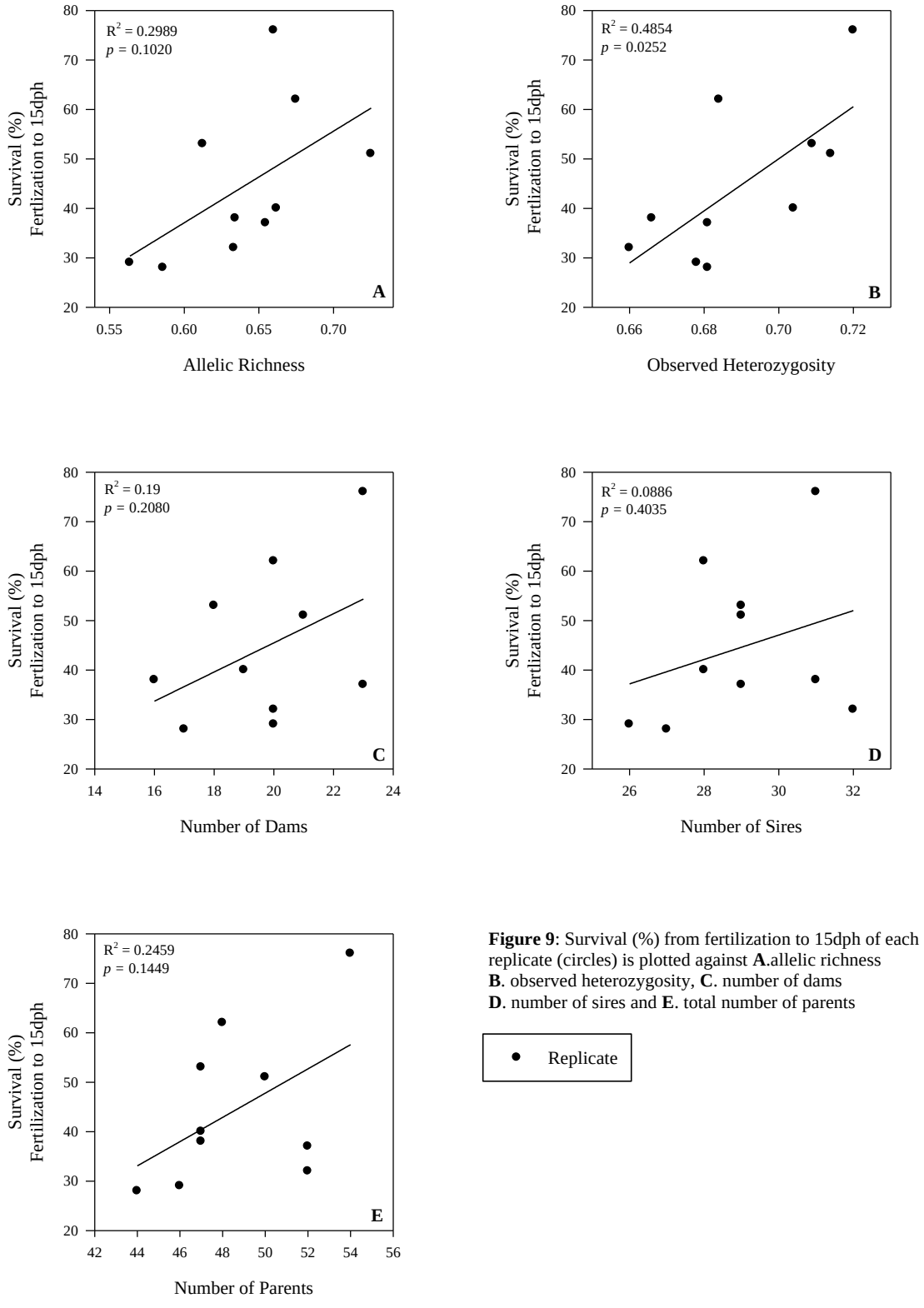

Figure 9: Survival (%) from fertilization to 15dph of each replicate (circles) is plotted against **A**.allelic richness **B**. observed heterozygosity, **C**. number of dams **D**. number of sires and **E**. total number of parents

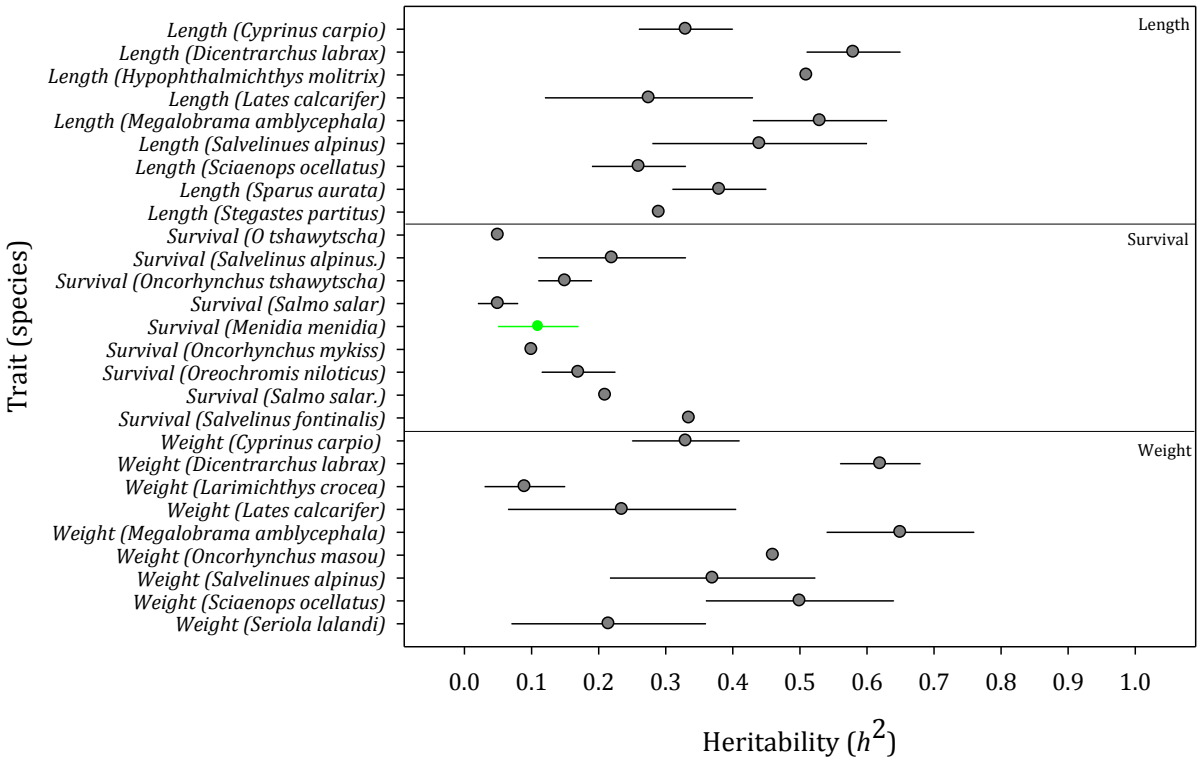

Figure 10: Mean heritability (SE) is plotted for three life history traits (length, weight and survival) and 27 species of fish. Heritability of larval CO_2-resistance is plotted in green.

Days Post-Hatch (dph)																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			
Rep	**Number of Mortalities (recovered)**															**Dead**	**Survivors**	**Unhatched**
1	2	7	13	10	7	4	4	1	-	-	-	1	-	-	-	49	28	21
2	-	1	3	5	2	1	2	2	1	1	-	-	-	-	-	18	52	20
3	7	7	6	12	5	4	1	-	1	-	1	-	-	-	-	44	32	16
4	9	2	3	9	7	2	1	-	-	-	-	-	-	-	-	33	28	34
5	4	-	-	2	6	5	1	1	-	-	-	-	1	-	-	20	60	14
6	11	3	4	3	7	2	-	2	-	-	1	-	-	-	-	33	51	7
7	12	6	3	10	6	5	1	1	-	-	-	1	-	-	-	45	37	10
8	-	6	15	9	1	1	2	1	1	-	1	-	-	-	-	37	36	10
9	5	2	1	2	-	1	-	1	-	-	2	-	-	-	-	14	76	15
10	17	4	2	6	5	4	-	-	-	-	4	1	-	-	-	43	36	10
Total	**67**	**38**	**50**	**68**	**46**	**29**	**12**	**9**	**3**	**1**	**9**	**3**	**1**	**0**	**0**	**336**	**436**	**157**

Table 3: Number of daily mortalities (1 – 15dph) in each replicate from OA experiment 1 (4.26.13-5.17.13) and amount of survivors, dead larvae and unhatched eggs per replicate.

Experiment	Treatment	pH(NBS)	pCO2	Total DIC	Total Alkalinity	Aragonite
1-2012	Ambient	8.03±0.02	660.3	2226	2281	0.97
	High	7.48±0.05	2153	2338	2249	0.32
1-2013 (Heredity)	Ambient	8.11±0.07	458.9±72.85	2175±125.5	2262±122.5	1.96±0.15
	High	7.45±0.04	2294±133.6	2324±165.5	2235±178.5	0.43±0.04

Table 4: pH, pCO2, Total dissolved inorganic carbon, total alkalinity and aragonite is shown for experiment 1 of 2012 and experiment 1 of 2013 – heredity experiment

Trait	model	V_A(SE)	V_R(SE)	V_P(SE)	V_M(SE)	h^2	m^2
Survival	2	3.56±2.38	27.55±2.15	32.15±1.86	1.03±1.13	0.11±0.07	0.03±0.03

Table 5: Heritability (h^2) and maternal effect (m^2) is shown for survival at high CO_2, including the single trait animal model used. Variance components (±SE) are indicated as: additive genetic variance ($\mathbf{V_A}$), residual variance ($\mathbf{V_R}$), Total phenotypic variance ($\mathbf{V_P}$), maternal variance ($\mathbf{V_M}$).

Chapter 2:

Introduction:

Genetic variation among populations of fish facing selection pressure from climate change stressors (i.e. ocean acidification) is crucial to facilitate adaptation throughout evolutionary timescales.

High CO_2-induced mortality may be a selective process, removing certain genotypes that may not be able to cope with rapid changes in water chemistry from generation to generation. In chapter 1, the heritability and maternal effects of larval survival at high CO_2 was calculated for *Menidia menidia* and determined to be low; (0 to 0.11) and (0.03±0.03), respectively. Low heritability may be an indication of slower adaptive responses in long-term evolutionary timescales. Our lab has set out to test this notion by designing a CO_2 survival-selective experiment using the offspring of survivors of high and low CO_2 early life exposure (Figure 12). *M.menidia* has been a long-standing model species used in a myriad of informative laboratory experiments in the last 35 years.

Many of these experiments have revealed important insights about life history evolution including observations of temperature-dependent sex determination (Conover & Fleisher 1986), countergradient variation in growth at different latitudes (Conover & Present 1990, Baumann & Conover 2011), the evolution of somatic growth rate in populations faced with size-selective fishing pressure (Conover & Munch 2002) and genetic variation in early-life growth (Gao & Munch 2013). *M. menidia* are now being used to provide the first estimates of quantifiable evolutionary potential for a marine fish faced with ocean acidification by way of experimental evolution. Experimental evolution can encompass a wide range of experiments; however, one process that may be informative in measuring the evolutionary potential of fish is "laboratory culling," a process which requires exposing a population to a deadly stressor (i.e. high CO_2) and allowing the survivors to become the parents of the next generation (Garland & Rose 2009). Direct observation of selection and evolutionary change through these experiments coupled with the estimates of heritability and maternal effects obtained in chapter 1 will provide a comprehensive analysis of the realized potential for *M. menidia* to genetically adapt to anthropogenic increases in CO_2. While these experiments are species specific, they are meant to provide researchers with methodological approaches combining quantitative genetics and experimental evolution to explore the potential for quantifying evolutionary adaptation for a variety of marine species.

Research Objective:

The objective of this experiment was to rear surviving *M.menidia* offspring from high CO_2 (~2,300 µatm) and low CO_2 (~600 µatm) early-life exposure experiments to sexual maturity and induce spawning in the laboratory to produce F_2 generation to test their survival when exposed to the same levels of CO_2 from fertilization to 10dph.

Materials and Methods:

Experimental Design:

Surviving fish (60 high CO_2 and 64 low CO_2) from experiment two (2012) early life CO_2 exposure experiments were grown out to sexual maturity (~one year) in ambient CO_2 conditions (~600ppm) in circular seawater baths (500L, 22ºC). During the first seven months of life, fish were kept on a daily photoperiod of (15L: 9D), at seven months of age the light cycle was changed to (12L: 12D), and finally at 10 months old the photoperiod was reverted back to (15L: 9D) to induce spawning (Conover & Munch 2002). Food in the form of newly hatched brine shrimp nauplii, *Artemia salina* (San Francisco strain, Brine Shrimp Direct) was fed *ad libitum* until one month old at which time they were fed progressive stages of Otohime fish food (Reed Mariculture®) and supplementary food in the form of frozen *A. salina* throughout the entirety of their life cycle.

Embryos from spawning adults were collected by placing a cluster of yarn in the center of each tank to mimic spawning substrate of a tidal marsh. Yarns were collected on a daily basis to quantify fertilized eggs to be hung into replicate containers (high CO_2 and low CO_2) filled with seawater. Food in the form of newly hatched brine shrimp nauplii, *Artemia salina*, (San Francisco strain, Brine Shrimp Direct, Inc) was provided *ad libitum* to all replicates immediately before hatching (~5-6 days post fertilization) and each day post-hatch (dph). Additional food in the form of commercial larval powder (Otohime Marine Weaning Diet, Size A, Reed Mariculture®) was provided 1 to 2 days post hatch to reduce mortality normally occurring around the time of first feeding (Baumann et al. 2011). Larval survival was quantified within each replicate at 1 dph and 10dph by gently scooping small groups of live larvae into new rearing containers.

The experiment was terminated after two weeks of spawning due to lack of laboratory space. Adult fish were immediately sexed and measured for total length (cm) and weight wet (g). Sub samples of surviving larvae were measured for standard length (mm) using calibrated pictures and image analysis software (ImagePro 4.5.1, Media Cybernetics). Surviving fish from both treatments were collected and transferred to ambient water conditions to grow out to sexually maturity to test the survival of future generations.

CO_2 treatments and measurements:

CO_2 delivery has been described in detail in chapter 1. For this experiment, two levels of CO_2 were administered: ambient (**~600 µatm)**, and high (**~2,300 µatm),** these treatment levels represent

current day CO_2 levels at our study site and open ocean CO_2 levels projected for the year 2300 according to IPCC 2013 predictions.

Results:

Over a period of 10 days of spawning in the laboratory, the high CO_2 stock of fish produced 1720 eggs while the low CO_2 stock of fish produced 3590 fertilized eggs. Upon termination of the experiment, sex determination revealed that there were 12 females and 48 males in the high CO_2 stock and 23 females and 41 males in the low CO_2 stock.

The high CO_2 stock of spawning males and females had a mean total length (cm (±SE)) of 10.3 (±0.1) and 11.3 (±0.18) and a mean wet weight (g (±SE)) of 6.3 (±0.2) and 8.0 (±0.35) respectively. The low CO_2 stock of spawning males and females had a mean total length (cm (±SE)) of 10.0 (±0.08) and 10.6(±0.15) and a mean wet weight (g (±SE)) of 6.1(±0.15) and 7.0(±0.3) respectively.

Mean survival (±SE) at 10dph for offspring from the high CO_2 stock in the high CO_2 and low CO_2 treatments was 43% (±20%) and 46%(±17%) respectively. Mean survival (±SE) at 10dph for offspring from the low CO_2 stock in high CO_2 and low CO_2 treatments was 35% (±10%) and 37%(±10%) respectively (Figure 12). There was no statistically significant difference in survival of offspring from the high CO_2 or low CO_2 stock at either treatment.

Mean length (mm (±SE) at 10dph for offspring from high CO_2 stock in the high CO_2 and low CO_2 treatments was 8.9 (±0.13) and 10.28 (±0.17), respectively. Mean length (mm (±SE) at 10dph for offspring from low CO_2 stock in the high CO_2 and low CO_2 treatments was 10.3 (±0.17) and 10.2 (±0.10), respectively (Figure 13). High CO_2 broodstock length at 10dph was significantly lower (ANOVA, $F_{1, 113} = 37.929$, $p = 0.000$) than low CO_2 broodstock length at 10dph in the high CO_2 treatment.

Discussion:

Our results suggest that there is no difference in early-life survival of offspring reared from parents that survived high CO_2 (~2,200ppm) or low CO_2 (~600ppm) when introduced to identical treatments from fertilization to 10dph. There was also no observed difference in mean adult length (cm) or wet weight (g) of sexually mature fish grown in high and low CO_2 treatments. Low heritability (0 to 0.11) for survival at high CO_2 calculated in chapter 1 may explain why there was no difference in survival of F_2 offspring given that it may take multiple generations to observe adaptive genetic change, or natural selection. *M. menidia* have shown the ability for life-history traits (size at age, fecundity) to rapidly

evolve in as little as four generations under artificial-selection experiments (Conover & Munch 2002). Hence, this may be the case here if selected individuals were continually grown to sexual maturity, repeatedly introducing their offspring to high and low CO_2 to the observe the evolvable potential of survival through multiple generations exposed to selection. *M. menidia* are a unique model marine species of fish that can be harvested and reared in the laboratory with relative ease and their life cycle is short (~one year), enabling us to carry out experiments throughout multiple generations. Mean length (mm, ±SE) at 10dph for the high CO_2 broodstock in our high CO2 treatment was significantly less than the ambient broodstock, suggesting that we do see some sort of variability or difference in offspring length even after only one generation. Time constraints of two years of funded research limit the ability for me to carry out long-term selection experiments; however, surviving fish will continue to be grown to sexual maturity, allowing future students to observe the possibility of fish surviving high CO_2 environments producing offspring with decreased vulnerability to high CO_2 conditions.

This experiment was the result of only 12 total replicates (six high CO_2 stock offspring replicates– three high CO_2, three low CO_2) and (six low CO_2 stock offspring replicates– three high CO_2, three low CO_2). Lack of sufficient replication may be another reason for not distinguishing any statistical difference in early-life survival of offspring from selected parents. Chapter 1 displayed the need for extensive replication due to evident variability of survival within ten replicates (n=100, 28% to 76%). Unfortunately, this experiment was terminated after just 10 days of laboratory-induced spawning due to limited space needed to carry out 2013 biweekly survival experiments. Previous experimental research on *M.menidia* suggests they have the ability to spawn in captivity for long periods of time (Conover 1984). Methodologically, it is important to note that *M. menidia* sex determination is influenced by temperature during phases of larval development. High temperatures, (17-25ºC) produce higher proportions of males, which was observed in this experiment (12 females, 48 males in high CO_2 @ 22ºC and 23 females and 41 males in low CO_2 @ 22ºC) whereas low temperature (11-19ºC) produce a higher proportion of females (Conover & Fleisher 1986). In future experiments, fish should be kept at lower temperatures to ensure a significant proportion of females contributing to future generations.

This study is in its infancy; however, it provides evidence that observing evolutionary change from CO_2-induced mortality selection in the laboratory is possible and *M. menidia* is the ideal model species to carry out such experiments. We know from previous experiments discussed in chapter 1 that the heritability of survival at high CO_2 for a New York population of *M.menidia* takes a value from 0 to 0.11, an estimate that may suggest a slow evolutionary response. Keeping track of future generations of *M.menidia* offspring bred from parents exposed to early-life high and low CO_2 conditions will provide direct estimates of the speed in which evolutionary changes may be occurring. The goal of this

experiment is to determine whether there is potential for local evolutionary adaptation to ocean acidification for *M.menidia.* No studies to date have obtained quantifiable estimates for adaptive potential to OA for a marine fish but our lab has suggested evidence for approaches to measure and obtain values for the heritability and maternal effects of survival at high CO_2, ways to directly observe evolutionary change through experiment evolution and observations of the possibility for short-term plastic responses of phenotypic change.

Figures:

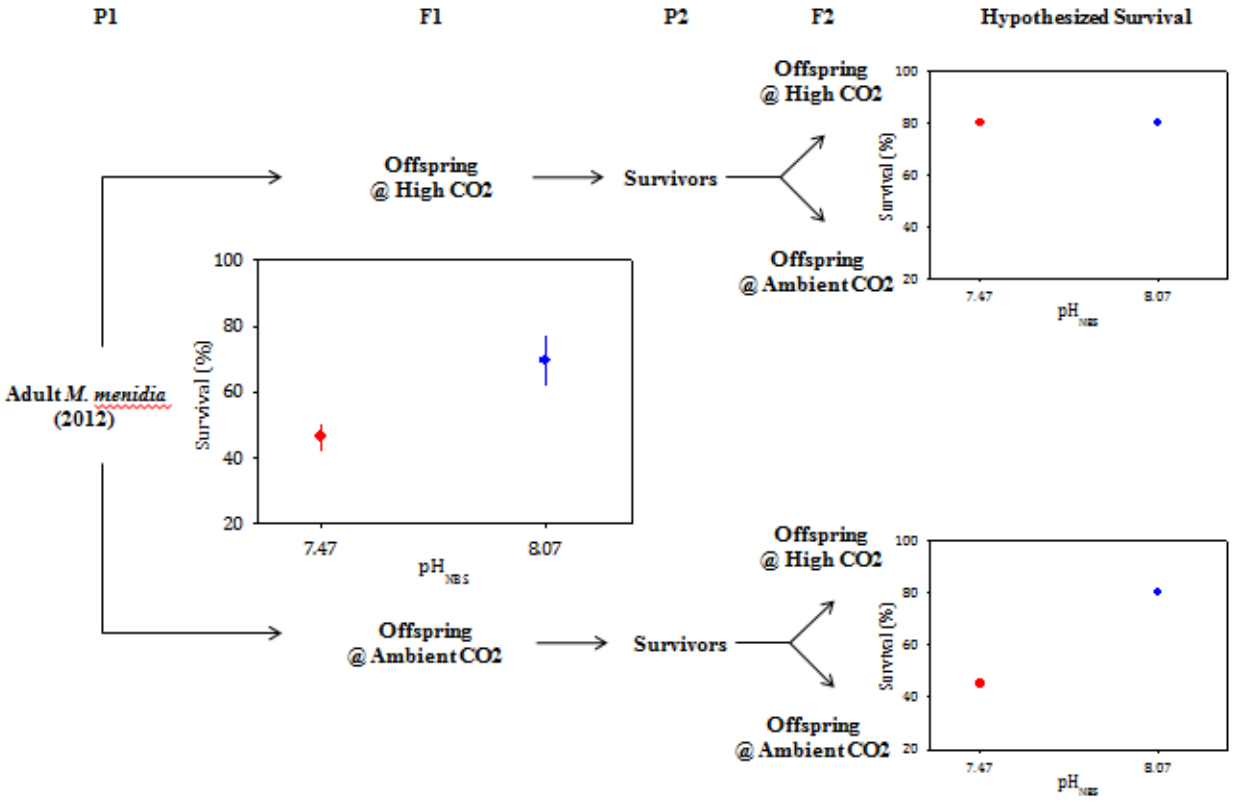

Figure 11: Display of parents and offspring used in CO_2 selection experiment and hypothesized survival plots for future generations. F_2 generation survivors are currently being reared to sexual maturity.

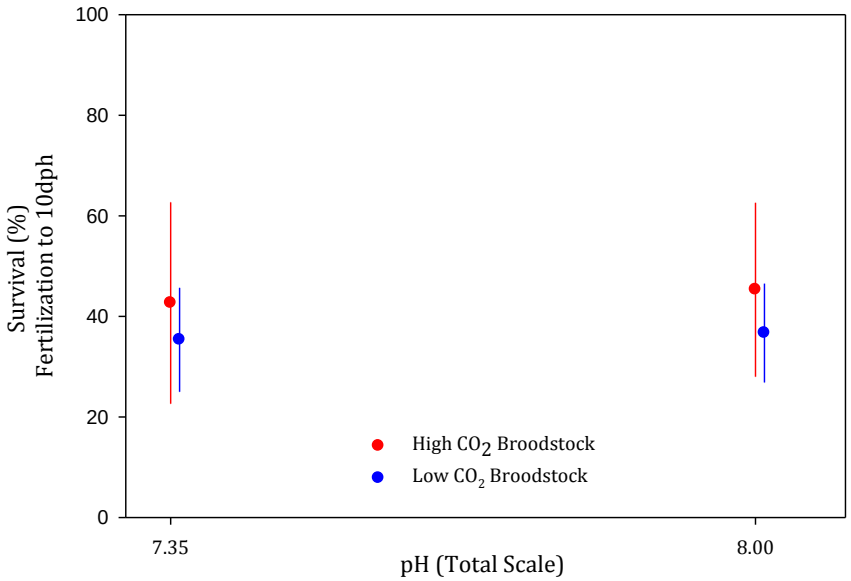

Fig 12: Mean Survival (±1 SD) is plotted for offpspring from high CO_2 broodstock (red) and low CO_2 broodstock (blue) in high CO_2 treatment (pH_{TS} = 7.35) and low CO_2 treatment (pH_{TS} = 8.00).

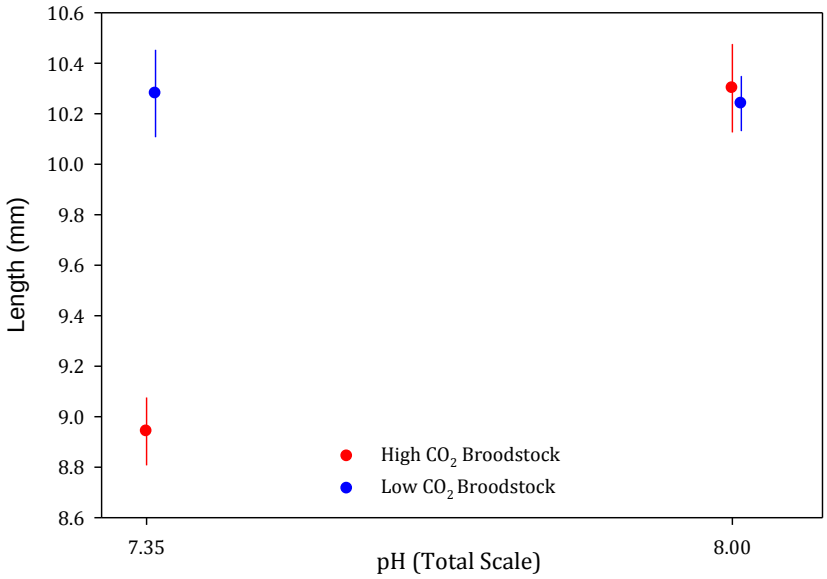

Fig 13: Mean length (mm, ±1 SD) is plotted for offpsring from high CO2 broodstock (red, N = 115) and low CO2 broodstock (blue, N = 119) in high CO2 treatment (pH_{TS} = 7.35) and low CO_2 treatment (pH_{TS} = 8.00).

Literature cited:

Åkesson M, Bensch S, Hasselquist D, Tarka M, Hansson B (2008) Estimating heritabilities and genetic correlations: comparing the 'animal model'with parent-offspring regression using data from a natural population. PLoS One 3:e1739

Allendorf FW (1986) Genetic drift and the loss of alleles versus heterozygosity. Zoo biology 5:181-190

Alley R, Berntsen T, Bindoff N, Chen Z, Chidthaisong A, Friedlingstein P, Gregory J, Hegerl G, Heimann M, Hewitson B (2007) IPCC 2007: summary for policymakers. Climate change:1-18

Barton NH, Keightley PD (2002) Understanding quantitative genetic variation. Nature Reviews Genetics 3:11-21

Baumann H, Conover DO (2011) Adaptation to climate change: contrasting patterns of thermal-reaction-norm evolution in Pacific versus Atlantic silversides. Proceedings of the Royal Society B: Biological Sciences 278:2265-2273

Baumann H, Talmage SC, Gobler CJ (2011) Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. Nature Climate Change

Beaufort L, Probert I, de Garidel-Thoron T, Bendif E, Ruiz-Pino D, Metzl N, Goyet C, Buchet N, Coupel P, Grelaud M (2011) Sensitivity of coccolithophores to carbonate chemistry and ocean acidification. Nature 476:80-83

Bernardo J (1996) Maternal effects in animal ecology. American Zoologist 36:83-105

Bignami S, Sponaugle S, Cowen RK (2013) Response to ocean acidification in larvae of a large tropical marine fish, Rachycentron canadum. Global Change Biology

Boag PT, Grant PR (1978) Heritability of external morphology in Darwin's finches.

Cai W-J, Hu X, Huang W-J, Murrell MC, Lehrter JC, Lohrenz SE, Chou W-C, Zhai W, Hollibaugh JT, Wang Y (2011) Acidification of subsurface coastal waters enhanced by eutrophication. Nature Geoscience 4:766-770

Caldeira K, Wickett ME (2005) Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J Geophys Res 110:C09S04

Chambers R, Candelmo A, Habeck E, Poach M, Wieczorek D, Cooper K, Greenfield C, Phelan B (2013) Ocean acidification effects in the early life-stages of summer flounder, Paralichthys dentatus. Biogeosciences Discussions 10:13897-13929

Change IPOC (2007) Climate change 2007: the physical science basis. Agenda 6:07

Checkley DM, Dickson AG, Takahashi M, Radich JA, Eisenkolb N, Asch R (2009) Elevated CO2 enhances otolith growth in young fish. science 324:1683-1683

Collins S, Bell G (2005) Evolution of natural algal populations at elevated CO2. Ecol Lett 9:129-135

Conner JK, Hartl DL (2004) A primer of ecological genetics. Sinauer Associates Incorporated

Conover DO (1984) Adaptive significance of temperature-dependent sex determination in a fish. American Naturalist:297-313

Conover DO (1998) Local adaptation in marine fishes: evidence and implications for stock enhancement. Bulletin of Marine Science 62:477-493

Conover DO, Arnott SA, Walsh MR, Munch SB (2005) Darwinian fishery science: lessons from the Atlantic silverside (Menidia menidia). Canadian Journal of Fisheries and Aquatic Sciences 62:730-737

Conover DO, Fleisher MH (1986) Temperature-sensitive period of sex determination in the Atlantic silverside, Menidia menidia. Canadian Journal of Fisheries and Aquatic Sciences 43:514-520

Conover DO, Munch SB (2002) Sustaining fisheries yields over evolutionary time scales. science 297:94-96

Conover DO, Present TM (1990) Countergradient variation in growth rate: compensation for length of the growing season among Atlantic silversides from different latitudes. Oecologia 83:316-324

Crozier L, Hendry A, Lawson P, Quinn T, Mantua N, Battin J, Shaw R, Huey R (2008) Potential responses to climate change in organisms with complex life histories: evolution and plasticity in Pacific salmon. Evolutionary Applications 1:252-270
Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean acidification: the other CO2 problem. J Mar Sci 1
Duarte CM, Hendriks IE, Moore TS, Olsen YS, Steckbauer A, Ramajo L, Carstensen J, Trotter JA, McCulloch M (2013) Is ocean acidification an open-ocean syndrome? Understanding anthropogenic impacts on seawater pH. Estuaries and Coasts:1-16
Dupont S, Pörtner H (2013) Marine science: get ready for ocean acidification. Nature 498:429-429
Eldridge WH, Bacigalupi MD, Adelman IR, Miller LM, Kapuscinski AR (2002) Determination of relative survival of two stocked walleye populations and resident natural-origin fish by microsatellite DNA parentage assignment. Canadian Journal of Fisheries and Aquatic Sciences 59:282-290
Estoup A, Gharbi K, SanCristobal M, Chevalet C, Haffray P, Guyomard R (1998) Parentage assignment using microsatellites in turbot (Scophthalmus maximus) and rainbow trout (Oncorhynchus mykiss) hatchery populations. Canadian Journal of Fisheries and Aquatic Sciences 55:715-723
Fabry VJ, Seibel BA, Feely RA, Orr JC (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. ICES J Mar Sci 65:414-432
Ferrari MCO, Dixson DL, Munday PL, McCORMICK M, Meekan MG, Sih A, Chivers DP (2011) Intrageneric variation in antipredator responses of coral reef fishes affected by ocean acidification: implications for climate change projections on marine communities. Glob Change Bio 17:2980-2986
Frommel AY, Maneja R, Lowe D, Malzahn AM, Geffen AJ, Folkvord A, Piatkowski U, Reusch TB, Clemmesen C (2011) Severe tissue damage in Atlantic cod larvae under increasing ocean acidification. Nature Clim Change
Futuyama D, Bennett AF, Garland Jr T, Rose M (2009) The importance of experimental studies in evolutionary biology. Experimental Evolution: Concepts, Methods, and Applications University of California Press, Berkeley:15-30
Gao J, Munch SB (2013) Genetic and maternal variation in early growth in the Atlantic silverside Menidia menidia. Marine ecology Progress series 485:211-222
Garland T, Rose MMR (2009) Experimental evolution: concepts, methods, and applications of selection experiments. University of California Pr
Green BS (2008) Maternal effects in fish populations. Advances in marine biology 54:1-105
Guo SW, Thompson EA (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics:361-372
Guo X, Elston R (1999) Linkage information content of polymorphic genetic markers. Human Heredity 49:112-118
Gutowska MA, Pörtner HO, Melzner F (2008) Growth and calcification in the cephalopod Sepia officinalis under elevated seawater pCO2. Marine Ecology Progress Series 373:303-309
Hauser L, Adcock GJ, Smith PJ, Ramírez JHB, Carvalho GR (2002) Loss of microsatellite diversity and low effective population size in an overexploited population of New Zealand snapper (Pagrus auratus). Proceedings of the National Academy of Sciences 99:11742-11747
Hendriks IE, Duarte CM, Álvarez M (2010) Vulnerability of marine biodiversity to ocean acidification: a meta-analysis. Estuarine, Coastal and Shelf Science 86:157-164
Hoegh-Guldberg O, Mumby P, Hooten A, Steneck R, Greenfield P, Gomez E, Harvell C, Sale P, Edwards A, Caldeira K (2007) Coral reefs under rapid climate change and ocean acidification. science 318:1737-1742
Hoffmann AA, Merilä J (1999) Heritable variation and evolution under favourable and unfavourable conditions. Trends in Ecology & Evolution 14:96-101
Hoffmann AA, Sgrò CM (2011) Climate change and evolutionary adaptation. Nature 470:479-485

Hofmann GE, Barry JP, Edmunds PJ, Gates RD, Hutchins DA, Klinger T, Sewell MA (2010) The effect of ocean acidification on calcifying organisms in marine ecosystems: an organism-to-ecosystem perspective. Ann Rev Ecol Syst 41:127-147

Hurst TP, Fernandez ER, Mathis JT, Miller JA, Stinson CM, Ahgeak EF (2012) Resiliency of juvenile walleye pollock to projected levels of ocean acidification. Aquatic Biology 17:247-259

Ishimatsu A, Hayashi M, Kikkawa T (2008) Fishes in high-CO2, acidified oceans. Marine Ecology Progress Series 373:295-302

Jin P, Gao K, Beardall J (2013) Evolutionary responses of a coccolithophorid Gephyrocapsa oceanica to ocean acidification. Evolution

Kelly MW, Padilla-Gamiño JL, Hofmann GE (2013) Natural variation and the capacity to adapt to ocean acidification in the keystone sea urchin Strongylocentrotus purpuratus. Global Change Biology

Klerks PL, Xie L, Levinton JS (2011) Quantitative genetics approaches to study evolutionary processes in ecotoxicology; a perspective from research on the evolution of resistance. Ecotoxicology 20:513-523

Kroeker KJ, Kordas RL, Crim R, Hendriks IE, Ramajo L, Singh GS, Duarte CM, Gattuso JP (2013) Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. Global Change Biology

Kruuk LEB (2004) Estimating genetic parameters in natural populations using the 'animal model'. Philosophical Transactions of the Royal Society of London Series B: Biological Sciences 359:873-890

Kurihara H (2008) Effects of CO2-driven ocean acidification on the early developmental stages of invertebrates.

Langer G, Nehrke G, Probert I, Ly J, Ziveri P (2009) Strain-specific responses of Emiliania huxleyi to changing seawater carbonate chemistry. Biogeosciences 6:2637-2646

Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits.

Mayo O (2008) A century of Hardy–Weinberg equilibrium. Twin Research and Human Genetics 11:249-256

Melzner F, Gutowska M, Langenbuch M, Dupont S, Lucassen M, Thorndyke MC, Bleich M, Pörtner H-O (2009) Physiological basis for high CO 2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences Discussions 6:4693-4738

Melzner F, Thomsen J, Koeve W, Oschlies A, Gutowska MA, Bange HW, Hansen HP, Körtzinger A (2012) Future ocean acidification will be amplified by hypoxia in coastal habitats. Marine Biology:1-14

Metz B, Davidson O, De Coninck H, Loos M, Meyer L (2005) IPCC special report on carbon dioxide capture and storage. Intergovernmental Panel on Climate Change, Geneva (Switzerland). Working Group III

Middaugh DP, Hemmer MJ, Goodman L (1987) Methods for Spawning, Culturing and Conducting Toxicity-Tests with Early Life Stages of Four Atherinid Fishes: The Inland Silverside,'Menidia beryllina', Atlantic Silverside,'M. menidia', Tidewater Silverside,'M. peninsulae' and California Grunion,'Leuresthes tenuis.

Mousseau TA, Fox CW (1998) The adaptive significance of maternal effects. Trends in Ecology & Evolution 13:403-407

Munday PL, Dixson DL, Donelson JM, Jones GP, Pratchett MS, Devitsina GV, Døving KB (2009a) Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. Proceedings of the National Academy of Sciences 106:1848-1852

Munday PL, Dixson DL, McCormick MI, Meekan M, Ferrari MC, Chivers DP (2010) Replenishment of fish populations is threatened by ocean acidification. Proceedings of the National Academy of Sciences 107:12930-12934

Munday PL, Donelson JM, Dixson DL, Endo GGK (2009b) Effects of ocean acidification on the early life history of a tropical marine fish. P Roy Soc Lond B Bio 276:3275-3283
Munday PL, Gagliano M, Donelson JM, Dixson DL, Thorrold SR (2011) Ocean acidification does not affect the early life history development of a tropical marine fish. Marine Ecology Progress Series 423:211-221
Munday PL, McCormick MI, Nilsson GE (2012) Impact of global warming and rising CO2 levels on coral reef fishes: what hope for the future? J Exp Biol 215:3865-3873
Munday PL, Warner RR, Monro K, Pandolfi JM, Marshall DJ (2013) Predicting evolutionary responses to climate change in the sea. Ecology letters 16:1488-1500
O'Leary SJ, Hice LA, Feldheim KA, Frisk MG, McElroy AE, Fast MD, Chapman DD (2013) Severe Inbreeding and Small Effective Number of Breeders in a Formerly Abundant Marine Fish. PLoS One 8:e66126
Orr JC, Fabry VJ, Aumont O, Bopp L, Doney SC, Feely RA, Gnanadesikan A, Gruber N, Ishida A, Joos F (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437:681-686
Pistevos JCA, Calosi P, Widdicombe S, Bishop JDD (2011) Will variation among genetic individuals influence species responses to global climate change? Oikos 120:675-689
Pörtner H-O (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. Mar Ecol Prog Ser 373:203-217
Riebesell U, Fabry VJ, Hansson L, Gattuso J-P (2010) Guide to best practices for ocean acidification research and data reporting.
Ries JB, Cohen AL, McCorkle DC (2009) Marine calcifiers exhibit mixed responses to CO2-induced ocean acidification. Geology 37:1131-1134
Rost B, Riebesell U (2004) Coccolithophores and the biological pump: responses to environmental changes. Coccolithophores—From Molecular Processes to Global Impact:76-99
Rost B, Zondervan I, Wolf-Gladrow D (2008a) Sensitivity of phytoplankton to future changes in ocean carbonate chemistry: current knowledge, contradictions and research directions. Mar Ecol Prog Ser 227
Rost B, Zondervan I, Wolf-Gladrow D (2008b) Sensitivity of phytoplankton to future changes in ocean carbonate chemistry: current knowledge, contradictions and research directions. Marine ecology progress series, 373237 227
Ruzzante DE, Taggart CT, Cook D (1996) Spatial and temporal variation in the genetic composition of a larval cod (Gadus morhua) aggregation: cohort contribution and genetic stability. Canadian Journal of Fisheries and Aquatic Sciences 53:2695-2705
Salisbury J, Green M, Hunt C, Campbell J (2008) Coastal acidification by rivers: a threat to shellfish? Eos, Transactions American Geophysical Union 89:513
Sbrocco EJ, Barber PH (2011) Ten polymorphic microsatellite loci for the Atlantic Silverside, Menidia menidia. Conserv Genet 3:585-587
Sunday JM, Crim RN, Harley CDG, Hart MW (2011) Quantifying rates of evolutionary adaptation in response to ocean acidification. PLoS One 6:e22881
Sunnucks P, England PR, Taylor AC, Hales DF (1996) Microsatellite and chromosome evolution of parthenogenetic Sitobion aphids in Australia. Genetics 144:747-756
Talmage SC, Gobler CJ (2010) Effects of past, present, and future ocean carbon dioxide concentrations on the growth and survival of larval shellfish. P Natl Acad Sci-Biol 107:17246-17251
Vandeputte M, Kocour M, Mauger S, Dupont-Nivet M, De Guerry D, Rodina M, Gela D, Vallod D, Chevassus B, Linhart O (2004) Heritability estimates for growth-related traits using microsatellite parentage assignment in juvenile common carp (< i> Cyprinus carpio</i> L.). Aquaculture 235:223-236

Visscher PM, Hill WG, Wray NR (2008) Heritability in the genomics era—concepts and misconceptions. Nature Reviews Genetics 9:255-266
Wilson AJ, Réale D, Clements MN, Morrissey MM, Postma E, Walling CA, Kruuk LEB, Nussey DH (2009a) An ecologist's guide to the animal model. Journal of Animal Ecology 79:13-26
Wilson AJ, Réale D, Clements MN, Morrissey MM, Postma E, Walling CA, Kruuk LEB, Nussey DH (2009b) An ecologist's guide to the animal model. J Anim Ecol 79:13-26
Wilson AJ, Réale D, Clements MN, Morrissey MM, Postma E, Walling CA, Kruuk LEB, Nussey DH (2010) An ecologist's guide to the animal model. Journal of Animal Ecology 79:13-26
Wootton JT, Pfister CA, Forester JD (2008) Dynamic patterns and ecological impacts of declining ocean pH in a high-resolution multi-year dataset. Proceedings of the National Academy of Sciences 105:18848-18853