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Effects of Ocean Acidification on the Early Life History of Two Pelagic Tropical Fish Species, Cobia (Rachycentron canadum) and Mahi-mahi (Coryphaena hippurus)

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Sean G. T. Bignami

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Effects of Ocean Acidification on the Early Life History of Two Pelagic Tropical Fish Species, Cobia (*Rachycentron canadum*) and Mahi-mahi (*Coryphaena hippurus*) (May 2013)

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Ocean acidification affects a wide diversity of marine organisms and is of particular concern for vulnerable larval stages critical to population replenishment and connectivity. In fishes, the existing body of ocean acidification research has focused mostly on small demersal species, which may be more resistant to acidification than offshore pelagic species that exhibit different life history strategies and are often highly valued. A better understanding of the effects of ocean acidification on these understudied and highly valuable species requires examination of biological factors that influence larval ability to successfully survive, navigate, and recruit to the adult population. This dissertation examines the impact of ocean acidification on the larval stages of two widely distributed, pelagic, tropical fish species, cobia (*Rachycentron canadum*) and mahi-mahi (*Coryphaena hippurus*). Larval cobia and mahi-mahi were raised for up to 21 d under several projected ocean acidification scenarios, including scenarios for year 2100 (800 μatm pCO_2), year 2300 (1700-2100 μatm pCO_2), comparative worst-case scenarios (up to 5400 μatm pCO_2), and multi-stressor experiments crossing ocean acidification with increased temperature and starvation stress (reduced ration). Treatment effects were evaluated throughout ontogeny using selected metrics including larval growth, size, development (flexion), swimming ability (U_{crit}), swimming activity, and otolith (ear

stone) formation. Larval cobia exhibited resistance to ocean acidification under year 2100 and 2300 "business as usual" scenarios as measured by most metrics, although significantly reduced size-at-age, growth rate, and delayed developmental progression was identified under the year 2300 scenario in one of three experiments. Comparative "worst-case" ocean acidification scenarios caused clearly reduced size-at-age and delayed development, but acidification combined with increased temperature or starvation stress did not increase larval susceptibility to acidification. Larval mahi-mahi were similarly robust and unaffected by ocean acidification as measured by most metrics, although they exhibited significantly larger size-at-age and a significant decrease in routine swimming speed during one of three experiments. Together, these results indicate that these pelagic species may not be more susceptible to ocean acidification than presumably more resistant nearshore demersal species. However, despite resistance to ocean acidification as measured by many of our chosen metrics, significant effects were detected in both species. Larval cobia at 1700 μatm pCO_2 (year 2300 scenario) exhibited more rapid mortality (lower LT_{50}) due to complete feeding cessation, indicating increased vulnerability to periods of suboptimal feeding conditions in a patchy marine feeding environment. Furthermore, cobia exhibited increased otolith size at the lowest pCO_2 reported to date (800 μatm, year 2100 scenario). Novel *in situ* analysis of larval cobia otoliths using three-dimensional micro-computed tomography also identified increased otolith volume, density, and relative mass. Similarly, larval mahi-mahi raised in acidified water exhibited increased otolith size when measured via light microscopy. Using a modeling approach, these changes to otoliths were shown to affect auditory sensitivity, including up to a ~50% increase in hearing range in a high-CO_2 ocean. Effects on larval

otoliths and hearing ability could substantially influence the dispersal, survival, and recruitment of larvae in nature, with implications for population replenishment, connectivity, and conservation efforts for valuable fish stocks. The presence of treatment effects on these discreet metrics, amidst many unaffected gross metrics (e.g. size), highlights the importance of improving assays to detect impacts of ocean acidification. Although the cumulative ecological and management implications of these effects are not yet clear, collectively, this dissertation provides unprecedented perspective on the potential impacts of ocean acidification on two high value pelagic fish species.

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CHAPTER 1. Introduction

Ocean acidification

Since the onset of the industrial revolution, anthropogenic emissions have increased atmospheric CO_2 concentration from 280 ppm to 390 ppm, reaching levels which are higher than at any point in the past 800,000 years (Lüthi et al. 2008). Approximately 25% of anthropogenic CO_2 has been absorbed by the world's oceans (Sabine et al. 2004), where it is hydrated to form carbonic acid, which dissociates into bicarbonate and carbonate, releasing H^+ ions and thereby reducing ocean pH. This process of ocean acidification has already reduced average ocean pH by 0.1 units since pre-industrial times (Meehl et al. 2007), caused water that is undersaturated with respect to aragonite to reach the ocean surface (Feely et al. 2008), and is one of the many aspects of global environmental change that pose a threat to marine ecosystems (Royal Society 2005). Under the Intergovernmental Panel on Climate Change (IPCC) SRES-A2 scenario, it is predicted that by the end of the 21st century, atmospheric CO_2 concentration will reach nearly 1000 ppm (Figure 1.1a), corresponding to a surface ocean pH reduction of ~ 0.45 units, which is greater than a 100 % increase in seawater [H^+] (Caldeira & Wickett 2005, Meehl et al. 2007). Over the next three centuries, unchecked increase in anthropogenic CO_2 could result in an atmospheric CO_2 concentration of nearly 2000 ppm (Figure 1.1b) — driving ocean pH down ~ 0.77 units at a rate of change faster than any experienced in the last 300 million years (Caldeira & Wickett 2003, 2005, Honisch et al. 2012). In addition to ocean acidification, increased atmospheric CO_2 has produced climatic changes including increased global temperatures, with a multitude of cascading effects on atmospheric, terrestrial, and oceanic systems (IPCC 2007). These rapid global

environmental changes will inevitably result in biological impacts, the severity and scope of which are only beginning to be understood.

Impacts on marine organisms

Although ocean acidification is often perceived as a future problem, impacts on marine life and the human economies they support are already being felt in some regions. Oyster hatchery production failures in the Northwest United States have been linked to the presence of low-pH water along the Pacific coast (Barton et al. 2012), and although upwelling naturally brings low-pH water to the surface, the shoaling of water with more drastically reduced pH has been exaggerated by anthropogenic CO_2 in the ocean (Feely et al. 2008). Experimentally, projected levels of ocean acidification have been shown to negatively influence a diversity of marine organisms, including coccolithophores (Riebesell et al. 2000), copepods (Mayor et al. 2007), corals (Langdon et al. 2003), molluscs (Michaelidis et al. 2005, Shirayama & Thornton 2005), echinoderms (Kurihara & Shirayama 2004, O'Donell et al. 2010), and fishes (Michaelidis et al. 2007, Frommel et al. 2011, Munday et al. 2012, and others). Most studies have focused on calcifying marine invertebrate species because altered carbonate chemistry has direct implications for calcification processes (Fabry et al. 2008). Comparatively, there is limited research on organisms that do not form calcium carbonate shells or skeletal structures, including fishes. This is likely due to the fact that organisms with higher metabolic rates are often able to efficiently regulate their internal acid-base balance in response to metabolic or environmental disturbances, and thus are considered to be more capable of resisting ocean acidification (Melzner et al. 2009a). However, this may not hold true for the early

life stages of fishes, which are more susceptible to elevated CO_2 than adults (reviewed by Pörtner et al. 2005).

Impacts on larval fishes

The days to month-long pelagic larval period is an ecologically vital ontogenetic phase in marine fishes because it constitutes the primary mode of dispersal in many species (Cowen & Sponaugle 2009) and represents the life stage most susceptible to mortality (Houde 1997). Slight changes in mortality or growth rates of larvae can have large cumulative effects on recruitment potential (Houde 1989a, 1997), which in turn influences population replenishment and connectivity, both of which are central to the ecological function and effective management of marine populations (Cowen & Sponaugle 2009). Given the importance of the larval stages, data on this ontogenetic stage are necessary for a more complete understanding of the impacts of ocean acidification on fish populations.

Early research on larval fishes demonstrated significantly reduced survival at low pH (Kikkawa et al. 2003, 2004, Ishimatsu et al. 2004), and verified that CO_2-induced acidification produces more negative effects than addition of acid alone (Hayashi et al. 2004, Kikkawa et al. 2004). However, treatments in these studies were based on concentrated CO_2 sequestration scenarios, with extremely low pH values, beyond what is projected to occur in the next several centuries. More recently, studies have focused on levels of ocean acidification within IPCC-projected ranges, as well as other modeled scenarios. These studies have produced conflicting inter- and intraspecific results. For some species, ocean acidification has detrimental effects on larval survival, growth (Baumann et al. 2011), tissue health (Frommel et al. 2011), metabolism (Franke &

Clemmesen 2011, Miller et al. 2012), condition (Franke & Clemmesen 2011), and behavior (Munday et al. 2009a, Dixson et al. 2010, Devine et al. 2011, Simpson et al. 2011, Ferrari et al. 2012a, 2012b, Domenici et al. 2012). However, some of the very same studies failed to find significant negative effects, or even found positive effects, on growth, development (Munday et al. 2009b, 2011a, Franke & Clemmesen 2011, Moran & Støttrup 2011, Frommel et al. 2012), and swimming ability (Munday et al. 2009b). Effects on otolith growth have been reported in some species (Checkley et al. 2009, Munday et al. 2011b, Hurst et al. 2012), but not others (Franke & Clemmesen 2011, Munday et al. 2011a, Simpson et al. 2011, Frommel et al. 2012), yet potential ecological consequences have not been addressed.

When testing the impact of acidification at levels projected to occur within the next one to two centuries, most studies have not detected direct increases in larval or juvenile mortality (except see Baumann et al. 2011, Miller et al. 2012). The majority of direct effects of ocean acidification are sublethal; altering the physiology, growth, sensory function, or behavior of the fish, but only causing higher mortality when other factors such as predation are introduced (Munday et al. 2010, Ferrari et al. 2011a, 2011b). It is likely that some of these sublethal effects of ocean acidification on fishes can be attributed to the same physiological capabilities that are thought to confer resistance to acidification. Increased environmental CO_2 results in the accumulation of CO_2 in the blood of fishes, which decreases blood pH but is quickly and persistently compensated for by the active uptake and retention of base-equivalent HCO_3^- ions (Claiborne & Heisler 1986, Claiborne et al. 2002, Esbaugh et al. 2012). This response to a disturbance in acid-base balance, via increased synthesis and activity of ATP-consuming ion

transporters, likely has an associated metabolic cost (Pörtner et al. 2004, Deigweiher et al. 2008). An increase in cellular metabolic demand may be challenging for larvae due to their narrow metabolic scope (Cunha et al. 2007) and could result in the reallocation of metabolic resources away from somatic growth. Increased metabolic rate correlating with decreased size was recently observed in juvenile tropical fish exposed to acidification (Miller et al. 2012) and a similar scenario is proposed to have caused reduced growth in larvae exposed to metabolically high-cost osmoregulatory challenges (Fielder et al. 2005). Conversely, acute exposure to severe hypercapnia has been shown to result in decreased metabolic rate and diminished protein synthesis in fish (Langenbuch & Pörtner 2003). If growth and development are impacted by altered metabolic demand or the repartitioning of metabolic resources, there are clear consequences for the survival of larval fishes. The growth-mortality hypothesis poses that fishes which grow more slowly, are smaller at-age, or have extended stage-durations will experience higher mortality rates (Anderson 1988), with cascading implications for recruitment magnitude and populations replenishment (Houde 1989a). Growth rates throughout the larval stages can also "carry-over" to influence juvenile condition and survival (Searcy & Sponaugle 2001, McCormick & Hoey 2004, Hamilton 2008), resulting in additional impacts after settlement occurs.

Other physiological consequences of acidification include the potential for altered blood-oxygen delivery, decreased aerobic scope, and diminished aerobic swimming ability under conditions of altered CO_2/pH (Pörtner et al. 2004, Michaelidis et al. 2007, Munday et al. 2009c). Swimming ability has direct relevance to the dispersal potential and settlement of fishes in the wild (Fisher 2005), particularly where late-stage larvae

must swim to reach suitable settlement habitat (Leis 2006). In addition, the same physiological response that compensates for reduced blood pH in fish may result in altered neurological function, possibly driving the observed impact of acidification on a wide range of behavioral traits and sensory functions (Nilsson et al. 2012). Changes in routine swimming activity are known to be a sublethal consequence of other stressors, such as exposure to toxins (del Carmen Alvarez & Fuiman 2005). Altered routine activity levels could help compensate for increased metabolic demand (Munday et al. 2009b), and possibly mask otherwise detrimental effects of acidification on the growth and development of larvae. Other effects of altered behavior include reduced prey encounter rate (Gerritsen & Strickler 1977) and a disruption of larval dispersal (Cowen & Sponaugle 2009).

While individual sublethal effects of ocean acidification may not have a clear negative effect on larvae under experimental laboratory conditions, they affect a number of factors that are critical to the successful survival, navigation, and recruitment of larvae. This is well illustrated in a series of studies by Munday et al. (2009a, 2010), who reported altered olfactory homing and predator detection capability, changes to routine behavior, and increased risk-taking activity in damselfish larvae raised under conditions of acidification projected to occur within this century; these changes resulted in up to a nine-fold higher *in situ* mortality of new recruits on the reef.

Knowledge gaps

Given the recent expansion of research on the impacts of ocean acidification on the early life history stages of fishes, is not surprising that there are many areas in which our current understanding is limited. Some of these areas include the study of species

with different life history strategies and physiological capabilities, the impact of acidification when combined with other stressors, the long term impacts of acidification on fish growth and reproduction, the response of fish to more accurate simulations of variable ocean acidification, and the potential for acclimatization and adaptation to acidification. Although the scope of this dissertation cannot encompass all of these areas, the following chapters address the first two.

An overarching theme throughout ocean acidification literature is the negative yet variable response of marine organisms (Kroeker et al. 2010, Hendriks et al. 2010). Though intriguing, this variability confounds our ability to formulate general conclusions regarding the fate of fish populations under future environmental scenarios. To better clarify the underlying factors driving patterns of susceptibility or resistance to ocean acidification, it is necessary to examine a diversity of species with distinct differences in life history strategy, physiological capability, and habitat use. Studies on temperate species (i.e. *Atractoscion nobilis, Clupea harengus, Gadus morhua, Menidia beryllina, Theragra chalcogramma*; Checkley et al. 2009, Franke & Clemmesen 2011, Baumann et al. 2011, Frommel et al. 2011, Hurst et al. 2012, among others) have focused on both pelagic and demersal species, but for tropical fishes, there has been little study of large pelagic species (Figure 1.2). Pelagic fish species that inhabit the offshore environment often share common life history strategies, similar high-performance physiological capabilities, and also include some of the highest-value fisheries species in the world.

Offshore pelagic environments generally lack the extreme diurnal cycles in pH and temperature observed in shallow nearshore environments (Ohde & van Woesik 1999, Perez-Dominguez et al. 2006, Hofmann et al. 2011, Frieder et al. 2012, Price et al. 2012).

It has been suggested that compared to demersal species regularly exposed to fluctuating environmental conditions, offshore pelagic species which have adapted to such a stable environment may be more susceptible to future environmental changes such as ocean acidification (Munday et al. 2008a, Pörtner 2008). This theory has not been thoroughly addressed in fishes, but the comparison of two studies on a single temperate species provides some insight. Baltic cod (*Gadus morhua*) is a species that likely has evolved adaptations to the high pCO_2 found in its natural habitat (i.e. fjords) and consequently demonstrates resistance to high levels of acidification (Frommel et al. 2012). In contrast, Atlantic cod, a conspecific not regularly exposed to high pCO_2 in its natural environment, is affected by acidification (Moran & Støttrup 2011, Frommel et al. 2011). Research on pelagic species is necessary if we hope to differentiate how various life history strategies, physiological characteristics, and previous adaptations to native environments influence the susceptibility of fishes to ocean acidification.

It is also important to evaluate how organisms respond to ocean acidification in combination with additional stressors associated with global climate change, such as increased temperature, more widespread hypoxia, or altered food availability (Pörtner et al. 2005, Fabry et al. 2008, Guinotte & Fabry 2008, Pörtner 2012). In tropical and subtropical waters, sea surface temperature (SST) is projected to rise 1 - 3°C by the end of this century (Figure 1.3; Sheppard & Rioja-Nieto 2005, Angeles et al. 2007). It has been well demonstrated that larval growth is directly related and pelagic larval duration inversely related to temperature (Houde 1989b, Sponaugle et al. 2006, O'Connor et al. 2007), although extreme temperatures can also limit growth in juvenile and adult fishes (Munday et al. 2008b). As discussed previously, a slight growth change has implications

for larval survival, dispersal, and recruitment magnitude (Anderson 1988, Houde 1989a). Furthermore, it is likely that exposure to increased temperature and ocean acidification will limit tolerance to both stressors, putting fishes at greater risk of impact than would individual stressors (Pörtner 2012).

This challenge could be magnified by a reduction in the availability and quality of prey. As increasing SST drives more intense water column stratification, nutrient availability in surface waters may become limited, resulting in decreased net primary productivity (Behrenfeld et al. 2006) and reduced zooplankton biomass (Roemmich & McGowan 1995). The effect of ocean acidification on primary production is less clear (Guinotte & Fabry 2008); increased pCO_2 may increase phytoplankton growth rates (Wolf-Gladrow et al. 1999), but lower pH may limit productivity by decreasing the bioavailability of limiting nutrients such as iron (Shi et al. 2010) or interacting synergistically with light stress (Gao et al. 2012). Ocean acidification may also impact the quality of biological production; it was recently demonstrated that increased pCO_2 reduces the nutritional value of producers and their zooplanktonic predators (e.g., copepods; Rossoll et al. 2012), the primary prey of many subtropical larval fishes (e.g., see Llopiz & Cowen 2009). Although synergistic interactions between ocean acidification and other stressors has been frequently addressed with invertebrates (e.g., see Byrne 2011), there has been limited research on the impacts on larval and juvenile marine fish (except see Nowicki et al. 2011). To more fully understand the effects of ocean acidification on fishes, it is necessary to examine scenarios that more accurately simulate the complexity of the future marine environment.

Broader significance

Although we have rapidly improved our understanding of ocean acidification impacts on a broad range of marine organisms, it remains challenging to scale up this information to effectively evaluate impacts at population and ecosystem levels (Le Quesne & Pinnegar 2011). In this regard, our progress is even more limited for fishes. Organism-level effects of CO_2-induced acidification, such as reduced growth rate, inhibited swimming ability, or decreased tolerance of other stressors, may exert population-level ecological impacts by altering the survival, dispersal, and recruitment events responsible for the replenishment of fish stocks and maintenance of biodiversity. These processes are directly tied to the success of conservation and management efforts, and while ocean acidification has been shown to potentially impact fisheries productivity and the economies that they support (Cooley & Doney 2009, Cheung et al. 2011), these evaluations are scarce and the magnitude of such impacts is not always clear. Thus it is important to focus research on topics which will enable the evaluation of broader impacts that contribute to adapting management strategies to future climate change scenarios (Doney 2009). This dissertation addresses several of the knowledge gaps that continue to limit our understanding of ocean acidification impacts on marine fishes and our subsequent ability to respond as a society.

Objectives

The overall goal of this research was to identify potential effects of projected CO_2-induced ocean acidification on the larvae of tropical pelagic marine fishes, thereby improving the broader understanding this environmental change. This will contribute to providing direction for future research and enable more informed adaptation of

management and conservation strategies. The following chapters address four main objectives:

(1) *Measure the effects of a range of projected future ocean acidification scenarios on the larvae of pelagic tropical fishes including growth, development, swimming ability, swimming activity, and otolith development.*

(2) *Assess the impact of acidification on otolith development in relation to sensory function and the use of otoliths as tools for fisheries biology research.*

(3) *Evaluate the response of larval fish to ocean acidification when combined with additional stressors.*

(4) *Address the general lack of ocean acidification research on tropical pelagic species by studying and comparing two of the largest, most economically valuable, pelagic species studied to date.*

The first objective was addressed in Chapter Two by examining the basic response to ocean acidification in larval cobia (*Rachycentron canadum*), a large, pelagic, tropical fish species. During two experiments, larvae were raised under a range of projected future ocean acidification scenarios and sampled throughout ontogeny to assess impacts on growth, development, swimming ability, swimming activity, and otolith development. These factors were evaluated by measuring standard length, developmental progression through flexion, critical swimming speed, routine and olfactory stimulated swimming activity (e.g., velocity, angle change during turns, net-to-gross displacement), the size of otoliths, and the widths of individual daily otolith growth increments. The second objective was addressed in Chapter Three, where micro-computed tomography was used to provide detailed evaluation of acidification impacts on larval cobia otolith size and

density. These data were applied to a mathematical model of otolith motion to reveal the potential impacts of enhanced otolith growth on auditory sensitivity and hearing range, and also discuss implications for the effective use of otoliths as tools for fisheries biology research. Chapter Four addresses the third objective via two experiments to measure the impact of multiple stressors on larval cobia. This was accomplished by combining exposure to acidification and increased temperature, as well as acidification and starvation stress. The metrics used to evaluate treatment effects were similar to those used in Chapter Two, but also included the use of RNA:DNA ratio as a measure of larval condition and a starvation mortality experiment as a proxy for starvation resistance under acidified conditions. The fourth objective was addressed in Chapter Five in the study larval mahi-mahi (*Coryphaena hippurus*), a widely distributed, large, tropical fish species with an offshore pelagic lifecycle and high economic value. During three experiments the response of mahi-mahi to projected future scenarios of ocean acidification was evaluated using the same metrics that were previously utilized to assess impacts on cobia.

In summary, this dissertation represents the first evaluation of the impacts of ocean acidification on the larvae of two large tropical pelagic fish species, the first consideration of the sensory and ecological implications of acidification-driven alteration of otolith growth, as well as the first report of impacts on larval fishes due to acidification in combination with other stressors. The results of these studies improve the breadth of our understanding of ocean acidification impacts on fishes, provide direction for future research, and are informative for adapting management and conservation strategies in response to future environmental change.

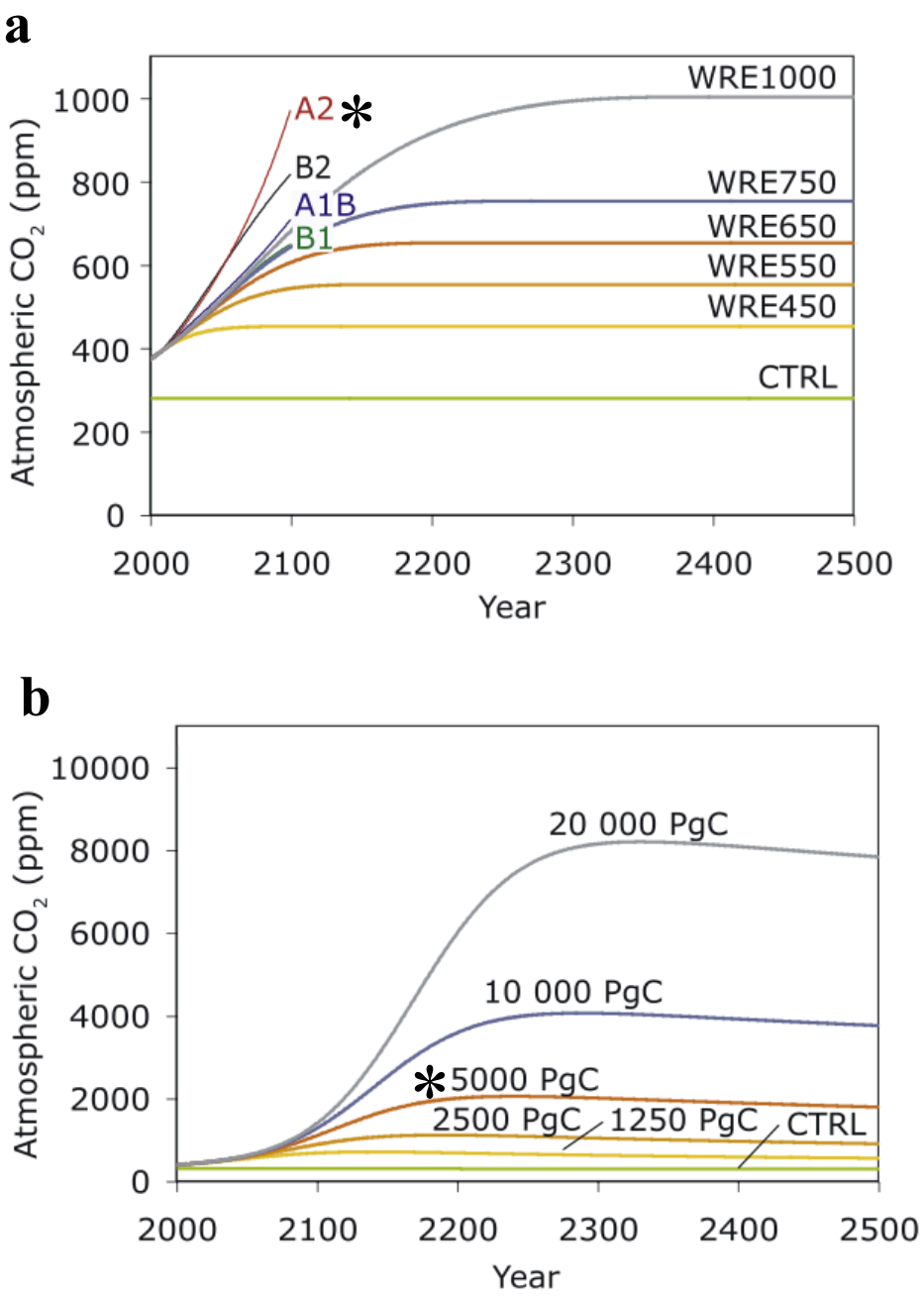
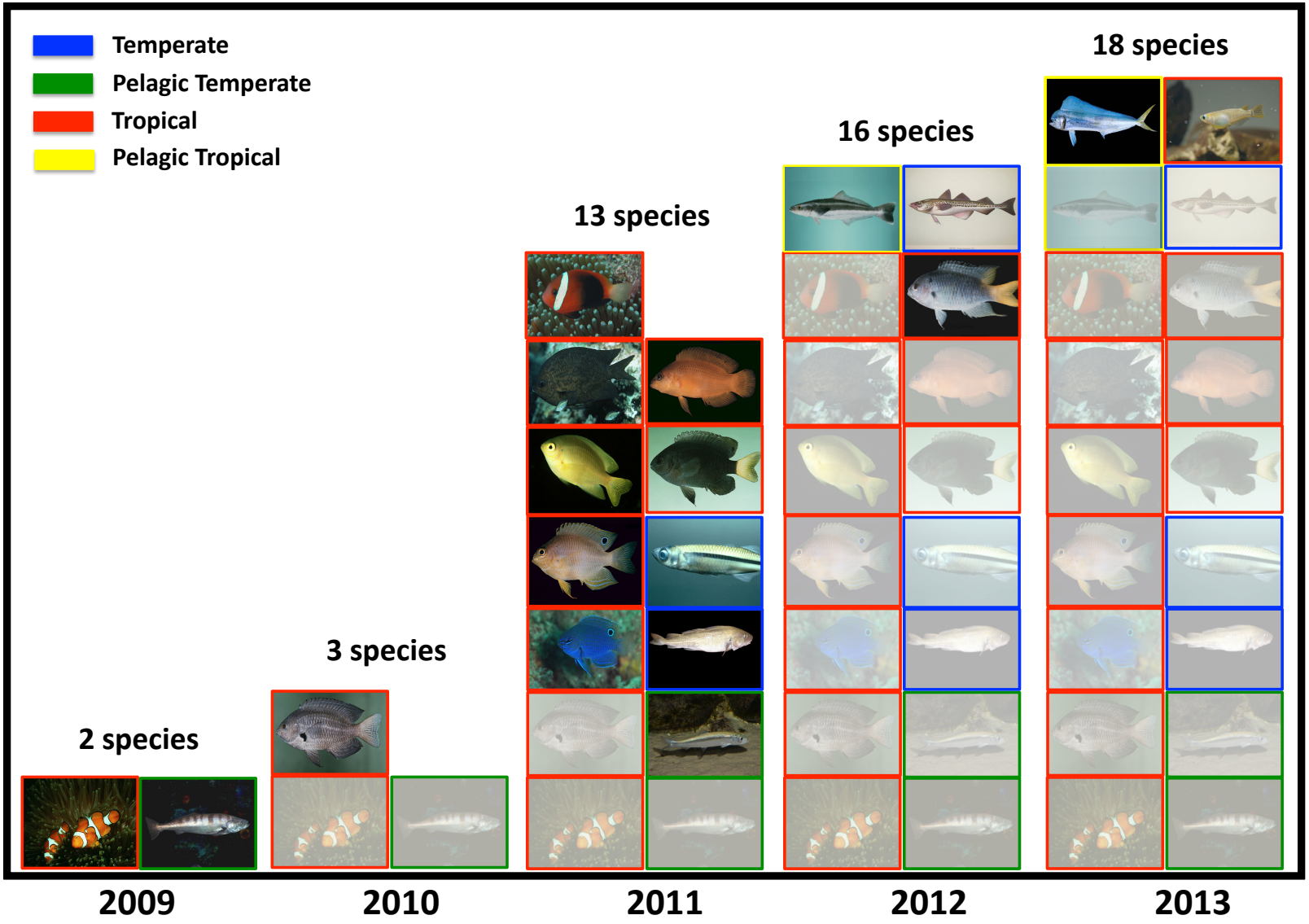


Figure 1.1 Projected scenarios of increased atmospheric CO_2 over the next several centuries. (**a**) The Intergovernmental Panel on Climate Change SRES-A2 scenario (marked with asterisk) projects atmospheric CO_2 concentrations of nearly 1000ppm by the end of the 21st century, whereas (**b**) models assuming the combustion of all known fossil fuel resources (5000 PgC, marked with asterisk) project concentrations as high as 2000ppm within the next two centuries. Figure adapted from Caldeira & Wickett (2005).

Figure 1.2 Depiction of the increase in total number of larval and juvenile fish species examined with regard to projected future ocean acidification. This dissertation contributes both pelagic tropical species to the literature. Study species are displayed as opaque images in years subsequent to their initial addition to the literature. Species habitat indicated by outline colors. Listed left to right, top to bottom in 2013, species include *Coryphaena hippurus*, *Oryzias latipes*, *Rachycentron canadum*, *Theragra chalcogramma*, *Amphiprion melanopsis*, *Neopomacentrus azysron*, *Acanthochromis polyacanthus*, *Pseudochromis fuscus*, *Pomacentrus moluccensis*, *P. Chrysurus*, *P. amboinensus*, *Menidia beryllina*, *P. nagasakiensis*, *Gadus morhua*, *P. wardi*, *Clupea harengus*, *A. percula*, and *Atractoscion nobilis*. Images from EOL (2013).


Temperate
Pelagic Temperate
Tropical
Pelagic Tropical
2 species
3 species
13 species
16 species
18 species
2009
2010
2011
2012
2013

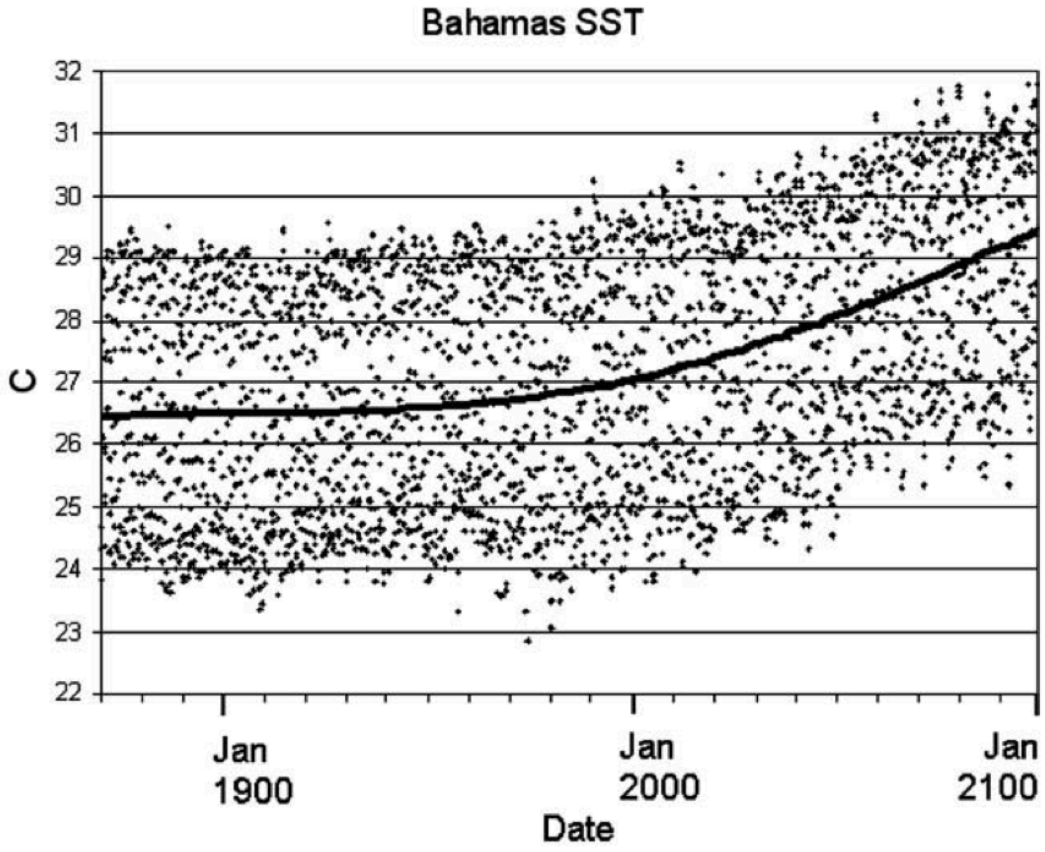


Figure 1.3 Projected rise in sea surface temperature in the Western Atlantic throughout the 21st century. From Sheppard & Rioja-Nieto (2005).

CHAPTER 2. Response to ocean acidification in larvae of a large tropical marine fish, *Rachycentron canadum*[1]

Summary

Currently, ocean acidification is occurring at a faster rate than at any time in the last 300 million years, posing an ecological challenge to marine organisms globally. There is a critical need to understand the effects of acidification on the vulnerable larval stages of marine fishes, as there is potential for large ecological and economic impacts on fish populations and the human economies that rely on them. We expand upon the narrow taxonomic scope found in the literature today, which overlooks many life history characteristics of harvested species, by reporting on the larvae of *Rachycentron canadum* (cobia), a large, highly mobile, pelagic-spawning, widely distributed species with a life history and fishery value contrasting other species studied to date. We raised larval cobia through the first three weeks of ontogeny under conditions of predicted future ocean acidification to determine effects on somatic growth, development, otolith formation, swimming ability, and swimming activity. Cobia exhibited resistance to treatment effects on growth, development, swimming ability, and swimming activity at 800 and 2100 μatm pCO_2. However, these scenarios resulted in a significant increase in otolith size (up to 25 % larger area) at the lowest pCO_2 levels reported to date, as well as the first report of significantly wider daily otolith growth increments. When raised under more extreme scenarios of 3500 μatm and 5400 μatm pCO_2, cobia exhibited significantly reduced size-at-age (up to 25 % smaller) and a 2-3 d developmental delay. The robust nature of cobia may be due to the naturally variable environmental conditions this species currently encounters throughout ontogeny in coastal environments, which may lead to an increased acclimatization ability even during long-term exposure to stressors.

1. Bignami S, Sponaugle S, Cowen RK (2013) Glob Change Biol 19:996-1006, reprinted with permission from John Wiley & Sons, Inc. License #3112101086158

Background

Since the onset of the industrial revolution, anthropogenic emissions have increased atmospheric CO_2 concentration from 280 ppm to 390 ppm, reaching levels which are higher than at any point in the past 800,000 years (Lüthi et al. 2008). Approximately 25% of anthropogenic CO_2 has been absorbed by the world's oceans (Sabine et al. 2004), where it is hydrated to form carbonic acid, which dissociates into bicarbonate and carbonate, releasing H^+ ions and thereby reducing ocean pH. This process of ocean acidification has already reduced ocean pH by 0.1 units since pre-industrial times (Meehl et al. 2007), and is one of the many aspects of global environmental change that pose a threat to marine ecosystems (Royal Society 2005). Under the Intergovernmental Panel on Climate Change (IPCC) SRES-A2 scenario, it is predicted that by the end of the 21st century, atmospheric CO_2 concentration will reach nearly 1000 ppm, corresponding to an surface ocean pH reduction of ~ 0.45 units, which is greater than a 100 % increase in seawater $[H^+]$ (Caldeira & Wickett 2005, Meehl et al. 2007). Over the next three centuries, unchecked increase in anthropogenic CO_2 could result in an atmospheric CO_2 concentration of nearly 2000 ppm— driving ocean pH down ~ 0.77 units at a rate of change faster than any experienced in the last 300 million years (Caldeira & Wickett 2003, 2005, Honisch et al. 2012). This rapid alteration of the marine environment will inevitably result in biological impacts, the severity and scope of which are unknown.

Many studies have shown that conditions of predicted ocean acidification can negatively influence marine organisms, including coccolithophores (Riebesell et al. 2000), copepods (Mayor et al. 2007), corals (Marubini et al. 2001, Langdon et al. 2003, Manzello et al. 2008), molluscs (Michaelidis et al. 2005, Shirayama & Thornton 2005),

echinoderms (Kurihara & Shirayama 2004, Shirayama & Thornton 2005), and fishes (Michaelidis et al. 2007, Munday et al. 2009a, 2009b, 2011a, 2011b, Dixson et al. 2010, Frommel et al. 2010, 2011, 2012, Nowicki et al. 2011). However, many ocean acidification studies report conflicting results within and among taxonomic groups, thereby challenging the general assumption of susceptibility to this perceived threat for untested organisms (Hendriks et al. 2010). As the effect of ocean acidification on larval and adult fishes has, until recently, received considerably less attention than calcifying invertebrates, fishes were not included among the 59 studies (49 species) analyzed by Hendriks et al. (2010).

The highly vulnerable larval stage of marine fish is a critical component of ontogeny. During this early life history, larvae face high cumulative mortality, driven mainly by subtle changes in growth, predation, and selective pressure (Houde 1989a). Ultimately, successful completion of the larval stage influences stock replenishment, population connectivity, and ecological interactions in the juvenile or adult habitat (Cowen & Sponaugle 2009). Fish larvae typically lack many of the complex physiological characteristics that allow juvenile and adult fishes to acclimatize to changing environmental conditions and are therefore presumably more sensitive to environmental perturbation (McKim 1977, Kikkawa et al. 2003, Munday et al. 2008a). Thus, ocean acidification might pose an ecological threat to fishes (Pankhurst & Munday 2011) and data on the effects of acidification on early stages are necessary for a more complete understanding of the effects on populations.

To date, research on larval fishes has demonstrated a significant reduction in survival at low pH (Kikkawa et al. 2003, 2004, Ishimatsu et al. 2004), and verified that

CO_2-induced acidification produces more negative effects than addition of acid alone (Hayashi et al. 2004, Kikkawa et al. 2004). However, treatments in these studies were based on concentrated CO_2 sequestration scenarios, with extremely low pH values beyond what is projected to occur in the next several centuries. More recently, studies have focused on levels of ocean acidification within IPCC-projected ranges, as well as other modeled scenarios. Similar to the responses of other marine organisms to ocean acidification, recent studies on larval fishes have produced conflicting inter- and intraspecific results. For some species, ocean acidification has detrimental effects on survival, growth (Baumann et al. 2011), tissue health (Frommel et al. 2011), metabolism (Franke & Clemmesen 2011), and behavior (Munday et al. 2009a, Dixson et al. 2010, Devine et al. 2011, Simpson et al. 2011, Ferrari et al. 2012a, 2012b, Domenici et al. 2012). However, some of the very same studies failed to find significant negative effects, or even found positive effects, on growth and development (Munday et al. 2009b, 2011a Franke & Clemmesen 2011, Moran & Støttrup 2011, Frommel et al. 2011, 2012), swimming ability (Munday et al. 2009b), and otolith growth (Franke & Clemmesen 2011, Munday et al. 2011a, Simpson et al. 2011, Frommel et al. 2012). Additional effects, such as enhanced otolith growth (Checkley et al. 2009, Munday et al. 2011b), have been reported, but are of unknown ecological consequence. These variable and sometimes conflicting results highlight the need for the inclusion of study species with a diversity of life history strategies and natural habitats, to further clarify what traits generally correspond to resistance or vulnerability.

In this study, we investigated the impact of ocean acidification on the early life history of cobia (*Rachycentron canadum*). In contrast to many previous study species,

cobia is a highly mobile, broadcast spawning species that inhabits a variety of habitats throughout its life history, ranging from estuarine to pelagic environments (Ditty & Shaw 1992). Cobia exhibit rapid growth rates, with adults attaining sizes > 1.5 m and 60 kg

(Shaffer & Nakamura 1989, Franks & Warren 1999), making it the largest tropical marine fish studied to date. In addition, cobia is a voracious predators of crustaceans, fishes, and other benthic and pelagic organisms, and is a popular fishery species for both recreational and commercial harvest (Shaffer & Nakamura 1989). Our study applied a variety of established, adapted, and novel metrics for identifying effects of CO_2-induced acidification on larval cobia, allowing for direct comparison with previous studies, as well as the development of new techniques.

Our metrics for evaluating treatment effects were chosen because growth and development have a direct relationship to mortality and recruitment magnitude (Anderson 1988, Houde 1989a), as well as for comparison to previous studies (e.g. Munday et al. 2009b). Otolith (ear stone) growth was measured because of contrasting results in previous studies (e.g. Checkley et al. 2009, Franke & Clemmesen 2011), and was expanded to include the analysis of daily otolith growth increments, a method often utilized by larval ecologists to measure environmental influences on early life history traits such as growth, size, and pelagic larval duration, as well as to identify trait-based selective mortality (Sponaugle 2010). Critical swimming speed (U_{crit}), an ecologically relevant measure of swimming ability (Plaut 2001), was measured to enable comparison with previously studied species (i.e. Munday et al. 2009b) and due to its direct relevance to the dispersal potential and settlement of fishes in the wild (Fisher 2005). Multiple metrics of routine swimming activity and response to olfactory stimulation were included

to examine the behavioral impacts of acidification on larval cobia, provide comparison to previous studies (e.g. Munday et al. 2009a, Dixson et al. 2010, Ferrari et al. 2012a, 2012b, and others), and provide a way to detect subtle indirect effects on metabolic demand, potential detection by predators, or feeding ability. We hypothesized that over a range of increased-pCO_2/decreased-pH treatment conditions, acidification would negatively affect larval growth, development, and swimming ability, while also altering otolith development and swimming activity.

Materials and methods

Study species

Cobia (*Rachycentron canadum)* has nearly circumglobal distribution in tropical to warm temperate regions, with adults primarily found in coastal and continental shelf waters over a variety of benthic habitats, and juveniles in a range of nearshore environments (Shaffer & Nakamura 1989). In the Gulf of Mexico and Western Atlantic, cobia spawns during the warmer months (25-30°C), releasing pelagic eggs that hatch in approximately 24 hrs at 29°C (Ditty & Shaw 1992). Planktonic larvae hatch at about 3 mm standard length (SL), undergo flexion at 5-10 mm SL, and develop via a gradual transition into the juvenile stage within 30 days, 15-30 mm SL (Shaffer & Nakamura 1989).

Experimental design and rearing

This study investigated the response of larval cobia to a broad range of pCO_2 conditions over the first two to three weeks of ontogeny. Two experiments, EX1 and EX2, were conducted to accommodate multiple pCO_2 scenarios. Treatments during EX1 included ambient water conditions (control) and two increased-pCO_2 treatments targeting

800 and 2000 μatm, based on the SRES-A2 and other modeled scenarios of projected "business as usual" changes in atmospheric CO_2 during the next one to three centuries (Caldeira & Wickett 2003, 2005, Meehl et al. 2007). Treatments during EX2 included ambient (control) and two increased pCO_2 treatments at 3500 and 5400 μatm, providing an experimental comparison to EX1 by targeting more extreme present day and future scenarios of acidification in coastal environments (Melzner et al. 2012) as well as projected scenarios of CO_2 production over the next several centuries (Caldeira & Wickett 2005).

Cobia eggs and larvae for these experiments were produced at the University of Miami Experimental Hatchery (UMEH) from a population of 10 F1-generation broodstock (six female, four male). The UMEH broodstock was raised from two wild-caught broodstock populations and maintained using established husbandry methods (Benetti et al. 2008a). Methodology for larval rearing was adapted from Benetti et al. (2008b). Briefly, naturally spawned eggs were collected within 12 h of fertilization and stocked into 1000-L flow-through incubator tanks at an approximate density of 400 eggs L^{-1}. Eggs immediately received a 100 ppm formalin sterilization treatment for 1 h, followed by rapid flushing of seawater. Larvae hatched in approximately 24 h and developed in incubators under ambient water conditions until 2 d post hatch (dph), at which point they were transferred into experimental rearing tanks.

Rearing was conducted outdoors under 95% shade cloth in 12 (EX1) and 9 (EX2) replicated 400-L flow-through experimental tanks. Tanks received filtered, UV-sterilized seawater at a daily exchange rate of 300 %, were aerated with a small amount (approx. 1 L min^{-1}) of bubbled air, and were partially submerged within a large water bath to

maintain minimal temperature differences (within ±0.3 °C) among replicate tanks. Tanks were fitted with translucent white polyethylene lids to prevent CO_2 off-gassing, intrusion of rain water, or introduction of contaminants. At 2 dph, larvae were stocked into experimental tanks at a density of 9-10 larvae L^{-1}, calculated volumetrically by counting larvae in five 2-L subsamples from the incubator tank immediately prior to removal. Treatments were applied upon stocking, reaching full effect within 24 h. Larvae were fed enriched rotifers at total densities of 5 - 10 mL^{-1} d^{-1} between 2 dph and 12 dph, gradually increasing with age. Beginning at 8 dph, larvae were also provided enriched *Artemia* nauplii at total densities of 0.25 - 2 mL^{-1} d^{-1}, gradually increasing with age (Benetti et al. 2008b). Rearing continued until 22 dph (EX1) and 14 dph (EX2).

Water chemistry manipulation and measurements

Increased CO_2 treatments were applied by addition of equimolar HCl and $NaHCO_3$ solutions directly into seawater plumbing via peristaltic pump, simulating the same water conditions as direct CO_2 bubbling (Gattuso 2010). Tank pH was monitored daily using a handheld pH meter (pH11, Oakton) and Ross Electrode (Orion 9102BWNP, Thermo Scientific) calibrated daily with TRIS buffer. Water samples were taken every 5 d, collected in 250 mL PET bottles and fixed with 100 μL of saturated mercuric chloride. Total alkalinity (TA) and pH_T were measured using automated Gran titration, with accuracy comparisons performed using certified reference material provided by A. Dickson (Scripps Institution of Oceanography; Langdon et al. 2000), and pCO_2 was calculated in CO2SYS (Lewis & Wallace 1998). Tank temperatures and dissolved oxygen were measured with a combination meter (550A, YSI incorporated) twice and

once d^{-1}, respectively, and salinity was measured once daily using a refractometer (RHS-10 ATC, Premium Aquatics).

Larval size, development, and mortality

Subsamples of 10 - 30 larvae were removed from each replicate tank throughout ontogeny, with sampling days targeting specific developmental stages: pre-flexion, the several-day progression through flexion, and post-flexion (EX1: 2, 5, 8-12, 15, 17, 22 dph, EX2: 2, 6-10, 14 dph). Larvae were stored in 95% ethanol for a minimum of two weeks, then a digital image of each larva was obtained, from which standard length (SL) was measured to the nearest 0.1 mm using the software ImageProPlus (v7.0, MediaCybernetics). The completion of the pre-flexion stage, defined here as the initiation of flexion, was qualitatively assessed according to notochord position and development of caudal fin rays. Upon cessation of each experiment, total survivors were enumerated and daily mortality rate, z, was calculated for each tank using the equation $N_t=N_oe^{-zt}$, where N_o represents the population size at time zero (starting population minus sampled larvae, approx. 3600) and N_t represents mean population size after t days.

Otolith analysis

During EX1, left and right sagittal and lapillar otoliths were dissected from 10 larvae per replicate tank, stored in medium viscosity immersion oil, and imaged sulcus side down under a total magnification of 400 x. Using ImageProPlus (v7.0), each otolith was digitally outlined according to pixel contrast and measurement data were collected for dimensions including area, length, width, rectangularity, and roundness (otolith area divided by the area of the smallest rectangle able to contain it, and otolith perimeter divided by the circumference of the smallest circle able to contain it, respectively;

Munday et al. 2011a, 2011b). Otolith dimensions were scaled according to the SL of each larva. The widths of the first seven daily otolith growth increments from one sagittal otolith per larva were measured and analyzed for comparison of early larval growth between young larvae at 8 dph and surviving larvae at 22 dph (e.g. Rankin & Sponaugle 2011).

Swimming ability and activity:

Critical swimming speed was measured for post-flexion fish (17 and 22 dph) during EX1, using a six lane swimming flume similar to that described by Munday et al. (2009b). Prior to the first daily feeding, subsamples of six larvae were removed from each replicate tank and held in 1-L containers to allow acclimatization to flume water conditions for at least 1 h. Subsequently, individual larvae were placed in each flume lane and allowed to acclimatize to the flume for 5 min at a current speed of 2 cm s^{-1}. Due to logistical constraints, larvae from all treatments were tested under control (ambient) water conditions; water condition in the swim chamber has not been shown to affect swimming ability in other species (Munday et al. 2009b). U_{crit} was measured using methodology modified from Stobutzki & Bellwood (1997), with water current speed increased by 3 cm s^{-1} at 2-min intervals, until larvae failed to maintain position. Flume calibration was verified using dye immediately before and after each day of testing, and U_{crit} speeds were calculated in body lengths s^{-1} using the equation $U_{crit} = V_p + ((t_f / t_i) \times V_i)$, where V_p is the penultimate velocity increment successfully completed for the full time interval (t_i), prior to the velocity increment at which failure occurred (V_i) at a failure time (t_f) less than the full time interval (Hammer 1995).

During EX1, routine and olfactory-stimulated swimming activity were observed for pre-flexion (9 dph) larvae in a 15 cm diameter observation container. Prior to the first daily feeding, three subsamples of five fish each were removed from each replicate tank and allowed to acclimatize to the observation container for at least 1 h. Following acclimatization, each container of five larvae was placed individually inside an enclosed observation chamber, consisting of an opaque PVC housing with translucent white lid and clear acrylic shelf, and allowed to recover for 2 min. Routine swimming activity was subsequently recorded for 2 min using a low-light video camera (Hi-Res EXvision, Super Circuits) set 40 cm beneath the larvae. Directly following routine swimming observation, a pipette was used to gently add 1 ml of food-scented water (30 μm filtered rotifer culture water) to the center of the observation dish as an olfactory stimulant. Swimming activity in response to olfactory stimulation was then recorded for an additional 2 min, with the previous routine swimming used as a reference. Video observations were converted to digital files, reduced to 2 frames per second, and analyzed with the software ImageJ (v1.46p, National Institutes of Health) using the MTrackJ plug-in (Meijering et al. 2012). Individual larvae were tracked over the entire 2-min routine swimming and subsequent 2-min olfactory stimulation observation periods. Swimming track data were analyzed for total length, mean and maximum swimming speed, average angle change between 0.5-s observation points, and net-to-gross displacement (del Carmen Alvarez & Fuiman 2005). To capture periods of active swimming, and because larvae were sometimes stationary for extended periods of time, analyses of average angle change and net-to-gross displacement were performed on one randomly selected 20-s period of active swimming per larva (defined as a minimum of 5 cm gross distance traveled in 20 s).

Data analysis

Data were analyzed using tank means calculated from subsampled fish, following verification of the assumption of normality and homoscedasticity using Shapiro-Wilk and Bartlett tests. All analysis of variance (ANOVA) and Tukey HSD procedures were performed in the statistical program R (v2.12), except for ANOVAs on otolith shape, which were performed along with t-tests and analysis of covariance (ANCOVA) procedures in SYSTAT (v11, SYSTAT Software Inc.). Size-at-age, proportion of larvae having initiated flexion, mortality rate, otolith shape, between-age-group differences in otolith increment width, U_{crit}, and swimming activity data (e.g. track length, mean speed, etc.) were analyzed using ANOVA, with each respective measurement as the response variable and pCO_2 as a fixed factor. Transformations were applied to data for proportion of larvae having initiated flexion (arcsine), mean and maximum swimming speed (square root), and swimming track length (log), to correct for non-normal distributions prior to ANOVA procedures. Within-age-group otolith growth increment widths were compared between control and high-pCO_2 treatments using pooled two-sample t-tests, while otolith size data (e.g. length, width, and area) were tested using ANCOVA procedures, with each size measurement as a response variable, pCO_2 as a fixed factor, and standard length as a continuous covariate. Results were considered significant at $P < 0.05$.

Results

Water chemistry

Calculated average pCO_2 treatments during EX1 were 305, 796, and 2123 μatm (hereafter referred to as 300 μatm or control, 800, and 2100 μatm; Table 2.1). During EX2, calculated average pCO_2 treatments were 533, 3471, and 5438 μatm (hereafter

referred to as 500 μatm or control, 3500, and 5400 μatm). Although temperature varied by up to 1.5 °C diurnally, all tanks remained within ± 0.3 °C of each other at all times and mean temperature was consistent at ~ 27 °C and ~ 30 °C throughout the duration of EX1 and EX2, respectively (Table 2.1). TA levels were consistent between control and treatment tanks (Table 2.1), dissolved oxygen remained > 90% saturated, and salinity ranged from 34 to 37 throughout EX1 and EX2.

Larval size, development, and mortality

Throughout all sampling days of EX1, the SL of larvae raised at 800 and 2100 μatm pCO_2 did not differ significantly from larvae in control conditions (ANOVA, all $P > 0.05$, Fig. 2.1a). In contrast, larvae raised at 3500 and 5400 μatm pCO_2 during EX2 were significantly smaller at-age than control larvae on most days from 6 to 9 dph, compared to controls (Fig. 2.1b, Table 2.2). Developmental progression, quantified as the proportion of larvae that had initiated flexion, had comparable results to larval size. During EX1, flexion began at 9 dph and nearly all fish had initiated flexion by 12 dph, with no significant differences between treatments in the proportion of fish having initiated flexion on each day (ANOVA, all $P > 0.05$, Fig. 2.2a). During EX2, cobia began to initiate flexion at 7 dph, with a majority of larvae in all treatments having initiated flexion by 10 dph. However, both high-CO_2 treatments had significantly smaller proportions of fish that had initiated flexion between 7 and 9 dph, compared to controls (Fig. 2.2b, Table 2.2). Despite this 2 - 3 d developmental delay, there was no difference in size-at-flexion (ANOVA, $P > 0.05$), with most larvae initiating flexion at about 7.5 mm SL.

Mortality rates during EX1 did not differ among treatments, with mean (± SE) daily rates of 17.8 % ± 3.1, 13.1 % ± 0.7, and 17.7 % ± 3.4 for control, 800, and 2100 μatm pCO_2 treatments, respectively (ANOVA, $P > 0.05$). During EX2 there was a trend of higher mortality with higher pCO_2, however, this trend was not significant (ANOVA, $P = 0.150$). Larvae raised in control conditions experienced a mean (± SE) daily mortality rate of 17.5 % ± 1.8, those at 3500 μatm pCO_2 a rate of 38.1 % ± 6.3 , and those at 5400 μatm pCO_2 a rate of 49.3 % ± 9.2 .

Otolith growth, shape, and increments

During EX1, mean lapillar and sagittal otolith area at 8 dph and 22 dph were significantly larger in larvae from 800 and 2100 μatm pCO_2 treatments than control larvae (Fig. 2.3, Table 2.3). The adjusted mean areas of otoliths from high-CO_2 treatments were 10 - 33 % larger than comparable control otoliths. High-CO_2 treatment larvae also had significantly longer and wider otoliths, although rectangularity and roundness were not affected (Table 2.3).

Within-age-group (i.e. 8 or 22 dph) analysis of the first seven daily growth increments from the sagittal otoliths did not reveal a significant difference in the widths of comparable increments (CI, i.e. same increment day) between control and 800 μatm fish (t-test, $P > 0.05$). However, fish in 2100 μatm pCO_2 had significantly wider CIs than control fish on CI-days 4 and 6 for larvae in the 8 dph age group and CI-days 4-7 for larvae in the 22 dph age group (t-test, all $P < 0.05$, Fig. 2.4). Larvae surviving to 22 dph exhibited a trend of narrower mean increment widths during the first seven days of growth, relative to larvae in the 8 dph age group (Fig. 2.4, broken versus solid lines). The between-age-group difference in mean CI widths was calculated by subtracting 8 dph CI

widths from 22 dph widths within each treatment group, to test for treatment effects on the "selective" removal of slow (narrow width) or fast (wide width) early-life growth rates from tank populations between 8 and 22 dph. ANOVA comparison between treatments did not indicate a significant treatment effect on between-age-group CI widths (Table 2.4).

Swimming ability and behavior

During EX1, mean U_{crit} did not differ significantly among treatments at 17 or 22 dph (ANOVA, all $P > 0.05$, Fig. 2.5), although U_{crit} increased between 17 and 22 dph (significant for control and 800 μatm pCO_2 treatments only: ANOVA, both $P < 0.05$). The magnitude of U_{crit} increases between 17 and 22 dph larvae in each treatment were compared to determine if CO_2 affected the amount U_{crit} increased with age, but did not differ significantly by treatment (ANOVA, $P > 0.05$).

Swimming activity of 9 dph larvae did not differ significantly between treatments in track length, mean and maximum swimming speed, or net-to-gross displacement during routine swimming or olfactory stimulation observations (Table 2.5, light gray cells). Mean angle change during turns decreased significantly for larvae in 800 μatm pCO_2 during olfactory stimulation observations (Table 2.5), indicating that they turned less dramatically between observation points than control fish, however this was not evident for larvae in the 2100 μatm pCO_2 treatment. Olfactory stimulation did not significantly affect swimming activity by any measure within any treatment group (Table 2.5, dark gray cells).

Discussion

Our study demonstrates that larval cobia are capable of exhibiting some resistance to future ocean acidification scenarios. "Business as usual" levels of acidification, predicted to occur during the next several centuries (i.e. EX1), produced significant changes in otolith growth, as well as a marginal effect on swimming activity, while more extreme treatment scenarios in EX2 resulted in significantly decreased size-at-age, developmental delay, and a trend for higher mortality. Our results from EX2 may be indicative of subtle sublethal effects that were less easily detected during EX1. However, when interpreting our results, it should be noted that there are two ways by which we may have inadvertently reduced the susceptibility of the larvae to acidification. First, by delaying the application of treatments until 2 dph, we may have excluded a window of susceptibility during the embryonic and yolk-sac stages of development, which recently has been shown to be important in at least one other species (Baumann et al. 2011). Second, we did not monitor water chemistry conditions in broodstock tanks, and recent research with one species has shown that offspring of adult fish living under high-CO_2 conditions are more resistant to the impacts of acidification (Miller et al. 2012).

The lack of significant effects on size during EX1 are comparable to results reported for larvae exposed to pCO_2 treatments ranging from 450 to > 4500 μatm (Munday et al. 2011a, Frommel et al. 2012), while EX2 is comparable to other studies that report significantly reduced size in larval and juvenile fishes at 780 to 8500 μatm pCO_2 (Moran & Støttrup 2011, Baumann et al. 2011, Miller et al. 2012). Despite the high pCO_2 levels during EX2, our results are only the second to report negative impacts of ocean acidification on growth in a subtropical or tropical fish species. Similar to size-at-

age, a significant developmental delay was only detected during EX2. As this is the first study to utilize this metric for the identification of acidification effects, and because the altered developmental rate was not associated with a change in size-at-flexion, we attribute this effect to the observed reduction in growth rate. Although there was a seasonal difference in temperature during EX1 and EX2, this 3 °C range is narrow compared to the wide range cobia regularly experience in its natural habitats (17 – 32 °C; (Shaffer & Nakamura 1989).

The association of impacts on growth and development with the energy-intensive period of flexion points to increased susceptibility to additional stressors during this period of ontogeny, possibly due to the narrow metabolic scope larvae have while exhibiting rapid growth rates (Pedersen 1997, Cunha et al. 2007). The decreased growth observed in EX2 may be the result of changes in the production or activity of ion transporters used to maintain acid-base balance, thus increasing the metabolic demand (Pörtner et al. 2004, Deigweiher et al. 2008). A similar scenario is proposed for larvae exposed to metabolically high-cost osmoregulatory challenges (Fielder et al. 2005), and increased metabolic rate along with decreased size has recently been observed in larvae of another tropical fish species exposed to acidification (Miller et al. 2012). Even small increases in metabolic demand may result in long-term effects on growth and reproduction (Pörtner et al. 2004), with potentially broader ecological impacts than indicated by our experimental results alone.

During this study, acidification did not influence non-predator mediated mortality, although daily mortality rates in EX2 tended to be higher at higher pCO_2. These results are not unexpected; increased mortality is rarely attributed to acidification in the

scientific literature (except see Baumann et al. 2011, Miller et al. 2012), indicating that the impact of ocean acidification on fish populations may be less likely to be driven by direct increases in mortality rate, and more likely by sublethal effects that influence cumulative mortality within and across ontogenetic stages. For example, newly settled damselfish juveniles raised under acidified conditions experienced up to nine-fold greater *in situ* mortality than normal juveniles (Munday et al. 2010). The growth-mortality hypothesis poses that fishes which grow more slowly, are smaller at-age, or have extended stage-durations will experience higher mortality rates (Anderson 1988). Slight changes in mortality or growth rates of larvae can have large cumulative effects on recruitment potential (Houde 1989a, 1997), which in turn influences population replenishment and connectivity, both of which are central to the ecological function and effective management of marine populations (Cowen & Sponaugle 2009). Thus, it is important to understand the sublethal effects of ocean acidification on fishes, especially those of ecological or economic importance.

We also report the lowest CO_2 concentration (794 μatm) to elicit a significant change in otolith growth for any species of fish studied to date. Furthermore, our results demonstrate that this effect occurs early (8 dph) and persists until at least 22 dph. Enhanced larval otolith growth has previously been identified in two other species at pCO_2 treatments ranging from 993 – 2558 μatm (Checkley et al. 2009, Munday et al. 2011b), but pCO_2 treatments ranging from 600 - 3200 μatm failed to produce such effects in several other species (Munday et al. 2011a, Simpson et al. 2011, Frommel et al. 2012). Increased otolith size has been attributed to the physiological response of fish to high environmental CO_2, which causes an increase in the aragonite saturation state of the

blood (Esbaugh et al. 2012) and presumably the endolymph fluid surrounding the otoliths (Checkley et al. 2009). Variability in the occurrence of enhanced otolith growth may be explained by species-specific differences in the magnitude of their physiological response to increased pCO_2, and may correspond to physiological adaptations to particular environments or behaviors. However, despite these results and the importance of otoliths to the detection of sound, acceleration, and body position, there currently are no ecological impacts attributed to this effect. Simpson et al. (2011) reports that larval auditory response to reef sounds can be affected by increased pCO_2, but this effect has not been linked to otolith growth. Nonetheless, this does not preclude the possibility of other alterations to this complex mechano-sensory organ system, or ecological consequences due to such changes.

Otolith microstructure was analyzed to examine early life history traits that may differ between treatment and control larvae, or between young (i.e. 8 dph) and surviving larvae (i.e. 22 dph). These results confirmed the observed increase in overall otolith size at the scale of daily increments and identified a trend for non-predator mediated selective mortality in preference of slow growth during early ontogeny. Because there were no predators in these experiments, trait-based selective mortality was likely due to energetic or other physiological constraints during ontogeny. Otolith increment analysis is often used by ecologists to identify early life history traits such as growth, size-at-age, larval duration, and condition, which can underlie selective mortality in the ocean (Searcy & Sponaugle 2001, Sponaugle 2010). The direct relationship between otolith growth and somatic growth is critical to the interpretation of such data, thus the decoupling of this relationship has potentially important implications for our study of fish populations in the

future and in present day acidified environments such as fjords (Thomsen et al. 2010) or upwelling zones (Feely et al. 2008).

Our results indicated no direct effect of acidification on the swimming ability of larval cobia at 17 or 22 dph. These results are similar to those found for orange clownfish raised under acidified conditions (Munday et al. 2009b), and suggests that future ocean acidification may not alter the aerobic swimming ability of all larval fishes, despite changes in blood pCO_2, HCO_3^- concentrations, and muscle pH measured in fish at comparable treatment levels (1900 μatm pCO_2; Esbaugh et al. 2012). Swimming activity was also generally not impacted by acidification, an unexpected result considering the ubiquity of behavioral effects reported for several other species (Devine et al. 2011, Simpson et al. 2011, Ferrari et al. 2012a, 2012b, Domenici et al. 2012). However, since our methods failed to induce an olfactory response in any treatment group, the observed lack of effect on this aspect of behavior cannot be interpreted as a form of resistance to acidification, which would have been contrary to observed impacts in other species (Munday et al. 2009a, Dixson et al. 2010).

The wide range of habitats cobia use during its life history may explain this particular species' resistance to acidification. Coral reef and coastal environments are known to have wide natural fluctuations in pH, with reef environments varying diurnally by ~ 0.1 pH units or more, and some extreme tropical coastal locations varying by 0.9 pH units within a month (Hofmann et al. 2011). Fish in these variable environments may be more resistant to ocean acidification than those adapted to less variable environments (Pörtner et al. 2004, Munday et al. 2008a). The use of coastal habitats by young cobia has likely driven the evolution of adaptations which allow for rapid acclimatization to

variable environmental conditions. Baltic cod, although not tropical, is another species that has likely evolved adaptations to the high pCO_2 found in its natural habitat (i.e. fjords), and consequently demonstrates resistance to high levels of acidification (Frommel et al. 2012). In contrast, Atlantic cod, a conspecific not regularly exposed to high pCO_2 in its natural environment, is affected by acidification (Moran & Støttrup 2011, Frommel et al. 2011). Additionally, organisms with high metabolic rates require adequate physiological control of internal acid-base balance, which may correspond to a pre-adapted tolerance of high pCO_2 and thus reduced susceptibility to future ocean acidification (Melzner et al. 2009a). With ocean acidification occurring at a faster rate than at any point in the last 300 million years (Honisch et al. 2012), species that already possess such adaptations may continue to flourish, whereas species not as well equipped may be less successful.

To date, the cumulative body of research has only just begun to tease apart the various physiological and behavioral responses to ocean acidification, let alone the complex effects such responses will have on population, community, and ecosystem dynamics. As a large, broadcast-spawning, fisheries-targeted, tropical species, cobia possesses a combination of traits not previously examined in other acidification studies. With respect to the metrics used here, our study demonstrates that cobia is unlikely to experience a strong negative impact from CO_2-induced acidification predicted to occur within the next several centuries. However, this does not preclude the possibility of important sublethal impacts that were not measured, or may not have been detected, during our investigation. Our results provide some general insight into the potential resistance to ocean acidification by fishes adapted to variable environmental conditions, and advance our

understanding of the impacts of ocean acidification on tropical fish species of ecological, economic, and management interest.

Table 2.1 Temperature, pH, and total alkalinity (TA) measured during Experiments 1 and 2 (EX1 and EX2), and mean pCO_2 calculated with the software CO2SYS (Lewis & Wallace 1998). Values are means (± SE).

Experiment	**Treatment level** μatm pCO_2	**Temperature** °C	**pH** total scale	**TA** μmol kg^{-1}	**pCO_2** μatm
EX1	300 (control)	27.0 (± 0.1)	8.13 (± 0.01)	2291 (± 32)	305 (± 8)
	800	26.9 (± 0.1)	7.79 (± 0.02)	2291 (± 37)	796 (± 37)
	2100	27.0 (± 0.1)	7.40 (± 0.03)	2285 (± 34)	2123 (± 113)
EX2	500 (control)	29.7 (± 0.1)	7.95 (± 0.001)	2382 (± 22)	533 (± 3)
	3500	29.7 (± 0.1)	7.22 (± 0.02)	2372 (± 21)	3471 (± 187)
	5400	29.8 (± 0.1)	7.04 (± 0.03)	2363 (± 21)	5438 (± 381)

Table 2.2 Significant effects of acidification treatments on size, development, and otolith formation in larval cobia over several days post hatch (dph). Results are from analysis of variance (ANOVA) during Experiment 1 (EX1) and analysis of covariance (ANCOVA) during Experiment 2 (EX2).

Experiment	Test	Metric	dph	F-value	P-value
EX2	ANOVA	Size-at-age	6	8.97	0.020
			7	7.92	0.026
			8	15.88	0.005
			9	6.06	0.043
		Proportion flexed	7	13.02	0.009
			8	15.05	0.006
			9	6.10	0.043
EX1	ANCOVA	Lapillar otolith area	8	55.064	< 0.001
			22	47.190	< 0.001
		Sagittal otolith area	8	29.767	< 0.001
			22	31.458	< 0.001

* ANOVA: $F_{1,7}$, ANCOVA: $F_{2,8}$

Table 2.3 Results for size and shape analysis of lapillar (L) and sagittal (S) otoliths (Oto.) extracted from 8 and 22-day post hatch (dph) larvae during Experiment 1. F and P-values are from analysis of covariance of otolith length and width, and analysis of variance of otolith rectangularity and roundness. Tukey results indicate treatment effects tested among larvae from control (c), 800 μatm (1), and 2100 μatm (2) pCO_2 treatments. N = 4 per treatment.

Metric	dph	Oto.	F-value	P-value	Tukey
Length	8	L	56.40	< 0.001	c < 1 < 2
		S	30.84	< 0.001	c < 1 = 2
	22	L	28.76	< 0.001	c < 1 = 2
		S	27.90	< 0.001	c < 1 = 2
Width	8	L	59.16	< 0.001	c < 1 < 2
		S	6.85	0.018	c = 2 < 1
	22	L	29.41	< 0.001	c < 1 < 2
		S	28.28	< 0.001	c < 1 < 2
Rectan-gularity	8	L	0.38	0.696	c = 1 = 2
		S	1.29	0.323	c = 1 = 2
	22	L	1.18	0.350	c = 1 = 2
		S	0.04	0.964	c = 1 = 2
Round-ness	8	L	0.05	0.955	c = 1 = 2
		S	0.99	0.407	c = 1 = 2
	22	L	2.55	0.133	c = 1 = 2
		S	2.95	0.010	c = 1 = 2

*Length & width: ANCOVA, $F_{2,8}$, Rect. & round.: ANOVA: $F_{2,9}$

Table 2.4 Analysis of variance (ANOVA) results comparing the difference in mean otolith comparable increment (CI) widths between age groups (8 and 22 days post hatch; dph) in larval cobia from Experiment 1. Width-differences were calculated by subtracting 8 dph CI widths from 22 dph CI widths. Thus, negative values represent narrower increment widths in otoliths from larvae surviving until 22 dph. CI width-differences were analyzed by ANOVA to test for treatment effects on the magnitude of selective removal of slow (narrow width) or fast (wide width) early-life growth rates from tank populations between 8 and 22 dph. Values are means (± SE).

CI	Width-difference between age-groups (µm)			Width-difference ANOVA p-value
	control	800 µatm	2100 µatm	
1	-0.448 (± 0.175)	-0.268 (± 0.327)	-0.698 (± 0.319)	0.662
2	-0.662 (± 0.111)	-0.395 (± 0.107)	-1.080 (± 0.387)	0.499
3	-0.606 (± 0.095)	-1.048 (± 0.278)	-0.949 (± 0.298)	0.206
4	-0.525 (± 0.225)	-0.918 (± 0.505)	-0.606 (± 0.274)	0.433
5	-0.399 (± 0.142)	-0.649 (± 0.135)	-0.272 (± 0.291)	0.403
6	-0.350 (± 0.190)	-0.316 (± 0.267)	-0.520 (± 0.114)	0.904
7	-0.616 (± 0.130)	-0.419 (± 0.337)	-0.333 (± 0.258)	0.586

*ANOVA: $F_{1,10}$

Table 2.5 Results and analysis of 9-day post hatch larval cobia swimming activity observations from Experiment 1, including within-treatment/between-observation type analysis of variance (ANOVA; vertical comparisons, dark gray cells) and between-treatment/within-observation type ANOVA (horizontal comparisons, light gray cells). Track length, mean speed, and maximum speed were measured during the entire 2 min observation period, while angle change and net-to-gross displacement were measured during a randomly selected period of active swimming. Values are means (± SE).

	Metric	Observation type	control	800 µatm	2100 µatm	ANOVA p-value
Throughout 2 min observation period	Mean track length (cm)	Routine	42.27 (± 4.42)	39.27 (± 2.18)	40.15 (± 3.15)	0.958
		Olfactory stim.	41.35 (± 1.02)	39.31 (± 2.07)	34.94 (± 3.50)	0.725
		ANOVA p-value	0.985	0.828	0.397	
	Mean speed (cm s^{-1})	Routine	0.35 (± 0.04)	0.327 (± 0.02)	0.34 (± 0.03)	0.655
		Olfactory stim.	0.35 (± 0.01)	0.328 (± 0.02)	0.29 (± 0.03)	0.613
		ANOVA p-value	0.886	0.903	0.323	
	Maximum speed (cm s^{-1})	Routine	3.79 (± 0.44)	3.39 (± 0.18)	3.70 (± 0.16)	0.464
		Olfactory stim.	4.00 (± 0.24)	3.47 (± 0.34)	3.43 (± 0.58)	0.396
		ANOVA p-value	0.600	0.902	0.684	
During 20 s of active swimming	Mean angle change (deg)	Routine	55.64 (± 2.19)	45.77 (± 4.33)	48.09 (± 3.44)	0.062
		Olfactory stim.	56.44 (± 3.21)	40.52 (± 3.90)	45.69 (± 5.23)	**0.019**
		ANOVA p-value	0.843	0.403	0.714	
	Net-to-gross displacement	Routine	0.32 (± 0.02)	0.34 (± 0.02)	0.32 (± 0.03)	0.503
		Olfactory stim.	0.38 (± 0.03)	0.32 (± 0.05)	0.41 (± 0.04)	0.337
		ANOVA p-value	0.131	0.733	0.121	

*within-treatment/between-observation type (dark gray) ANOVA: $F_{1,6}$, within-observation type/between-treatment (light gray) ANOVA: $F_{1,10}$

Figure 2.1 Mean size-at-age (± SE) of cobia larvae throughout the duration of (**a**) Experiment 1 (EX1) and (**b**) Experiment 2 (EX2). Bars within each day post hatch (dph) that do not share a letter are significantly different (Tukey HSD, $P < 0.05$; $N = 4$ and 3 per treatment for EX1 and EX2, respectively). See Online Supplementary Information Table 2.3 for significant ANOVA analysis results. Low survival in some replicate tanks near the end of EX2 restricted our analysis to two 5400 μatm pCO_2 replicate tanks at 10 dph, and two 3500 μatm pCO_2 replicate tanks with the exclusion of 5400 μatm pCO_2 at 14 dph.

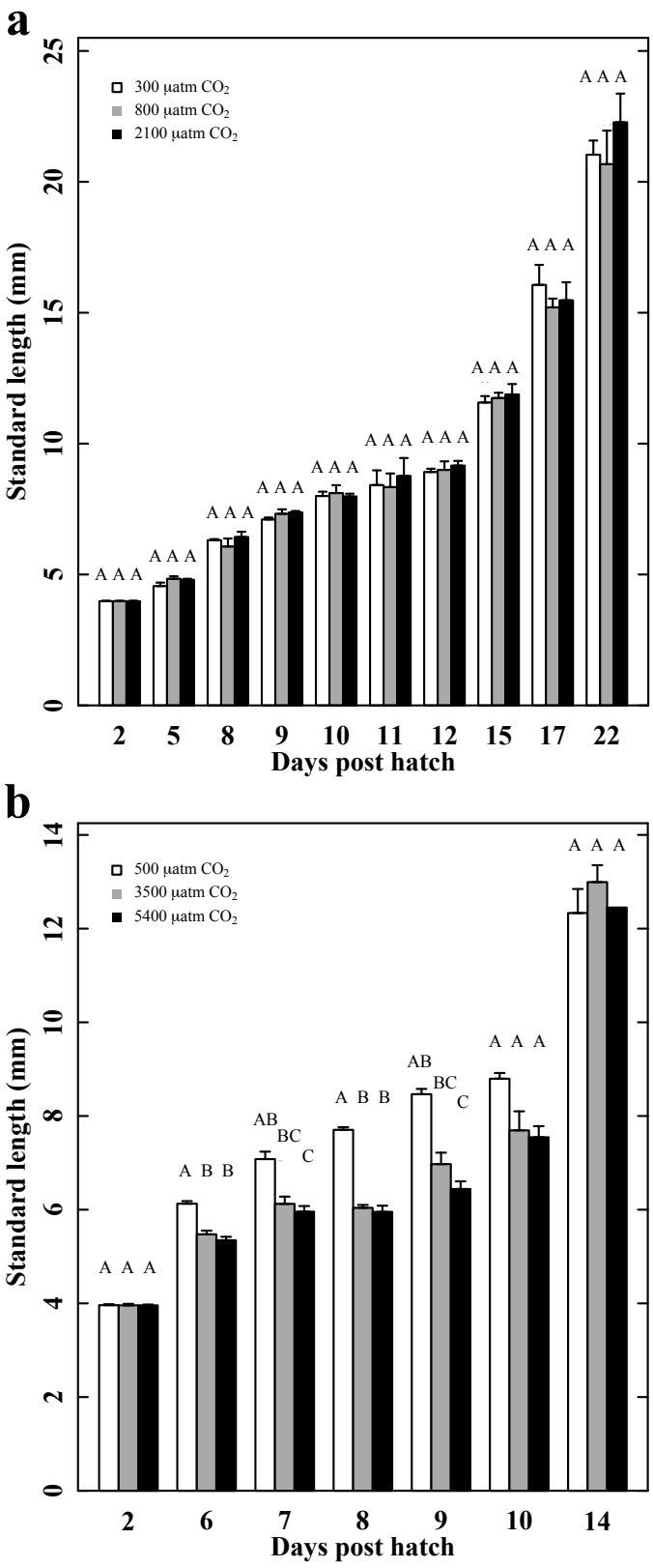
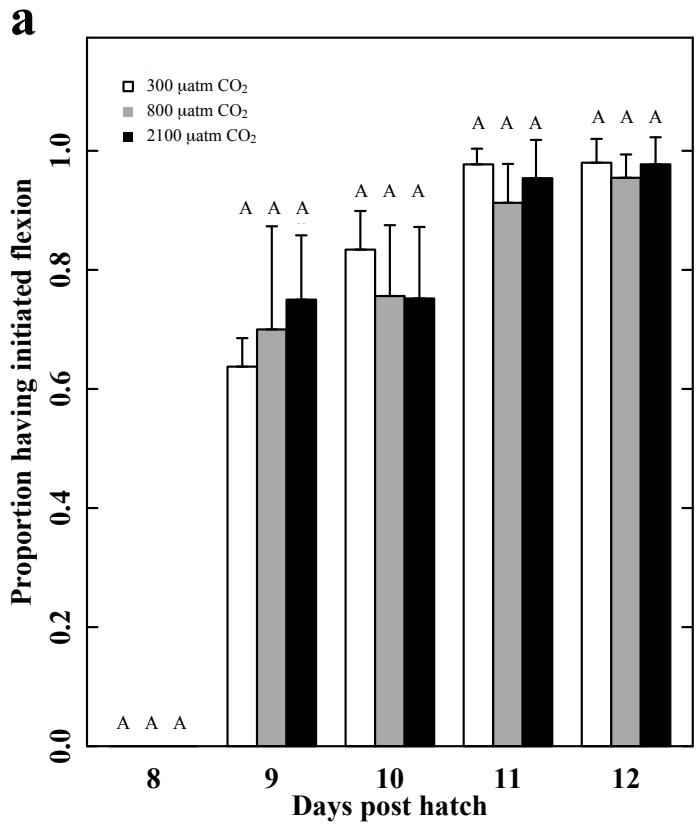
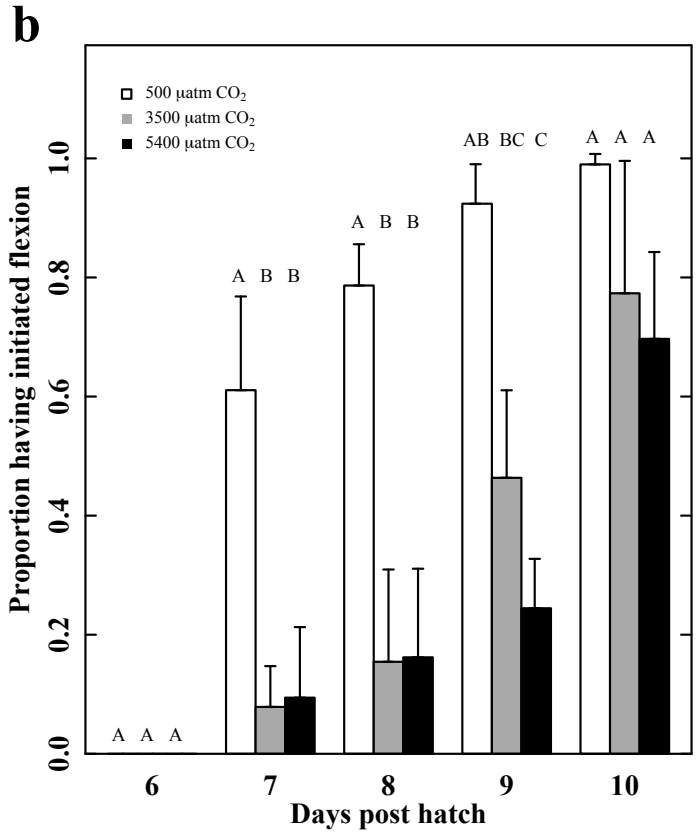

a
300 µatm CO2
800 µatm CO2
2100 µatm CO2
Standard length (mm)
Days post hatch
A A A
b
500 µatm CO2
3500 µatm CO2
5400 µatm CO2
Standard length (mm)
Days post hatch
A B B
AB
BC
C

Figure 2.2 Mean proportion of cobia larvae having initiated flexion (± SD) during (**a**) Experiment 1 (EX1) and (**b**) Experiment 2 (EX2). No larvae began to initiate flexion prior to 9 and 7 dph in EX1 and EX2, respectively. Bars within each dph that do not share a letter are significantly different (Tukey HSD, $P < 0.05$; $N = 4$ and 3 per treatment for EX1 and EX2, respectively). See Table 2.3 for significant statistical results. Low survival in some replicate tanks near the end of EX2 restricted our analysis to two 5400 μatm pCO_2 replicate tanks at 10 dph.


a
300 µatm CO2
800 µatm CO2
2100 µatm CO2
Proportion having initiated flexion
0.0
0.2
0.4
0.6
0.8
1.0
A A A
A A A
A A A
A A A
A A A
8
9
10
11
12
Days post hatch


b
500 µatm CO2
3500 µatm CO2
5400 µatm CO2
Proportion having initiated flexion
0.0
0.2
0.4
0.6
0.8
1.0
A A A
A B B
A B B
AB BC C
A A A
6
7
8
9
10
Days post hatch

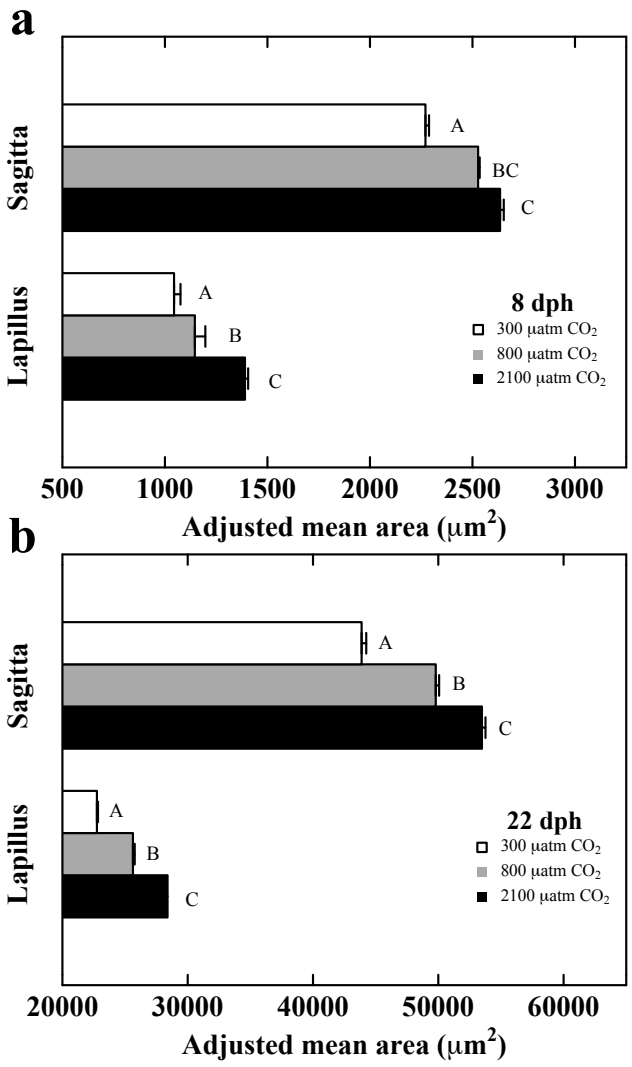


Figure 2.3 Adjusted mean otolith area (± SE) of cobia larvae at (**a**) 8 and (**b**) 22-days post hatch (dph) during Experiment 1. Bars that do not share a letter are significantly different (Tukey HSD, $P < 0.001$, $N = 4$ per treatment). See Table 2.3 for significant statistical results.

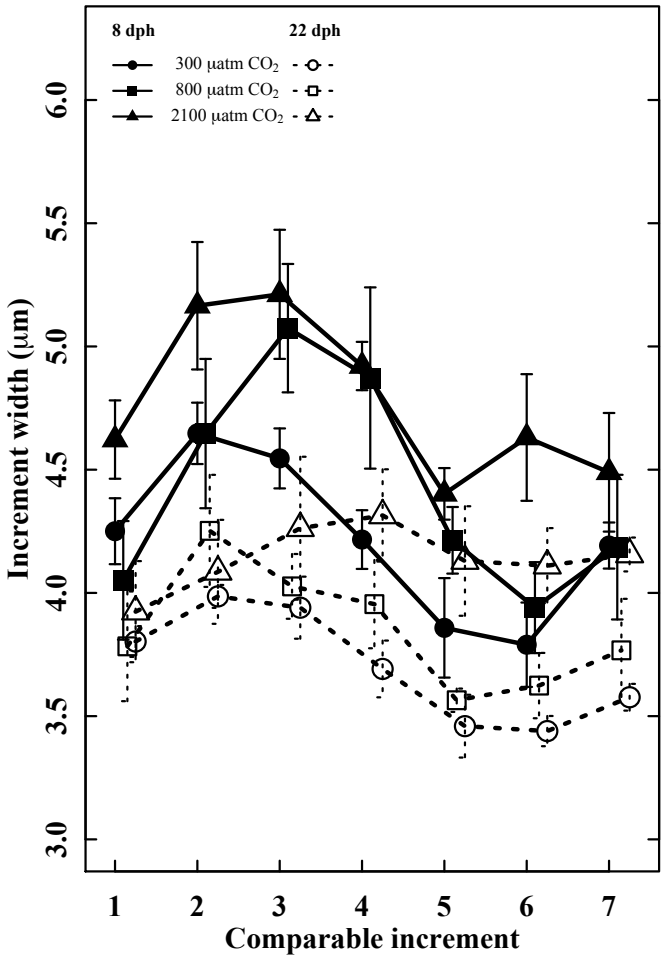


Figure 2.4 Mean width of daily otolith growth increments (± SE) of cobia larvae for the first 7 days post hatch (dph) during Experiment 1. Data from larvae at 8 dph denoted with solid points and lines, surviving larvae at 22 dph denoted with open points and broken lines. Significant differences between 300 and 2100 μatm pCO_2 comparable increment widths denoted by crosses for 8 dph and asterisks for 22 dph (t-test, $P < 0.05$, $N = 4$ per treatment, $df = 6$).

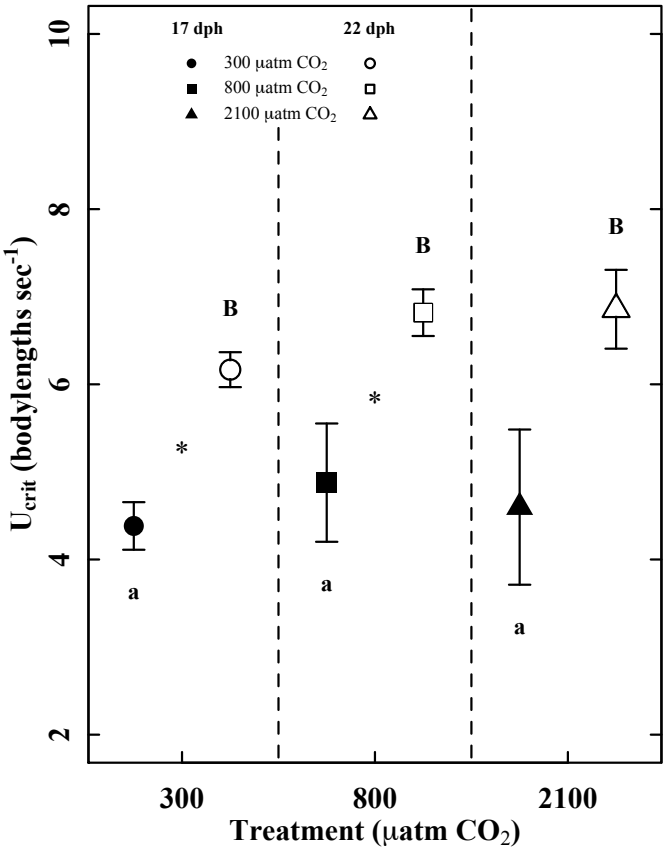


Figure 2.5 Mean critical swimming speed in bodylengths s^{-1} (± SE) of larval cobia during Experiment 1. Comparisons made within age group/between treatments at 17-days post hatch (dph; solid points, lowercase letters) and 22 dph (open points, uppercase letters). Within-age-group data sharing a letter indicates similar U_{crit} (ANOVA, $P > 0.05$). Significant differences in U_{crit} between age groups/within-treatment denoted with an asterisk (ANOVA, $P < 0.05$, $N = 4$ per treatment).

CHAPTER 3. Ocean acidification alters the otoliths of a pan-tropical fish species with implications for sensory function[2]

Summary

Ocean acidification affects a wide diversity of marine organisms and is of particular concern for vulnerable larval stages critical to population replenishment and connectivity. While it is well known that ocean acidification will negatively affect a range of calcareous taxa, the study of fishes is more limited in both depth of understanding and diversity of study species. We utilized new three-dimensional micro-computed tomography to conduct *in situ* analysis of the impact of ocean acidification on otolith (ear stone) size and density of larval cobia (*Rachycentron canadum*), a large, economically important, pan-tropical fish species that shares many life history traits with a diversity of high-value, tropical pelagic fishes. We show that 2100 μatm pCO_2 significantly increased not only otolith size (up to 50% greater volume and 58% greater relative mass) but also otolith density (6% higher). Estimated relative mass in 800 μatm pCO_2 treatments was 14% greater and there was a similar but non-significant trend for otolith size. Using a modeling approach, we demonstrate that these changes could affect auditory sensitivity including a ~50% increase in hearing range at 2100 μatm pCO_2, which may alter the perception of auditory information by larval cobia in a high-CO_2 ocean. Our results indicate that ocean acidification has a graded effect on cobia otoliths, with the potential to substantially influence the dispersal, survival, and recruitment of a pelagic fish species. These results have important implications for population maintenance/replenishment, connectivity, and conservation efforts for other valuable fish stocks that are already being deleteriously impacted by overfishing.

2. Bignami S, Enochs IC, Manzello DP, Sponaugle S, Cowen RK (2013) Ocean acidification alters the otoliths of a pan-tropical fish species with implications for sensory function. P Natl Acad Sci USA doi:10.1073/pnas.1301365110

Background

Present day atmospheric CO_2 concentration is higher than at any point in the past 800,000 years (Lüthi et al. 2008), driving unprecedented anthropogenic ocean acidification in pelagic (Byrne et al. 2010) and coastal environments (Feely et al. 2008). Future climate scenarios project further decline in ocean pH (Caldeira & Wickett 2003, 2005, Meehl et al. 2007) at a rate of change faster than any experienced in the last 300 million years (Honisch et al. 2012). Although ocean acidification is known to influence a diversity of marine organisms (Kroeker et al. 2010), it is a particular concern for vulnerable larval stages critical to population replenishment and connectivity (Cowen & Sponaugle 2009). Recently, the impact of ocean acidification on the larval stages of invertebrate and vertebrate marine species has attracted increased attention, however, experiments on larval fishes raised under projected ocean acidification scenarios have produced mixed results (Munday et al. 2011a, Nilsson et al. 2012). The days to month-long pelagic larval period is an ecologically vital ontogenetic phase in marine fishes because it constitutes the primary mode of dispersal in many species (Cowen & Sponaugle 2009) and represents the life stage most susceptible to mortality (Houde 1997). During this phase, the sensory abilities of larval fishes are important determinants of survival (Montgomery et al. 2006) and ultimately influence the persistence of viable populations. Therefore, the study of ocean acidification impacts on sensory function in fishes is of critical importance to our understanding of the cumulative effect of ocean acidification on fish populations.

To date, ocean acidification impacts on the sensory function of larval fishes have been documented in small, demersal study species through tests of olfactory

discrimination (Munday et al. 2009a, 2010, Dixson et al. 2010), and to a more limited extent, behavioral response to visual (Ferrari et al. 2012a) and auditory stimulus (Simpson et al. 2011). Although otoliths (ear stones) are an important part of the auditory and vestibular sense organs in fishes (Popper et al. 2005), their formation under ocean acidification conditions has received limited attention. Previous studies of larval fish otoliths have identified consistent ocean acidification effects across some species, but have been constrained by the use of two-dimensional measures of size, which limits further analysis of sensory consequences and the ability to examine the full extent of ocean acidification impacts (Checkley et al. 2009, Munday et al. 2011b, Bignami et al. 2013a). With this in mind, we used high-resolution micro-computed tomography (micro-CT) to measure the three-dimensional size and density of otoliths in fish larvae raised under acidified conditions (Fig. 3.1). This approach provided a more complete perspective of ocean acidification impacts on otoliths and enabled modeling of the sensory consequences of those effects.

The study species we utilized, *Rachycentron canadum* (cobia), is one of the largest and most widely distributed tropical species studied to date, and is also of significant ecological and economic value (Shaffer & Nakamura 1989, Fry & Griffiths 2010). It is a eurytopic top predator and the target of recreational and commercial fisheries throughout a nearly circumglobal distribution in the continental shelf waters of tropical to warm temperate regions (Shaffer & Nakamura 1989, Fry & Griffiths 2010). Global fishery landings were approx. 11,000 tons in 2000 and aquaculture production had a global value over USD 36 million in 2004 (Kaiser & Holt 2007). Cobia life history traits are shared by several high-value, pelagic, tropical fishes, thus their use in these experiments provides a

useful perspective of possible ocean acidification impacts to other pelagic species of high ecological and economic value.

Materials and methods

Study species

Cobia (*Rachycentron canadum)* is a highly mobile marine fish that reaches sizes > 1.5 m and over 60 kg (Shaffer & Nakamura 1989, Fry & Griffiths 2010). Pelagic spawning occurs during warmer months (25-30°C) and planktonic larvae hatch at ~3 mm standard length (SL), undergo flexion at 5-10 mm SL, and develop via a gradual transition into the juvenile stage within 30 d at 15-30 mm SL (Shaffer & Nakamura 1989). Cobia eggs and larvae for this experiment were produced at the University of Miami Experimental Hatchery from a population of 10 F1-generation broodstock (six females, four males). Eggs were collected and allowed to hatch and develop until 2 d post hatch (dph), then stocked into 12 replicated 400 L flow-through experimental tanks at a density of 9-10 larvae L^{-1} and raised according to established methods (Benetti et al. 2008b, Bignami et al. 2013a). Treatments were applied upon stocking and reached full effect within 24 h. Larvae were sampled at 22 dph, preserved in 95 % ethanol, and the SL of each larva was measured to the nearest 0.1 mm using digital calipers (M0006, Avenger).

Water chemistry

Treatments represented ocean acidification scenarios for the year 2100 (800 μatm pCO_2; Meehl et al. 2007) and 2300 (2100 μatm pCO_2; Caldeira & Wickett 2005), but also are found presently in fjords (Thomsen et al. 2010) and upwelling zones (Table 3.1; Feely et al. 2008). Seawater carbonate chemistry was manipulated via the addition of equimolar

HCl and $NaHCO_3$ prior to introduction into tanks (Gattuso 2010). Tank pH was monitored daily using a handheld pH meter (pH11, Oakton) and Ross Electrode (Orion 9102BWNP, Thermo Scientific) calibrated daily with TRIS buffer. Water samples were collected every 5 d in 250 mL PET bottles and fixed with 100 µL of saturated mercuric chloride. Total alkalinity (TA) and pH_T were measured using automated Gran titration checked for accuracy with Dickson standards (Scripps Institution of Oceanography). CO2SYS was used to solve the carbonate system using the two measured parameters (pH_T and TA; Lewis & Wallace 1998). Temperature and dissolved oxygen were measured with a combination meter (550A, YSI Inc.) twice and once d^{-1}, respectively, and salinity was measured once daily using a refractometer (RHS-10 ATC, Premium Aquatics). See Table 3.1 for summary water chemistry results.

Micro-CT procedures

Three larvae per tank (12 per treatment) were randomly selected and individually scanned in the micro-CT scanner (Skyscan 1174v2, 13 µm resolution, 0.3° step, 180° total rotation). X-ray attenuation was standardized across scans using hydroxy-apatite bone mineral density (BMD) standards. Two-dimensional x-ray images were reconstructed into three-dimensional image stacks using NRecon (v1.6.6.0, Bruker-microCT) and analyzed using CTan (v1.12.4.3, Bruker-microCT). Grey-scale x-ray images were thresholded to isolate regions of interest (ROIs) containing otoliths. Mean BMD was recorded from within these regions, three-dimensional volumes were interpolated across image stacks using a "shrink-wrap" function, and both volume and surface area were measured. Relative density was determined by comparing the mean X-ray attenuation coefficients from micro-CT scans between control and treatment otoliths.

Mathematical modeling

The mathematical model adapted to simulate otolith displacement is based on an elliptical otolith oscillating in response to a plane sinusoidal wave (Lychakov & Rebane 2000, 2005). Otolith displacement, Δ_x, relative to the closely associated bed of sensory hair cells (macula) is described by

$$\Delta x = a_x\omega^2(m - \rho_e V)\cos[\omega t + \tan^{-1}(-\gamma_x\omega/A)][1/A^2 + (\gamma_x\omega)^2]^{0.5}$$

where

$$A = -k_x + \omega^2(m - \rho_e V + \delta_x)$$

and a_x is amplitude of water particle displacement, ω is angular frequency of the wave ($\omega = 2\pi v$, v is frequency), m is otolith mass in mg, ρ_e is density of the endolymph fluid (assumed to be 1000 mg cm^{-3}), V is otolith volume in cm^3, t is time in seconds, γ_x is a coefficient of friction ($\gamma_x = 0.0029535 \times 10^6 m^{0.6356}$), k_x is a stiffness coefficient of hair cell bundles ($k_x = 21.2652 \times 10^6 m^{0.6356}$), and δ_x is the apparent additional mass of an object moving irregularly in liquid ($\delta_x = 0.0241m$; (Lychakov & Rebane 2000, 2005). Control otolith density was set at a standard value of 2900 mg cm^{-3} (Lychakov & Rebane 2005) and used along with micro-CT volume and relative density data to calculate simulated otolith density and mass.

We designated a water particle displacement amplitude (a_x) of 1.0 nm at 100 Hz as the threshold sound level for control fish. This is reported to be the behavioral threshold for detection of particle motion in some adult fishes without gas bladders (Fay & Megela Simmons 1999), and is a frequency representative of sounds produced by coastal fishes (Fish & Mowbray 1970). This was chosen in contrast to thresholds measured using the pressure component of sound and neuronal response, which may not

accurately describe the detection of particle motion (Fay & Megela Simmons 1999) and can underestimate behavioral response thresholds (Montgomery et al. 2006). Since thresholds have been shown to decrease with age (Kenyon 1996), 1.0 nm is likely a conservative estimate for a larval fish. The otolith displacement magnitude (Δx) needed for auditory detection (hearing threshold) was obtained by calculating control otolith displacement (~0.002 nm) under modeled conditions. Modeling was then repeated with particle displacement reduced to 0.8 nm, where the simulated control otolith no longer reached the hearing threshold but the 2100 μatm pCO_2 treatment otolith did. Hearing range was calculated assuming cylindrical spreading of sound with distance (r) from the source and amplitude decreasing as $1/(\sqrt{r})$ (Montgomery et al. 2006).

Data analysis

Data were analyzed in SYSTAT (v11, SYSTAT Software Inc.) using tank means, following verification of normality and homoscedasticity by Shapiro-Wilk and Bartlett tests. Data from the right or left sagittal and lapillar otoliths were randomly selected from each fish to test volume and surface area using analysis of covariance (ANCOVA), for which surface area or volume was the response variable, pCO_2 a fixed factor, and SL the continuous covariate. Adjusted means of surface area and volume data calculated during ANCOVA were subsequently used to produce SA:V and relative mass data, using mean mass of control treatments as a reference. Relative densities of all otoliths were calculated in reference to mean control treatment otolith density. Larval SL, relative otolith density, SA:V, and relative mass were tested using analysis of variance (ANOVA), with each as the response variable and pCO_2 as a fixed factor. SA:V and relative otolith mass data

were log transformed to correct for lack of homoscedasticity. See Table 3.2 for summary statistics.

Results and discussion

The sagittal and lapillar otoliths of larval cobia raised for 22 d in acidified conditions expected for the years 2100 and 2300 (800 and 2100 μatm pCO_2, respectively; Caldeira & Wickett 2005, Meehl et al. 2007) differed significantly from otoliths of larvae raised under control conditions (300 μatm pCO_2, Table 3.1). Otoliths from larvae raised in seawater at 2100 μatm pCO_2 had significantly greater volume, surface area, and density relative to controls (Fig. 3.2a-c, see Table 3.2 for statistical summary). There was also a significant decrease in the surface area to volume ratio (SA:V) of otoliths in both elevated-CO_2 treatments, as well as a significant increase in the estimated relative mass of sagittal otoliths under both elevated-CO_2 treatments (Fig. 3.2d, e). Otoliths from larvae raised in seawater at 800 μatm pCO_2 exhibited a trend of increased volume and surface area, but these patterns were not significant. Relative density of 800 μatm pCO_2 treatment otoliths was also not significantly different from controls. No treatment effect was detected for the SL of larvae at any treatment level ($P = 0.809$, $n = 4$). These results are the first direct measurements of otolith volume, surface area, and density in larval fish, and expand upon previous reports of increased otolith size measured using two-dimensional microscopy techniques (Checkley et al. 2009, Munday et al. 2011a, 2011b, Bignami et al. 2013a).

Alteration of otolith size, density, and mass has direct impacts on otolith mechanics and influences sensory function (Lychakov & Rebane 2000, 2005, Kondrachuk 2003). To simulate the mechanics of CO_2-altered otoliths, we applied the

size and relative density data from sagittal otoliths in our experiment to a mathematical model of otolith motion in response to an 0.8 nm amplitude sinusoidal acoustic wave (Lychakov & Rebane 2000, 2005). Our simulation demonstrated that when subjected to the same sound stimulus, the estimated CO_2-driven increase in relative otolith mass results in an increased displacement amplitude compared to control otoliths (Fig. 3.3a). Increased otolith displacement amplitude would enable larvae developing in high-CO_2 water to detect sounds that fish in low-CO_2 water cannot detect. For otolith displacement to reach the hearing threshold that was attained by control otoliths in response to a sound amplitude of 1 nm, 800 μatm pCO_2 treatment otoliths required 5 % less sound amplitude (0.95 nm) and 2100 μatm pCO_2 treatment otoliths required nearly 20 % less sound amplitude (0.80 nm). As sound amplitude decreases with distance from the source (Montgomery et al. 2006), heightened auditory sensitivity leads to detection of sounds at a greater distance from the source. We calculated the relative hearing ranges for larval fish with the auditory sensitivities of high-CO_2 (0.80 nm sound amplitude threshold), intermediate-CO_2 (0.95 nm threshold), and control otoliths (1 nm threshold) from our mathematical model assuming cylindrical spreading of sound (Montgomery et al. 2006) and determined that the more massive otoliths from high-CO_2 larvae produced ~50 % greater hearing range compared to control larvae, while otoliths from intermediate-CO_2 larvae produced ~10 % greater hearing range (Fig. 3.3b).

Increased auditory or vestibular sensitivity has important implications for the utilization of these sensory functions by fishes; it could influence a fish's ability to navigate to a desired habitat, detect predators or prey, perceive changes in water turbulence or current speeds, or maintain proper kinesthetic awareness. These changes

would be most relevant near the periphery of hearing ability, such as at distance from a sound source or when otolith displacement amplitude approaches the threshold for detection. Altered sensory ability could prove to be beneficial or detrimental depending on how a fish perceives this increased sensitivity. Improved detection of useful auditory information (e.g., distant nearshore sounds) would be advantageous to navigating coastal fishes, however, increased sensitivity to disruptive background noise (e.g., sea state) may mask useful auditory information. The need for auditory or vestibular sensitivity may also be life history specific. Many bottom dwelling fish species possess large otoliths relative to their body size, which may indicate an ecological need for high auditory and vestibular sensitivity (Lychakov & Rebane 2000). In contrast, highly mobile pelagic species often possess small otoliths relative to their body size, implying less sensitivity (Lychakov & Rebane 2000). Since these traits have likely evolved to suit the particular ecological needs of a species, benthic species may find increased otolith mass advantageous whereas such changes may be detrimental for pelagic species. Of course, it is also conceivable that increased otolith size could impinge upon the closely associated sensory hair cells of the macula and be detrimental to the function of the otolith organ regardless of species. These effects should apply to fishes of all ages, however, younger fish have less sensitive hearing ability (Kenyon 1996) and any sensory advantage or disadvantage during the larval stage could be particularly influential to survival, with cascading effects on recruitment, population connectivity, and stock replenishment. Although ocean acidification is typically considered a future threat, these implications already may influence the dispersal and distribution of fishes currently developing in

high-CO_2 water in habitats such as fjords (Thomsen et al. 2010) and upwelling zones (Feely et al. 2008).

The mechanistic cause of increased otolith size with ocean acidification has not been determined empirically, but has been attributed to the physiological response of fish to high environmental CO_2 (i.e., HCO_3^- retention; Claiborne & Heisler 1986), which likely causes an increase in the aragonite saturation state of the endolymph fluid surrounding the otoliths (Checkley et al. 2009). This physiological mechanism is sustained for the duration of high-CO_2 exposure (Claiborne & Heisler 1986), therefore it can be assumed that effects on otoliths will persist with age. Additionally, ocean acidification is known to alter neurological function in fishes (Nilsson et al. 2012) and there is evidence for neurological control of otolith mineralization (Anken 2006). Therefore, CO_2-induced neurological disruption may indirectly contribute to increased otolith size and density, either by changing the chemical composition of endolymph fluid or by altering neurologically controlled expression of genes which influence the crystalline or lattice structure of otoliths (Anken 2006). The results of either mechanism of change have important implications for the function of otoliths as sense organs, but there are also implications for their use as tools for fisheries biology research and conservation. Fisheries oceanographers and ecologists rely on otoliths to study fisheries stocks and the early life dynamics of fishes, often using the widths of daily otolith increments as a proxy for daily somatic growth (Sponaugle 2009). This method depends on a consistent correlation between otolith growth and somatic growth, but an increase in otolith size without a corresponding increase in somatic growth disrupts this relationship and may confound the use of this technique under variable CO_2 conditions. Also, otoliths

formed in high-CO_2 water may have a different mineralogical composition, thereby interfering with stock identification methodologies such as those using otolith microchemistry analysis (Thorrold & Swearer 2009). Similar to the ecological effects discussed above, present day occurrence of high-CO_2 water in fjords (Thomsen et al. 2010) and upwelling zones (Feely et al. 2008) makes this a current problem, and may already influence the interpretation of data collected using these techniques.

Our results indicate a graded impact of ocean acidification on cobia otoliths, similar to previously reported effects on two-dimensional otolith surface area under identical treatment conditions (Bignami et al. 2013a). This is evident in the end-of-century 800 μatm pCO_2 acidification treatment, where effects on otolith size followed a similar but non-significant trend. This is a potentially optimistic result, indicating some resistance to acidification and suggesting that under near-future scenarios these impacts may be most relevant in habitats already experiencing high pCO_2 levels. However, the trend for larger otoliths with increased CO_2 still produced a ~10 % increase in hearing range and it is not yet clear at what point these effects will become ecologically significant. Empirically, it is also unclear if natural exposure to variable environmental conditions leads to pre-adaptation, and thus resistance, to acidification in fishes (Munday et al. 2008a). Since cobia is eurytopic, inhabiting environments ranging from epipelagic to estuarine waters, this may affect the intensity of their response to ocean acidification whereas species restricted to more constant environments (e.g., entirely pelagic species) may respond differently to similar acidification scenarios.

It is widely accepted that the impact of ocean acidification on marine organisms varies along a gradient from obvious to subtle effects. Our observation of CO_2-induced

increases in otolith size and relative density is an unexpected subtle effect with important implications for the sensory abilities of fishes. Whether these sensory changes are ultimately positive or negative will depend on the species, but they have the potential to influence the survival, dispersal, and recruitment of a diversity of marine fishes, with subsequent population consequences. Because many ecologically and economically important species have characteristics similar to cobia, such population changes are expected to produce substantial ecologic and economic effects. These results contribute to a fuller understanding of the complex suite of direct and indirect ocean acidification effects on fishes as well as the broader ecological and economic consequences that may challenge fishery populations and conservation efforts in the future.

Table 3.1 Summary water chemistry results including temperature, pH, and total alkalinity measured during larval rearing, and mean pCO_2 calculated with the software CO2SYS (Lewis & Wallace 1998). Values are means (±SEM).

	Temperature	**pH**	**TA**	$\boldsymbol{pCO_2}$
Treatment	°C	total scale	mmol kg^{-1}	matm
Control	27.0 (± 0.1)	8.13 (± 0.01)	2291 (± 32)	305 (± 8)
Year 2100	26.9 (± 0.1)	7.79 (± 0.02)	2291 (± 37)	796 (± 37)
Year 2300	27.0 (± 0.1)	7.40 (± 0.03)	2285 (± 34)	2123 (± 113)

Table 3.2 Results of analysis of covariance (ANCOVA) and analysis of variance (ANOVA) of tank mean otolith data. Larval standard length was a continuous covariate in all ANCOVAs, and resulting adjusted mean surface area and volume were utilized in ANOVA procedures for surface area to volume ratio (SA:V) and relative mass. ANOVA procedures for relative density included a single value from each fish, determined across all three otolith pairs (sagittal, lapillar, and asterisci). $n = 4$ per treatment.

Otolith	Test	Variable	F-value	P-value
Lapillus	ANCOVA	Volume	22.16	0.001
		Surface area	22.67	< 0.001
	ANOVA	SA:V	36.82	< 0.001
		Relative mass	34.37	< 0.001
Sagitta	ANCOVA	Volume	21.43	0.001
		Surface area	5.12	0.037
	ANOVA	SA:V	1275.38	< 0.001
		Relative mass	78.76	< 0.001
Combined	ANOVA	Relative density	14.26	0.002

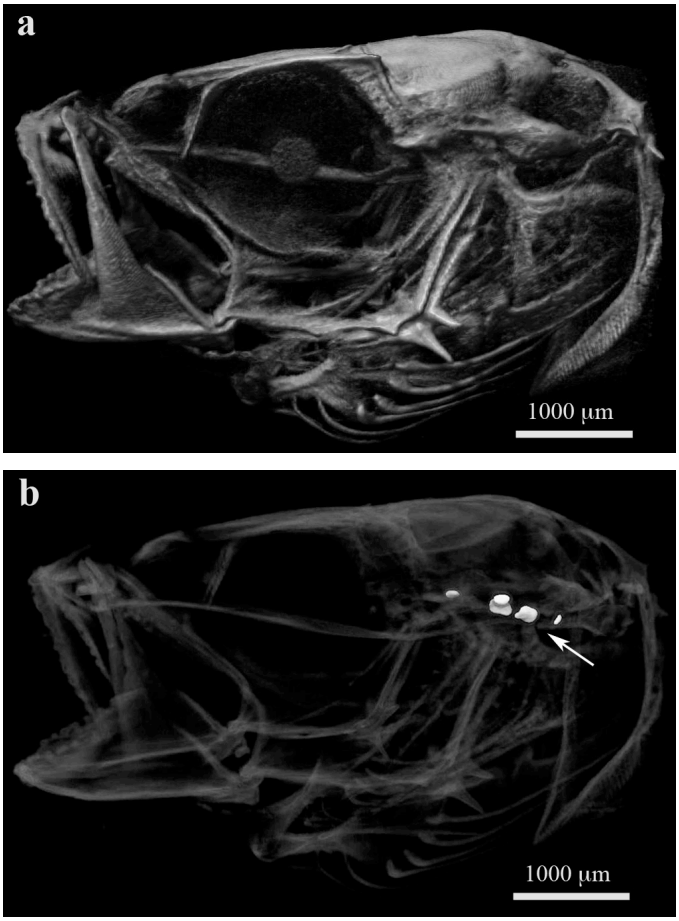
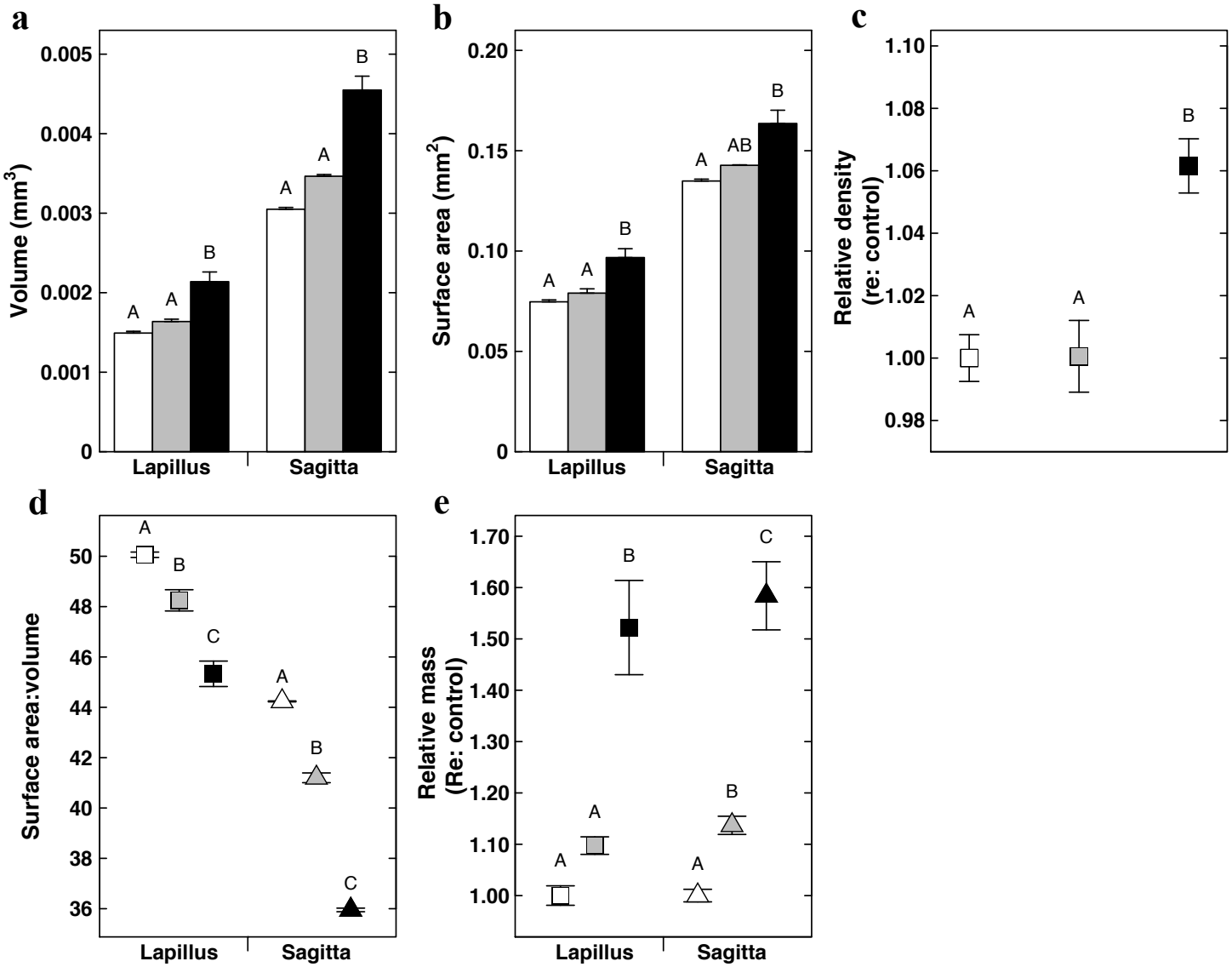


Figure 3.1 Lateral view micro-CT imagery of a 22-day post hatch larval cobia head. Three-dimensional data were filtered to produce imagery of (**a**) the complete skeletal structure of the cobia skull and (**b**) only more dense material, such as otoliths (marked with arrow).

Figure 3.2 Change in larval cobia otoliths as a result of elevated pCO_2. When raised in seawater with 300 μatm, 800 μatm, or 2100 μatm pCO_2 (white, grey, and black bars and symbols, respectively), larvae in the highest CO_2 treatment had lapillar and sagittal otoliths with up to (**a**) 49 % greater volume, (**b**) 37 % greater surface area, (**c**) 6 % greater relative density, (**d**) 19 % lower surface area to volume ratio (SA:V), and (**e**) 58 % greater relative mass. 800 μatm treatment only had a significant effect on otolith SA:V and the relative mass of sagittal otoliths. Within each otolith type, bars or symbols not sharing a letter are significantly different ($P < 0.05$, $n = 4$ per treatment). Values are (**a, b**) adjusted means (±SEM) and (**c-e**) means (±SEM).


a
Volume (mm3)
0.005
0.004
0.003
0.002
0.001
0
A
A
B
A
A
B
Lapillus
Sagitta
b
Surface area (mm2)
0.20
0.15
0.10
0.05
0
A
A
B
A
AB
B
Lapillus
Sagitta
c
Relative density
(re: control)
1.10
1.08
1.06
1.04
1.02
1.00
0.98
A
A
B
d
Surface area:volume
50
48
46
44
42
40
38
36
A
B
C
A
B
C
Lapillus
Sagitta
e
Relative mass
(Re: control)
1.70
1.60
1.50
1.40
1.30
1.20
1.10
1.00
A
A
B
A
B
C
Lapillus
Sagitta

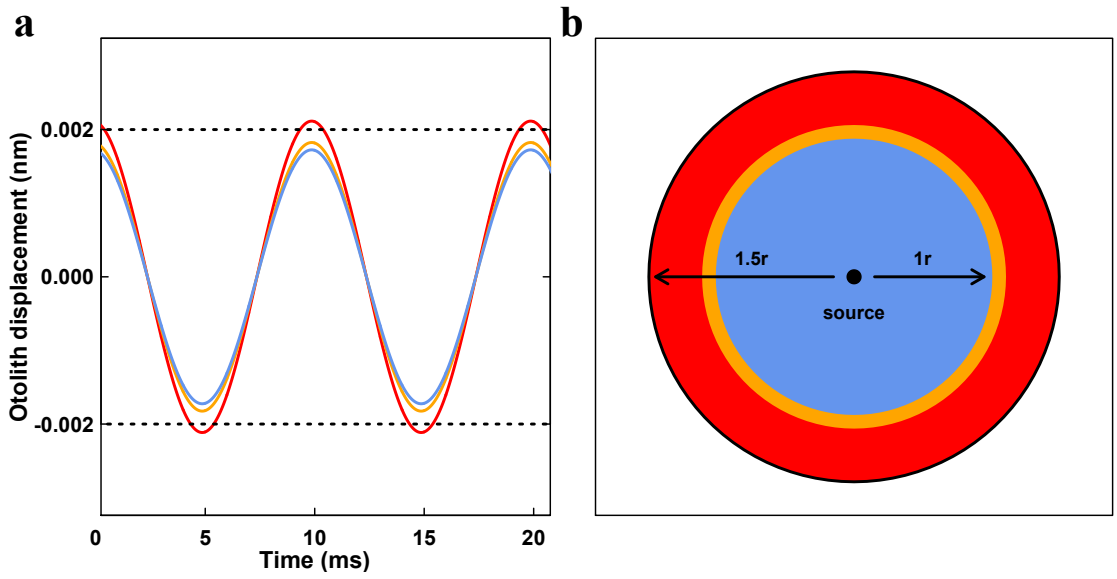


Figure 3.3 Simulated otolith displacement amplitude and hearing range for larval cobia under elevated-pCO_2 conditions. (**a**) When exposed to a simulated 0.8 nm amplitude 100 Hz sound wave, otoliths at 2100 µatm pCO_2 (red line) had greater otolith displacement than those at 800 µatm pCO_2 (orange line) or controls (300 µatm pCO_2, blue line), thereby reaching the hearing threshold (dotted horizontal line) when 800 µatm and control otoliths did not. (**b**) Assuming loss of sound amplitude by cylindrical spreading (13), 300 µatm pCO_2 fish (blue) had hearing range r and 800 µatm pCO_2 fish (orange) had a 10 % greater range, but those at 2100 µatm pCO_2 (red) had 50 % greater range due to the lower sound amplitude necessary for threshold otolith displacement.

CHAPTER 4. Combined effects of ocean acidification, increased temperature, and starvation stress on larvae of a large tropical marine fish[3]

Summary

The combination of ocean acidification and other environmental changes poses an ecological challenge to marine organisms globally. There is particular concern for the larval stages of these organisms, but few studies of larval fishes have investigated multiple stressors. We address this deficit by reporting on two experiments on larval cobia (*Rachycentron canadum*) raised under combined exposure to ocean acidification and increased temperature (CO_2/Temp) or starvation stress (CO_2/Ration). We found few synergistic effects of these combined stressors, but larvae responded to CO_2, temperature, and rationing treatments when tested individually. During CO_2/Temp, larvae exhibited significantly reduced standard length (SL), growth rate, and development rate due to high CO_2 (1700-2100 μatm pCO_2), and increased SL, development rate, and swimming ability due to increased temperature (32 vs. 27 °C). During CO_2/Ration, elevated CO_2 (1700 μatm pCO_2) did not significantly impact most metrics, including larval condition measured by RNA:DNA ratio, and larvae fed a reduced ration (25 % ration) had decreased SL, growth rate, and development rate. However, larvae in high-CO_2 seawater demonstrated lower starvation resistance when subjected to complete feeding cessation, indicating that ocean acidification may increase larval susceptibility to starvation in a naturally patchy marine feeding environment.

Background

Anthropogenic production of CO_2 has driven atmospheric CO_2 concentration to the highest level (390 ppm) experienced in the past 800,000 years (Lüthi et al. 2008), and would be much higher if not for the absorption of approximately 25% of all

3. Bignami S, Sponaugle S, Cowen RK, Hauff M (in review) Combined effects of ocean acidification, increased temperature, and starvation stress on larvae of a large tropical marine fish.

anthropogenic CO_2 into the oceans (Sabine et al. 2004). Consequently, CO_2-induced ocean acidification has reduced average ocean pH by 0.1 units since pre-industrial times and is projected to further decrease pH over the next one to three centuries (Caldeira & Wickett 2005, Meehl et al. 2007) at a rate faster than any experienced in the last 300 million years (Caldeira & Wickett 2003, 2005, Honisch et al. 2012). Climatic changes due to increased atmospheric CO_2, such as increased global temperatures, also produce a multitude of cascading effects on atmospheric, terrestrial, and oceanic systems (IPCC 2007). These changes are of particular concern for the vulnerable larval stages of marine organisms, a critical ontogenetic phase during which most species naturally experience high mortality (Cowen & Sponaugle 2009). For larval fishes, the combined effects of ocean acidification and additional stressors could impact a suite of factors and processes that influence the replenishment and connectivity of fish populations (Pankhurst & Munday 2011).

In tropical and subtropical waters, sea surface temperature (SST) is projected to rise 1 - 3°C by the end of this century (Sheppard & Rioja-Nieto 2005, Angeles et al. 2007). As temperature rises, marine organisms will likely experience an increase in their metabolic demand (Gillooly 2001) and might be forced to endure conditions which fall outside their optimal physiological range (Pörtner 2012). Ocean acidification could further increase the metabolic demand of fishes by challenging the maintenance of internal acid-base balance (Deigweiher et al. 2008), which, in turn, may reduce their tolerance to thermal challenges (Pörtner 2012). For larvae, this increase in metabolic demand coincides with an ontogenetic period of limited metabolic scope (Pedersen 1997,

Cunha et al. 2007) and may result in the reallocation of valuable metabolic resources away from growth, development, or activity, to basic maintenance of homeostasis.

This challenge could also be magnified by a reduction in the availability and quality of prey. As increasing SST drives more intense water column stratification, nutrient availability in surface waters may become limited, resulting in decreased net primary productivity (Behrenfeld et al. 2006) and reduced zooplankton biomass (Roemmich & McGowan 1995). The effect of ocean acidification on primary production is less clear (Guinotte & Fabry 2008). Increased pCO_2 may increase phytoplankton growth rates (Wolf-Gladrow et al. 1999), but lower pH may limit productivity by decreasing the bioavailability of limiting nutrients such as iron (Shi et al. 2010) or interacting synergistically with light stress (Gao et al. 2012). Ocean acidification may also impact the quality of biological production; it was recently demonstrated that increased pCO_2 reduces the nutritional value of producers and their zooplanktonic predators (e.g., copepods; Rossoll et al. 2012), the primary prey of many subtropical larval fishes (e.g., see Llopiz & Cowen 2009). Although starvation stress is likely experienced by larvae in the wild due to the spatial and temporal patchiness of prey (Steele 1978), it is often not well simulated in experimental settings where prey are typically overabundant.

Our understanding of the impact of ocean acidification on larval fishes has improved as studies have identified negative impacts on growth, survival (Baumann et al. 2011), neurological function (Nilsson et al. 2012), and behavior (Munday et al. 2010, Ferrari et al. 2012b), as well as potentially detrimental effects on otolith (ear stone) development (Checkley et al. 2009, Munday et al. 2011b, Bignami et al. 2013a, b) and

tissue health (Frommel et al. 2011). However, there has been little focus on the effects of ocean acidification and additional stressors on larval fishes, which may interact synergistically and have greater cumulative impact. Thus, to better understand how fish in nature may respond to projected climatic conditions it is necessary to examine combinations of these stressors which more accurately simulate the marine environment.

In this study, we utilized two experiments to investigate the response of larval cobia (*Rachycentron canadum*) to combined treatments of ocean acidification and either increased temperature or starvation stress. We hypothesized that increased temperature and acidification would interact synergistically to cause reduced growth and development, as well as altered behavior, and diminished swimming ability. Furthermore, we hypothesized that nutrient limitation combined with ocean acidification would induce a similar interactive response and that larvae subjected to complete feeding cessation would demonstrate decreased time to starvation under acidified conditions. Our metrics for evaluating treatment effects were chosen because growth and development have a direct relationship to mortality and recruitment magnitude (Anderson 1988, Houde 1989a). Swimming activity and olfactory response were included to allow detection of subtle indirect effects on metabolic demand and enable comparison with previous studies (e.g., Dixson et al. 2010, Munday et al. 2010), while critical swimming speed (U_{crit}), an ecologically relevant measure of swimming ability (Plaut 2001), was included due to its direct relevance to the dispersal potential and settlement of fishes in the wild (Fisher 2005). Finally, starvation resistance was evaluated as a way to measure the susceptibility of larval fishes to periods of inadequate food supply in the naturally patchy marine

environment, as well as to serve as an indirect measure of metabolic demand under acidified conditions.

Materials and methods

Experimental design and rearing

We conducted two experiments in which we crossed elevated pCO_2 with increased temperature (CO_2/Temp) and elevated pCO_2 with starvation stress (CO_2/Ration). During CO_2/Temp, we altered water temperature by submerging a 250 watt aquarium heater (EHEIM-JÄGER) into each of six 400 L high temperature experimental tanks or by diverting seawater through polyethylene tubing submerged in a cool water bath prior to delivery to six low temperature tanks. This produced a low temperature treatment near the natural spawning temperature of cobia (~ 27 °C; Stieglitz et al. 2011) and a high temperature treatment near the upper end of the natural range for cobia (~ 32 °C; Shaffer & Nakamura 1989) but within the 1-3 °C range of projected future increase in Western Atlantic and Caribbean summer SST (Sheppard & Rioja-Nieto 2005, Angeles et al. 2007). During CO_2/Ration, tanks received ambient temperature seawater and were partially submerged within a large water bath to maintain minimal temperature differences (within ± 0.1 °C) among replicate tanks.

We chose an elevated CO_2 scenario of approximately 1800 μatm pCO_2 (high-CO_2) for both experiments, according to modeled projections of increased atmospheric CO_2 over the next two centuries (Caldeira & Wickett 2003, 2005) as well as present day conditions in some marine environments (Thomsen et al. 2010, Melzner et al. 2012). Ambient water conditions (ambient-CO_2) served as controls. Treatments were applied and monitored as described by Bignami et al. (2013a). See Appendix 1 for detailed

methods. During CO_2/Temp, ambient pCO_2 of seawater was 200 to 350 μatm higher than desired, likely due to respiration in source water (Biscayne Bay) and within facility plumbing, but was well below high-CO_2 treatment levels. This did not occur during CO_2/Ration. See water chemistry parameters in Table 4.1. We determined that the difference in elevated pCO_2 treatments (~ 1800 vs. ~ 2100 μatm, respectively) was driven entirely by temperature and that both were ~ 1800 μatm when calculated at the same temperature using CO2SYS (Table 4.1; Lewis & Wallace 1998).

We conducted both experiments using larval rearing methodology adapted from Benetti et al. (2008b) and Bignami et al. (2013a). See Appendix 1 for full rearing methods. To ensure adequate survival through first feeding, we followed a standard feeding regiment during CO_2/Ration until 5 days post hatch (dph; four to five feedings of enriched rotifers d^{-1}). Subsequently, reduced ration treatments were fed approximately 25 % of a full ration, applied by reducing prey concentration (i.e., fewer prey during any one feeding) and skipping mid-day feedings (i.e., only fed in morning and evening). Although alternation of feeding days has been used previously to simulate starvation (Green & McCormick 1999), our preliminary trials indicated unacceptably high mortality. Rearing under treatment conditions began at 2 dph and continued until 21 dph (CO_2/Temp) and 14 dph (CO_2/Ration).

Growth and development

We collected subsamples of 5 - 20 larvae from each replicate tank throughout ontogeny and specifically targeted a 5-d period during the transition from pre-flexion through post-flexion. Larvae were stored in 95% ethanol, a digital image of each larva was captured, and standard length (SL) was measured to the nearest 0.1 mm using the

software ImageProPlus (v7.0, MediaCybernetics). Daily growth rates were calculated for the 5-d sampling period during flexion by finding the difference in tank mean SL between each two consecutive days. These four daily rates were averaged to produce the mean within-tank growth rate used for analysis. We qualitatively assessed progression through flexion according to notochord position (i.e., flexing upwards) and development of caudal fin rays, scoring each larva as "pre-flexion" or "undergoing/post-flexion".

Swimming activity and ability

Swimming activity tests were performed at 7 and 9 dph during CO_2/Temp and CO_2/Ration, respectively, according to previously published methods (Bignami et al. 2013a). Briefly, routine swimming activity of larvae was video recorded in an observation dish for 2 min, after which 1 ml of food-scented water (30 μm filtered rotifer culture water) was gently added via pipette as an olfactory stimulant. Swimming activity in response to olfactory stimulation was then recorded for an additional 2 min. Larvae were tracked using the software ImageJ (v1.46p, National Institutes of Health) with the MTrackJ plug-in (Meijering et al. 2012), and data were analyzed for total length, mean and maximum swimming speed, average angle change between 0.5-s observation points, and net-to-gross displacement (del Carmen Alvarez & Fuiman 2005). Analyses of average angle change and net-to-gross displacement were performed on one randomly selected 20-s period of active swimming per larva (defined as a minimum of 5 cm gross distance traveled in 20 s) while other analyses utilized each entire 2 min observation period. We measured U_{crit} during CO_2/Temp for post-flexion fish at 20 dph using a six lane swimming flume similar to that described by Munday et al. (2009b). U_{crit} was measured using methodology from Bignami et al. (2013a), modified from Stobutzki &

Bellwood (1997), with U_{crit} calculated in body lengths s^{-1} according to Hammer (1995). Larvae were tested in their respective CO_2 treatment conditions at a common intermediate temperature (30.5 ±0.5 °C). See Appendix 1 for full swimming activity and ability methods.

Larval condition and starvation resistance

During CO_2/Ration, larval nutritional condition was gauged by measuring RNA:DNA ratio (Buckley et al. 1999). RNA:DNA ratios of individual larvae from a subset of 9-12 larvae sampled from each tank at the outset of rationing treatments (5 dph) and then again at 10 dph. On each of the sampling days, live larvae were removed from rearing tanks at dawn (before feeding) to avoid contamination of samples by larval gut contents. At the time of sampling, individual larvae were placed in 1.5 mL cryovials, immediately flash frozen in liquid nitrogen, and stored at -80°C.

In preparation for RNA:DNA ratio assays, frozen larvae were thawed one at a time, and SL was measured to the nearest 0.01 mm using the stage micrometer on a Leica MZ12 stereomicroscope. The head of each larva was excised with a microscalpel and preserved in 95% EtOH for later otolith extraction, while the trunk was immediately homogenized in 150 µL ice-cold 1M NaCl. Homogenized samples were maintained at -80°C until nucleic acid quantification could be carried out. Individual RNA:DNA ratios were obtained using a fluorometric microplate assay. The methodology followed that of Westerman and Holt (1988) but used the fluorophore SYBR Green II (SGII, Molecular Probes) instead of Ethidium Bromide. After larval homogenates were treated with SGII, fluorescence levels were determined with a Tecan GENios plate reader (ex: 485 nm, em: 535 nm). Fluorescence levels were converted to nucleic acid concentrations using RNA

and DNA standard curves generated from fluorescence measurements of known quantities of type III RNA from baker's yeast (Sigma, R-6750) and type I DNA from calf thymus (Sigma, D-1501). The initial fluorescence measurement of each sample corresponded to the total quantity of nucleic acids in that sample (RNA and DNA combined). DNA alone was measured by selectively digesting RNA with RNase (Sigma, R-4875) and reading fluorescence again. RNA concentration could then be determined by calculating the difference between the total nucleic acid and DNA concentrations.

We tested starvation resistance of cobia larvae at the end of CO_2/Ration by measuring time to 50 % mortality (LT_{50}) upon complete cessation of feeding. This was conducted utilizing larvae raised to 14 dph with a full ration feeding regiment. Thirty larvae (270 total) were haphazardly redistributed into each of four (ambient-CO_2) or five (high-CO_2) replicate 400 L tanks that had been flushed of all prey. Larvae did not receive subsequent feedings and starvation mortalities were collected and recorded at dawn, dusk, and approximately every 4 h during daylight hours until 100 % mortality was attained (approx. 3.5 d).

Data analysis

All statistical analyses were conducted in R (v2.15.1), except starvation resistance, which was analyzed in CETIS (v1.8.0, Tidepool Scientific), and results were considered significant at $P < 0.05$. Standard length, growth rate, proportion of fish having entered flexion, RNA:DNA ratio, U_{crit} data, and routine swimming activity metrics were analyzed using two-way analysis of variance (ANOVA), with the respective measurement as the response variable and CO_2 and temperature (or ration) as fixed factors. One-way ANOVA was used for 5 dph data from CO_2/Ration, with CO_2 as the

only fixed factor, because rationing was not applied prior to that day. One-way ANOVA was also used to test differences between routine and olfactory stimulation swimming activity observations for data pooled across temperature treatments, within each CO_2 treatment. Time to 50 % mortality (LT_{50}) and 95 % confidence intervals (CI) were calculated following log-probit transformation for control treatment data, however, high-CO_2 treatment data did not conform to the assumption of homoscedasticity, therefore LT_{50} and CI for those data were determined using Steel's many-one rank test.

Prior to ANOVA procedures, data transformations were applied and normality and homoscedasticity were verified using Shapiro-Wilk and Bartlett's tests, respectively. Transformations were applied to CO_2/Temp data for proportion of fish having entered flexion (arcsine) and also to swimming activity data from CO_2/Temp and CO_2/Ration, including mean swimming speed (log), maximum swimming speed (square root), and swimming track length (square root during CO_2/Temp, log during CO_2/Ration). Some data did not conform to assumptions of normality and homoscedasticity even with transformation (Table 4.2), however, all underlying distributions of subsampled fish were normal and homoscedastic. ANOVA procedures were conducted because of this underlying normality and because F-values are robust to departures from normality and homoscedasticity, with negligible to modest inflation of Type I error (Harwell et al. 1992, Underwood 1997).

Results

CO_2 and temperature experiment

Growth and development- During CO_2/Temp, larvae from high temperature treatments had significantly longer SL at 8-11 and 20 dph (Figure 4.1a). Larvae in high-CO_2

treatments had significantly shorter SL than those in ambient-CO_2 treatments at 10 and 11 dph, but there was no significant interaction. Daily growth rate throughout flexion was significantly slower for larvae in high-CO_2 treatments, compared to ambient-CO_2 treatments (Figure 4.1b), however there was only a non-significant trend of increased growth rate with temperature and no treatment interaction. Larvae began to enter flexion on 8 dph and from 8 to 11 dph the proportions of larvae undergoing flexion were significantly greater in high temperature treatments than in low temperature treatments (Figure 4.1c). The proportions of larvae undergoing flexion were significantly lower for high-CO_2 treatments on 10 and 11 dph, and there was no interaction between temperature and CO_2 on any dph. See Table 4.3 for statistical summary of CO_2/Temp results.

Swimming activity and ability- Data from CO_2/Temp did not indicate significant effects of CO_2, temperature, or any interaction, on the mean track length, mean swimming speed, maximum swimming speed, mean angle change, or net-to-gross displacement of larvae during routine swimming observations. In addition, there was no effect of any treatment on swimming activity metrics between routine and olfactory stimulation observations within ambient- or high-CO_2 treatment groups. Swimming ability (U_{crit}) of larvae in high temperature treatments was significantly higher than larvae in lower temperature treatments (Figure 4.1d), but there was no significant effect of CO_2 or treatment interaction.

<u>CO_2 and rationing experiment</u>

Growth and development- During CO_2/Ration, larvae from reduced ration treatments had significantly shorter SL than full ration treatments on 10 and 12 dph (Figure 4.2a), with a similar but nonsignificant trend on 9 and 11 dph. Increased CO_2 did not significantly

impact SL, but there was a significant effect of treatment interaction on SL at 8 dph. Larvae from reduced ration treatments had significantly slower growth rates than controls, but neither CO_2 nor treatment interaction impacted growth rate (Figure 4.2b). Larvae had already begun to enter flexion on the first day of sampling targeting flexion (8 dph) and nearly all larvae in every treatment had entered flexion by 12 dph. On 10 and 12 dph, reduced ration treatments had significantly lower proportions of larvae undergoing flexion (Figure 4.2c), but there was no effect of CO_2 or treatment interaction. See Table 4.4 for statistical summary of CO_2/Ration results.

Swimming activity- Swimming activity results during CO_2/Ration were similar to CO_2/Temp, we detected no significant impact on any metric of swimming activity and no significant olfactory response.

Larval condition and starvation resistance- There were no significant differences in RNA:DNA ratio between treatments on 5 dph (Figure 4.2d). After 5 d of rationing (10 dph), RNA:DNA ratios in reduced ration treatments tended to be lower than in full ration treatments, but this pattern was not significant, and there was no effect of CO_2 or treatment interaction. Following CO_2/Ration, larvae subjected to complete feeding cessation under high-CO_2 conditions demonstrated a shorter LT_{50} (43.9 h ± 4.5 h 95% CI) than those under ambient-CO_2 conditions (49.8 h ± 1.3 h 95% CI; Figure 4.3).

Discussion

Our results demonstrate that larval cobia may exhibit a variety of responses to ocean acidification under future environmental conditions. When CO_2 and temperature treatments were crossed (CO_2/Temp), elevated CO_2 and temperature negatively influenced larval SL, growth rate, and development, while increased temperature

influenced U_{crit}, but there were no effects of any treatment on swimming activity. Contrary to our original hypothesis, there was a lack of detectable interactions between temperature and CO_2. The presence of main effects and lack of interaction between temperature and acidification treatments corresponds to similar results found for the aerobic scope of adult cardinalfish (Munday et al. 2009c), but contrasts the synergistic effects of temperature and CO_2 on the foraging behavior of juvenile damselfish (Nowicki et al. 2011). Since we utilized larval stages and did not evaluate the same metrics, it is difficult to conclude whether our results are stage-, species-, or metric-specific.

It is not unusual that SL, developmental rate, and U_{crit} all increased due to increased temperature, or that growth rate showed a similar trend. Larval growth is known to increase with temperature, even in subtropical regions (Sponaugle et al. 2006), as does metabolic rate, assuming temperature does not exceed the oxygen- and capacity-limited thermal tolerance (OCLTT), at which point metabolic depression may occur (Pörtner 2012). Bignami et al. (2013a) previously concluded that altered size-at-age, not direct impact on development, caused delayed flexion in larval cobia exposed to high-CO_2. It is likely that this also explains the precocious flexion of significantly larger larvae in our high temperature treatments during CO_2/Temp. Increased swimming ability with increased temperature was not surprising because U_{crit} has been shown to increase with temperature in other species (Koumoundouros 2002, Green & Fisher 2004). Although contrary to our initial hypothesis, the lack of CO_2 effect on U_{crit} corresponds to previous results reported for cobia (Bignami et al. 2013a) and damselfish (Munday et al. 2009b). The lack of significant change in swimming activity as a result of elevated CO_2 contrasts the variety of impacts to other behavioral measures in several different fish species (e.g.,

Dixson et al. 2010, Munday et al. 2010, Ferrari et al. 2011a), but is similar to the general lack of effect on swimming kinematics reported for Atlantic cod larvae (Maneja et al. 2012). Thus, impacts on swimming activity may be species specific and the ability to detect such impact likely depends upon the selected methodology.

Ocean acidification had negative impacts on SL, growth rate, and developmental rate during CO_2/Temp, as well as reduced starvation resistance when CO_2 and complete starvation were combined. These findings contrast to previous results for cobia, which demonstrated resistance to significant levels of elevated CO_2 as measured by SL and development (Bignami et al. 2013a), as well as the general resistance to CO_2-induced impacts on those metrics when CO_2 and reduced ration (CO_2/Ration) were crossed. This inter-experimental variation may be due to differences in egg quality and larval condition between different spawning events. It is possible that adult condition declines over the extensive spawning season of captive cobia (approx. April through September) and maternal condition has been shown to impact egg quality (e.g., yolk allocation) and early life history traits (e.g., size at hatch) in other species (Gagliano & McCormick 2007, Donelson et al. 2008). Therefore, "late-season" larvae in CO_2/Temp (August) may have originated from lower quality eggs, and been more vulnerable to acidification compared to "early-season" larvae in CO_2/Ration (June). However, recent research on captive spawning cobia at UMEH did not indicate a reduction in egg quality as measured by egg viability (Stieglitz et al. 2011). Furthermore, it is likely that both these experiments, and previous experiments by Bignami et al. (2013a), utilized eggs that originated from different combinations of males and females. Larval quality can be affected by parentage (Trippel et al. 2005, Sogard et al. 2008) and this could have influenced the response of

cobia larvae to experimental conditions. If either of these factors impacted the susceptibility of larval cobia to ocean acidification during CO_2/Temp or CO_2/Ration, it may be indicative of this species' ability to adapt to future environmental changes via natural selection. Alternatively, it is possible that the lack of CO_2 effects could be due to the unintentional exclusion of an early window of susceptibility (Baumann et al. 2011) because larvae were not subjected to treatment conditions until 2 dph (Bignami et al. 2013a). Similarly, some amount of transgenerational acclimation to elevated CO_2 is possible is adults are exposed to elevated pCO_2 (Miller et al. 2012), but broodstock tank carbonate chemistry was not closely monitored during this study. However, due to the occurrence of several significant CO_2 effects, we conclude that these latter two scenarios are unlikely.

Since larval condition is correlated with somatic effects and survival in fishes (Buckley et al. 1999, Searcy & Sponaugle 2001, Booth 2002, Hoey & McCormick 2004), it is understandable that rationing had a significant effect on SL, growth rate, and development. However, rationing did not increase susceptibility to ocean acidification and we did not observe any consistent trends in larval condition (RNA:DNA ratio) due to elevated CO_2. Reduced larval condition was expected to occur due to the reallocation of metabolic resources from growth (i.e., protein synthesis) to the maintenance of acid-base balance. Acidification-driven reduction in RNA:DNA ratio has been identified in one other species (i.e., Atlantic herring; Franke & Clemmesen 2011), but two other studies have failed to observe this effect (i.e., Atlantic and Baltic cod; Frommel et al. 2011, 2012). It may be possible for RNA:DNA ratio to increase under stressful pCO_2 conditions due to an increase in the transcription and translation of genes needed for the production

of ion transporters used in pH balance and ionoregulation. For example, Deigweiher et al.(2008) demonstrated an increase in mRNA expression for select genes in eelpout (*Zoarces viviparous*) exposed to elevated CO_2 (10,000 ppm). This may hinder the detection of acidification-driven reduction in condition and RNA:DNA ratio, but it is unclear if stress-induced impact on RNA levels would cause a large enough signal to be detected using a relatively low-resolution index such as RNA:DNA ratio. Stress can also cause a reduction in the expression of some genes, for example, Esbaugh et al. (2012) found reduced expression of select genes in gulf toadfish (*Opsanus beta*) under 1900 μatm pCO_2 treatment conditions. Reduced expression of some genes could offset increased expression of others, resulting in no net change to RNA:DNA ratio. These contrasting results may be treatment- or species-dependent, and verification of such processes in cobia would require specific investigation of gene expression under acidified conditions.

Despite no significant decrease in larval condition due to high-CO_2 conditions, our test of starvation resistance provided compelling evidence that cobia larvae are less capable of withstanding periods of starvation in combination with ocean acidification. Reduced starvation resistance may be due to an increase in metabolic demand as a result of acidification, which has been observed in another fish species (Miller et al. 2012). This increased metabolic demand could represent a significant challenge for fishes in tropical and subtropical oceans, as starvation is thought to be an important process influencing larval survival and recruitment magnitude (Houde 1989a). In contrast to high-latitude environments with more abundant prey, low-latitude oceans are more oligotrophic, with variable productivity that is difficult to predict in space and time (Longhurst & Pauly

1987). In addition, the larvae of many fishes are highly selective predators, with some genera limiting their diets to one or two types of prey (e.g., calanoid copepods, appendicularians) and many undergoing specific ontogenetic dietary shifts (Llopiz & Cowen 2009). Although subtropical field collections indicate high feeding incidence by larval fishes (Llopiz & Cowen 2008, 2009, Sponaugle et al. 2009), the presence of food in the gut does not ensure a larva's ability to sustain metabolic demand in warm tropical and subtropical water (Houde 1989b). For example, despite high feeding incidence in most studies, Tanaka et al. (2008) classified up to 25 % of wild-caught subtropical Pacific bluefin tuna larvae as being in "starving condition" based on comparisons of RNA:DNA ratios with lab-reared/starved larvae. Future climate change- and/or ocean acidification-driven changes in productivity (Gao et al. 2012) and prey quality (Rossoll et al. 2012) may stress an already tenuous balance between prey availability and metabolic demand for larval fishes, and according to this study, could be further exaggerated by concurrent impact of acidification on starvation resistance.

From a broader perspective, CO_2 effects on SL, growth rate, developmental rate, and starvation resistance in larval cobia have the potential to influence the basic ecological processes that govern the replenishment and connectivity of fish populations. The growth-mortality hypothesis poses that fishes which grow more slowly, are smaller at-age, or have extended stage-durations will experience higher mortality rates (Anderson 1988). Slight changes in mortality or growth rates of larvae can have large cumulative effects on recruitment potential (Houde 1989a, 1997), which in turn influences population replenishment and connectivity, both of which are central to the ecological function and effective management of marine populations (Cowen & Sponaugle 2009).

Although the results reported here demonstrate few direct impacts of ocean acidification on these selected metrics, it does not preclude the potential for critical but less obvious impacts to metrics that were not included in this study. Seemingly subtle effects, such as sensory or behavioral effects observed in cobia and other species (Munday et al. 2009a, Ferrari et al. 2012b, Bignami et al. 2013b) could greatly influence larval survival, dispersal, and recruitment, with cascading effects at the population level.

Even in the absence of synergistic interactions between treatments, the response of larval cobia to individual temperature, ration, and CO_2 treatments highlights the need to consider the large variety of possible environmental stressors when attempting to understand the response of fish to projected climate scenarios. It is increasingly evident that caution should be used when drawing conclusions from "no effects" studies, especially when those studies investigate individual stressors, since the combined impact of many small effects may overwhelm even the most experimentally robust and resilient species. Our understanding of how fishes respond to ocean acidification and other environmental changes will continue to improve as more complex experiments are conducted for a diversity of species, enabling the formulation of broader and more accurate conclusions, and facilitating the proper application of this knowledge to pressing management and conservation concerns.

Table 4.1 Water chemistry conditions during two experiments crossing CO_2 with increased temperature and CO_2 with reduced rationing. Temperature, pH, and total alkalinity (TA) were measured and mean pCO_2 calculated with the software CO2SYS (Lewis & Wallace 1998). For CO_2/Temp, pCO_2 values adjusted to a standard temperature are also reported. Values are means (± SE).

Treatment	Temp. °C	pH total scale	TA µmol kg^{-1}	pCO_2 µatm	pCO_2 at 27°C µatm
CO_2/Temp					
Ambient-CO_2/Cool	27.1 (± 0.1)	7.91 (± 0.01)	2393 (± 21)	608 (± 26)	621 (± 27)
Ambient-CO_2/Warm	32.1 (± 0.2)	7.84 (± 0.01)	2395 (± 22)	745 (± 30)	619 (± 24)
High-CO_2/Cool	27.1 (± 0.1)	7.50 (± 0.02)	2379 (± 25)	1767 (± 79)	1816 (± 75)
High-CO_2/Warm	32.1 (± 0.2)	7.44 (± 0.03)	2397 (± 27)	2113 (± 93)	1806 (± 83)
CO_2/Ration					
Ambient-CO_2/Full ration	27.6 (± 0.1)	8.10 (± 0.02)	2227 (± 25)	284 (± 12)	
Ambient-CO_2/25% ration	27.6 (± 0.1)	8.10 (± 0.02)	2229 (± 25)	283 (± 13)	
High-CO_2/Full ration	27.6 (± 0.1)	7.44 (± 0.04)	2210 (± 26)	1673 (± 173)	
High-CO_2/25% ration	27.6 (± 0.1)	7.45 (± 0.03)	2223 (± 23)	1685 (± 152)	

Table 4.2 Summary of assumption violations for tank mean two-way analysis of variance procedures on standard length (SL), proportion of larvae undergoing flexion (flexion), and critical swimming speed (U_{crit}) during the CO_2/Temp and CO_2/Ration experiments. Days post hatch (dph) and treatments affected are listed. Analysis of underlying subsamples did no violate any assumptions and ANOVAs were conducted because it is robust to such deviations (Harwell et al. 1992, Underwood 1997).

Assumption violated	Metric	dph: treatment affected
CO_2/Temp		
Normality	SL	20: High CO_2/High temp.
	Flexion	8: Low CO_2/High temp. 9: all except Low CO_2/Low temp.
	U_{crit}	High CO_2/High temp., High CO_2/Low temp. Low CO_2/High temp., high and low temp. treatments.
Homoscedasticity	SL	8 & 20: between temperature treatments
CO_2/Ration		
Normality	Flexion	8, 9: Low CO_2/Full ration

Table 4.3 Summary of statistical results for two-way analysis of variance of larval cobia standard length (SL), growth rate, flexion, U_{crit}, and routine swimming activity over multiple days post hatch (dph) during an experiment in which CO_2 and increased temperature were crossed.

Metric	F-value (CO_2 - Temp - Interaction)			P-value (CO_2 - Temp. - Interaction)			df
SL (dph)							
7	1.647	0.699	1.467	0.235	0.428	0.260	1,8
8	0.648	10.184	0.309	0.444	**0.013**	0.593	1,8
9	0.178	15.648	0.614	0.684	**0.004**	0.456	1,8
10	5.446	12.347	0.712	**0.048**	**0.008**	0.423	1,8
11	8.363	6.094	0.225	**0.020**	**0.039**	0.648	1,8
20	0.000	51.246	3.001	0.984	**<0.001**	0.121	1,8
Growth Rate							
	6.420	2.281	0.568	**0.035**	0.169	0.473	1,8
Flexion (dph)							
8	0.230	7.910	0.230	0.644	**0.023**	0.644	1,8
9	0.503	83.209	1.696	0.498	**<0.001**	0.229	1,8
10	10.408	10.599	1.757	**0.012**	**0.012**	**0.222**	1,8
11	6.640	8.084	0.001	**0.033**	**0.022**	0.974	1,8
U_{crit}							
	0.156	21.950	0.697	0.703	**0.002**	0.428	1,8
Routine swimming activity							
Track length	0.072	1.572	0.003	0.795	0.245	0.957	1,8
Mean velocity	0.077	1.569	0.004	0.789	0.246	0.952	1,8
Max velocity	0.001	2.821	0.135	0.976	0.622	0.948	1,8
Mean angle change	1.388	0.696	2.977	0.273	0.428	0.123	1,8
Net-to-gross displacement	< 0.001	0.152	0.118	0.989	0.707	0.740	1,8

Table 4.4 Summary of statistical results for two-way analysis of variance (ANOVA) of larval cobia standard length (SL), growth rate, flexion, routine swimming activity, and RNA:DNA ratio over multiple days post hatch (dph) during an experiment in which CO_2 and reduced rationing were crossed. SL was analyzed with one-way ANOVA on 5 dph because rationing treatment was not applied yet.

	F-value (CO_2 - Ration - Interaction)			**P-value (CO_2 - Ration - Interaction)**			**df**
SL (dph)							
5	1.929	NA	NA	0.195	NA	NA	1,10
8	0.832	1.330	7.448	0.389	0.282	**0.026**	1,8
9	0.129	3.623	0.696	0.728	0.094	0.428	1,8
10	0.069	9.922	0.695	0.799	**0.014**	0.429	1,8
11	3.151	4.949	0.387	0.114	0.057	0.551	1,8
12	0.376	6.406	0.202	0.557	**0.035**	0.665	1,8
Growth Rate							
	0.581	9.890	3.452	0.468	**0.014**	0.100	1,8
Flexion (dph)							
8	3.624	2.832	3.551	0.094	0.131	0.096	1,8
9	0.006	2.847	0.163	0.939	0.130	0.697	1,8
10	1.299	15.938	1.223	0.287	**0.004**	0.301	1,8
11	3.306	0.005	0.530	0.107	0.948	0.487	1,8
12	0.025	6.348	1.741	0.878	0.358	0.224	1,8
Routine swimming activity							
Track length	1.303	0.123	0.006	0.287	0.735	0.938	1,8
Mean velocity	1.311	0.122	0.006	0.285	0.736	0.939	1,8
Max velocity	4.267	0.040	0.243	0.073	0.846	0.635	1,8
Mean angle change	1.109	2.502	1.291	0.323	0.152	0.289	1,8
Net-to-gross displacement	0.518	0.611	2.336	0.492	0.457	0.165	1,8
RNA:DNA							
5	0.152	2.582	0.152	0.707	0.147	0.707	1,8
10	0.007	2.487	0.275	0.935	0.153	0.654	1,8

Figure 4.1 Impact of ocean acidification on larval cobia during a fully crossed CO_2/Temp experiment, including (**a)** standard length (SL), (**b**) growth rate during flexion, (**c)** proportion of larvae undergoing flexion, and (**d**) critical swimming speed (U_{crit}). See Table 4.1 for CO_2 treatment specifications. Asterisks indicate significant temperature effects and daggers indicate significant CO_2 effects. Results are from two-way analysis of variance. (Values are means ±SE, N = 3 per treatment, statistical summary found in Table 4.3)

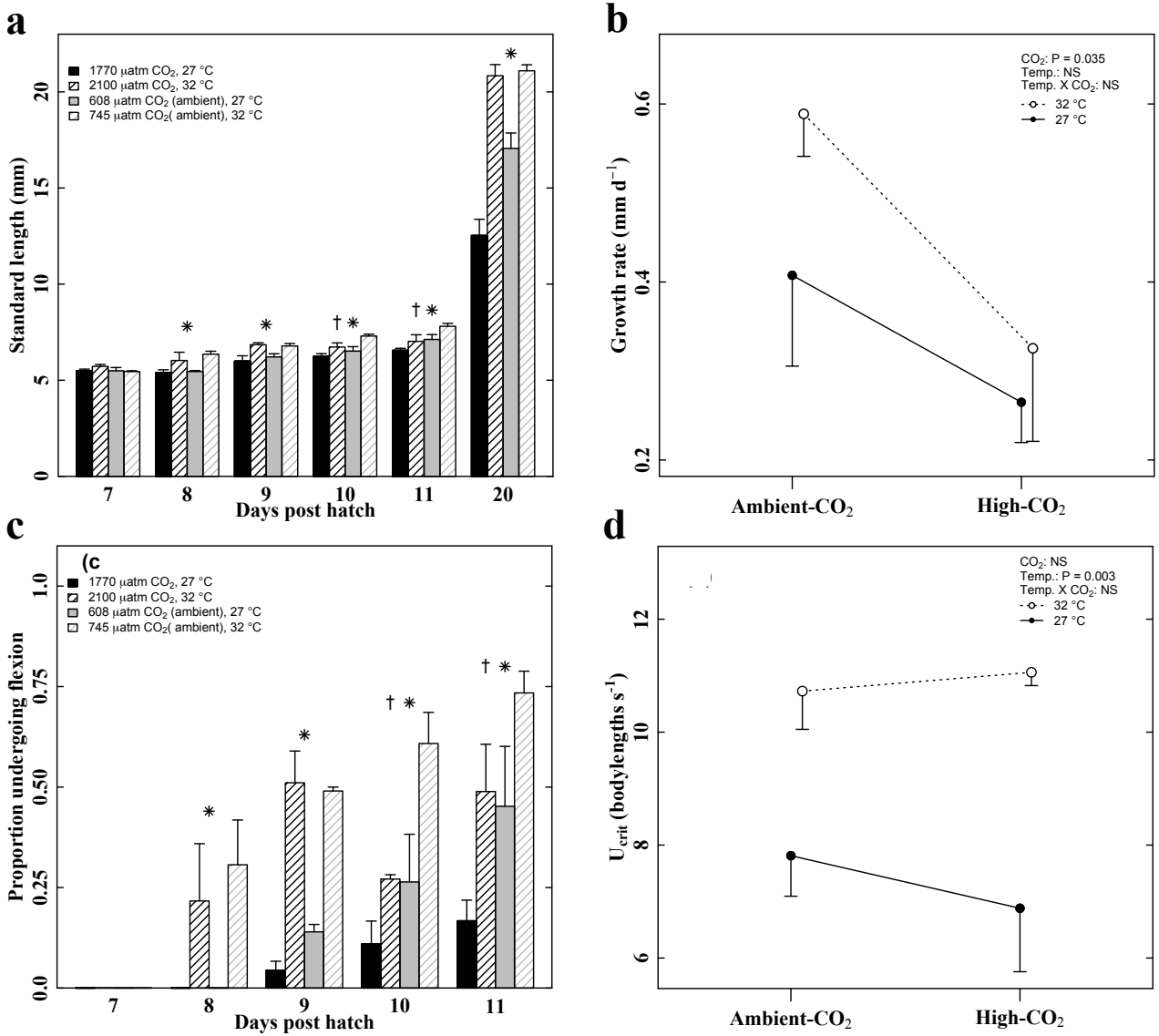
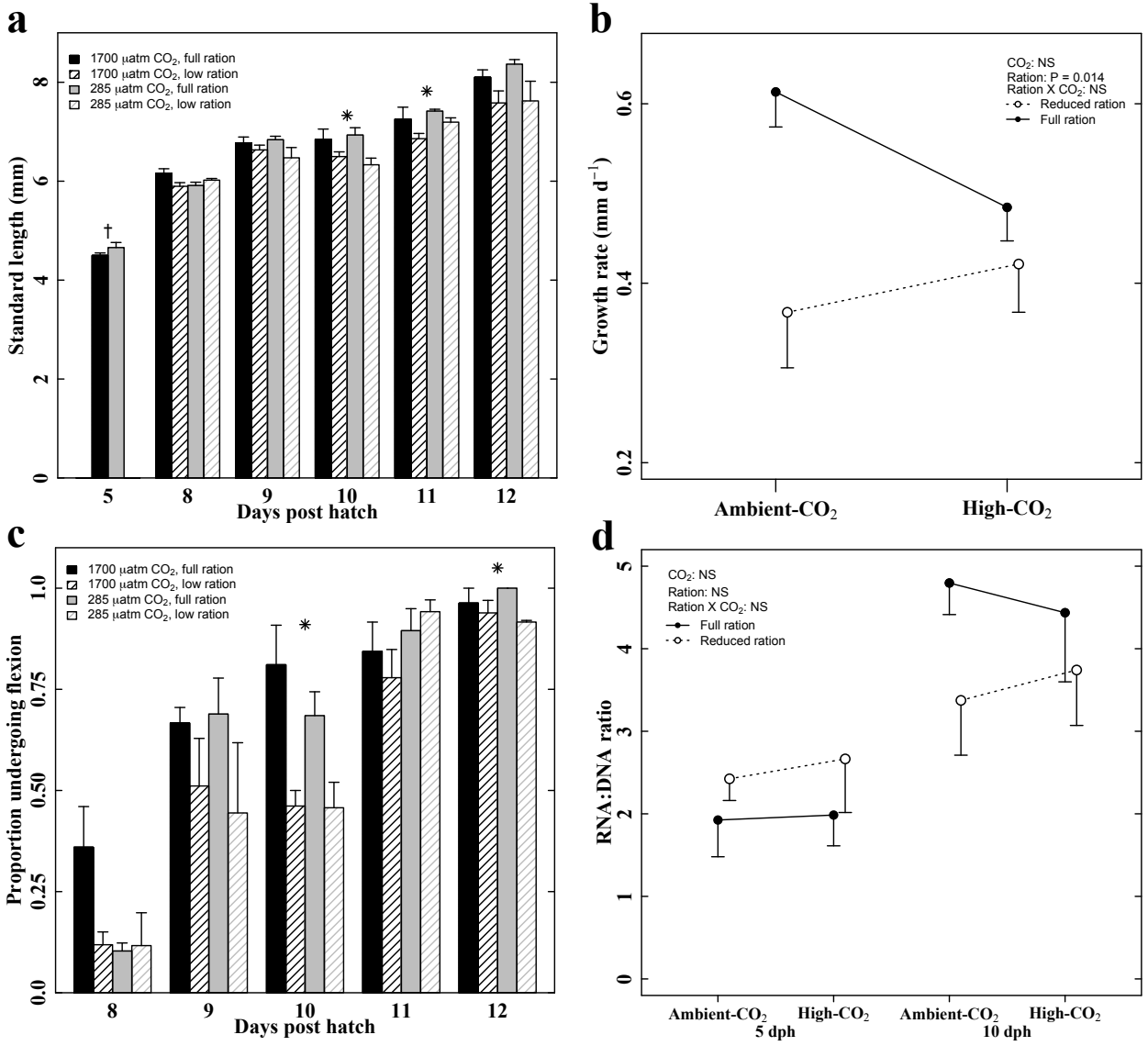

a
1770 µatm CO2, 27 °C
2100 µatm CO2, 32 °C
608 µatm CO2 (ambient), 27 °C
745 µatm CO2(ambient), 32 °C
Standard length (mm)
Days post hatch
b
CO2: P = 0.035
Temp.: NS
Temp. X CO2: NS
32 °C
27 °C
Growth rate (mm d-1)
Ambient-CO2
High-CO2
c
(c
Proportion undergoing flexion
Days post hatch
d
CO2: NS
Temp.: P = 0.003
Temp. X CO2: NS
Ucrit (bodylengths s-1)
Ambient-CO2
High-CO2

Figure 4.2 Impact of ocean acidification on larval cobia during a fully crossed CO_2/Ration experiment, including (**a)** standard length (SL), (**b**) growth rate during flexion, (**c)** proportion of larvae undergoing flexion, and (**d**) RNA:DNA ratio at 5 and 10 days post hatch (dph). See Table 4.1 for CO_2 treatment specifications. Asterisks indicate significant ration effects and double dagger indicates a significant CO_2 x ration interaction. Results are from two-way analysis of variance (ANOVA) except for testing of SL by one way ANOVA at 5 dph. (Values are means ±SE, N = 3 per treatment for two way ANOVA and 6 per treatment for one way ANOVA, statistical summary found in Table 4.4)


a
1700 µatm CO2, full ration
1700 µatm CO2, low ration
285 µatm CO2, full ration
285 µatm CO2, low ration
Standard length (mm)
Days post hatch
b
CO2: NS
Ration: P = 0.014
Ration X CO2: NS
Reduced ration
Full ration
Growth rate (mm d−1)
Ambient-CO2
High-CO2
c
Proportion undergoing flexion
Days post hatch
d
CO2: NS
Ration: NS
Ration X CO2: NS
RNA:DNA ratio
5 dph
10 dph

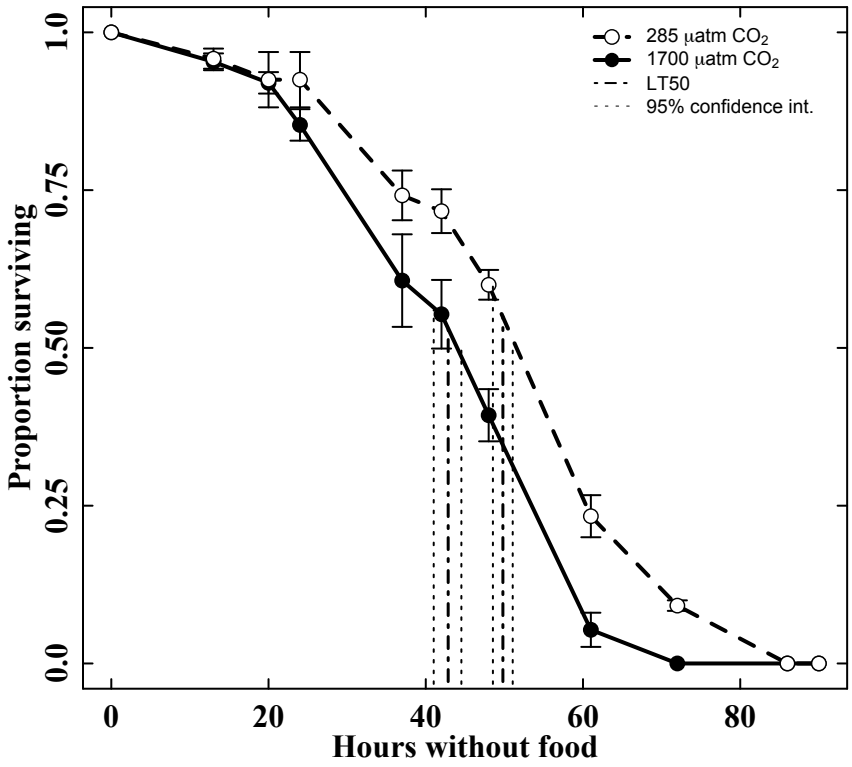


Figure 4.3 Proportion of 14-day post hatch cobia larvae surviving over time with complete feeding cessation following the CO_2/Ration experiment. Larvae in the high-CO_2 treatment had shorter time to 50% mortality (LT_{50}, left vertical dashed line) than the LT_{50} (right vertical dashed line) of those in the ambient-CO_2 treatment, with non-overlapping 95 % confidence intervals (vertical dotted lines). LT_{50} values and surrounding confidence intervals were calculated using log-probit (ambient-CO_2) and Steel's many-one rank test (high-CO_2). (Values are means ±SE, N = 4 for ambient-CO_2 and 5 for high-CO_2 treatments)

CHAPTER 5. Effects of ocean acidification on larvae of a high-value pelagic fisheries species, mahi-mahi (*Coryphaena hippurus*)[4]

Summary

Negative impacts of CO_2-induced ocean acidification on marine organisms have proven to be variable both among and within taxa. For fishes, these inconsistencies confound our ability to draw conclusions that apply across taxonomic groups and highlights the limitations of a nascent field with a narrow scope of study species. We present data from a series of experiments on the larvae of mahi-mahi (*Coryphaena hippurus*), a large pelagic tropical species of high economic value. Mahi-mahi larvae were raised for up to 21 d under projected scenarios of ocean acidification ranging from 770 to 2170 μatm pCO_2. Evaluation of hatch rate, size, development, swimming activity, swimming ability (U_{crit}), and otolith (ear stone) formation produced few significant effects. However, larvae unexpectedly exhibited significantly larger size-at-age and faster developmental rate during one experiment, possibly driven by metabolic compensation to elevated pCO_2 via a corresponding decrease in routine swimming velocity. Furthermore, microscopy analysis of otoliths revealed significantly larger otoliths at 2170 μatm pCO_2, and a similar but non-significant trend at 1200 μatm pCO_2, with potential implications for hearing sensitivity. According to most variables measured in our study, these data provide an optimistic indication that this large tropical species, which inhabits the offshore pelagic environment, may not be overtly susceptible to ocean acidification. However, the presence of some treatment effects on growth, swimming activity, and otolith formation also suggests the possibility of other unmeasured or undetected impacts of acidification on larval mahi-mahi, the cumulative consequences of which are still unknown.

4. Bignami S, Sponaugle S, Cowen RK. (submitted) Effects of ocean acidification on larvae of a high-value pelagic fisheries species, mahi-mahi (*Coryphaena hippurus*).

Background

A diversity of marine organisms are negatively affected by CO_2-induced ocean acidification, yet the occurrence and severity of such effects are highly variable between taxa and life history stages (Kroeker et al. 2010). While much research has focused on calcifying marine invertebrates, until recently there has been relatively little consideration of acidification impacts on fishes. This is due, in part, to the fact that fishes and other organisms with high metabolic rates are known to be relatively efficient at regulating their internal acid-base balance in response to metabolic or environmental disturbances, and thus are considered to be more capable of resisting ocean acidification (Melzner et al. 2009a). Unfortunately, there is little comparable information on the ability of early life history stages of fishes to cope with increased environmental CO_2; embryonic and larval fishes are not equipped with the same physiological machinery as juveniles and adults, but are known to be capable of some internal pH regulation (Brauner 2008). Nonetheless, existing literature indicates that the early life stages of fishes are more susceptible to elevated CO_2 relative to adults (reviewed by Pörtner et al. 2005). As the primary period of dispersal for many marine fishes, the larval stage is critical to population replenishment and connectivity (Cowen & Sponaugle 2009), therefore our understanding of the broader population- and ecosystem-level impacts of ocean acidification requires consideration of this life stage.

The impact of ocean acidification on larval fishes is highly variable among studies and taxa, with reported effects ranging from reduced growth and survival (Baumann et al. 2011) to altered neurological function (Nilsson et al. 2012) and behavior (Munday et al. 2010, Ferrari et al. 2012b). Additionally, more discrete effects have been found, such as

altered otolith (ear stone) development (Checkley et al. 2009, Munday et al. 2011b, Hurst et al. 2012, Bignami et al. 2013a, b) and impaired tissue health (Frommel et al. 2011), as well as several reports of no effects (e.g.,Munday et al. 2011a, Frommel et al. 2012). Though intriguing, this inter- and intra-specific variability in response to ocean acidification confounds our ability to formulate general conclusions regarding the fate of fish populations under future environmental scenarios. To better clarify the underlying factors driving patterns of susceptibility or resistance to ocean acidification, it is necessary to examine a diversity of species with distinct differences in life history strategy, physiological capability, and habitat. For tropical fishes, there has been little representation of large pelagic species in the literature, and although there are studies on large species such as tropical cobia (*Rachycentron canadum*; Bignami et al. 2013a, b, in review) and temperate white sea bass (*Astractoscion nobilis*; Checkley et al. 2009), their use of nearshore environments may cause them to differ from other pelagic species which exclusively inhabit offshore pelagic waters.

Fishes with an offshore pelagic life history spawn pelagic eggs, undergo planktonic larval development, and live as juveniles and adults in an offshore environment that lacks the extreme diurnal cycles in pH and temperature observed in shallow coastal environments (Ohde & van Woesik 1999, Perez-Dominguez et al. 2006, Hofmann et al. 2011, Frieder et al. 2012, Price et al. 2012). It has been suggested that compared to demersal species regularly exposed to fluctuating environmental conditions, pelagic species which have adapted to such a stable environment may be more susceptible to future environmental changes such as ocean acidification (Munday et al. 2008a, Pörtner 2008). This concept is consistent with the limited capacity for acclimation

to environmental change observed in other organisms adapted to stable conditions (Somero 2012), but is in contrast to the concept of increased resistance to acid-base disturbance due to the ability for tight physiological regulation in species with high metabolic rates (Melzner et al. 2009a). With regard to ocean acidification, our knowledge of impacts on the larvae of tropical pelagic fishes is limited to recent reports on cobia, which indicate that this species is somewhat resistance to most direct impacts of projected ocean acidification on growth, development, swimming activity, and swimming ability (Bignami et al. 2013a, in review). However, cobia is not completely unaffected; larvae exhibited altered otolith formation with implications for auditory sensation (Bignami et al. 2013a, b) and also have increased susceptibility to starvation under acidified conditions (Bignami et al. in review). Although cobia is the most comparable study species to a diversity of other tropical pelagic fishes, its life history includes the use of nearshore environments and therefore does not conform to the strictly offshore pelagic life history of some other species. This utilization of nearshore habitats may have resulted in evolved adaptations to variable environmental conditions and thus reduced its susceptibility to acidification (Bignami et al. 2013a). Research on species with entirely pelagic life cycles is necessary to differentiate how various life history strategies and corresponding physiological characteristics influence the susceptibility of fishes to ocean acidification.

This study provides the first report of larval response to acidification in mahi-mahi (*Coryphaena hippurus*), a widely distributed, large tropical species of high economic value which inhabits offshore pelagic waters. We present data from a series of experiments that investigated a suite of factors which are known to influence larval

ability to successfully survive and recruit to the adult population. Our chosen metrics are comparable to those reported for larval cobia and other species, including hatch rate, growth, development, swimming activity, critical swimming speed (U_{crit}), and otolith formation (Munday et al. 2009b, Baumann et al. 2011, Bignami et al. 2013a). Growth and development have a direct relationship to mortality and recruitment magnitude (Anderson 1988, Houde 1989a), while swimming activity may allow detection of subtle effects on metabolic demand. Critical swimming speed, an ecologically relevant measure of swimming ability (Plaut 2001), is of interest due to the high-performance swimming capability of mahi-mahi as well as its direct relevance to the dispersal potential of fishes in the wild (Fisher 2005). Finally, otolith formation was evaluated because impacts on otoliths have been identified in several other species (Checkley et al. 2009, Munday et al. 2011b, Bignami et al. 2013a), with implications for auditory sensory function (Bignami et al. 2013b). We hypothesized that mahi-mahi would be more susceptible to acidification than species adapted to more variable natural environments, and thus when exposed to elevated CO_2 treatments would exhibit reduced growth and development, altered swimming activity, diminished swimming ability, and enhanced otolith growth.

Materials and methods

Study species

Mahi-mahi (*Coryphaena hippurus)* is a highly migratory, epipelagic, predatory marine fish with circumglobal distribution in tropical to subtropical waters (Palko et al. 1989, FAO 2013). Spawning of pelagic eggs occurs throughout the year, primarily during warmer months (>24 °C, April-November), with planktonic larvae that hatch at ~3.5 mm SL, undergo flexion at ~7 to 9 mm SL, and develop via a gradual transition into the

juvenile stage (Ditty et al. 1994). Mahi-mahi grow rapidly and are capable of hatching, developing, and reaching reproductive maturity in as little as 4 mo (Beardsley 1967, Oxenford 1999). Maximum size is as large as 2 m and over 30 kg, however, longevity is short compared with other pelagic species, with an average of 2 yrs and maximum of 4 - 5 yrs (Beardsley 1967, FAO 2013). Mahi-mahi is highly targeted by commercial, artisanal, and recreational fisheries throughout its distribution (Oxenford 1999, Potoschi et al. 1999, Folpp & Lowry 2006, Zúñiga Flores et al. 2008, FAO 2013) and is one of the top seven harvested pelagic species in the western central Atlantic (Oxenford 1999). It has high commercial value, with global fishery landings over 57,000 tons in 2008 (FAO 2013).

Experimental design and rearing

We conducted three experiments (EX1, EX2, and EX3) to assess the effects of elevated pCO_2 on mahi-mahi larvae. In each experiment, we utilized ambient seawater as a control and applied one (EX1) or two (EX2 & EX3) elevated pCO_2 treatments ranging from 770 to 2100 μatm pCO_2 (Table 5.1) to simulate projected scenarios of ocean acidification over the next two centuries (Caldeira & Wickett 2003, 2005, Meehl et al. 2007), as well as present day conditions in some nearshore marine environments (Feely et al. 2008, Thomsen et al. 2010, Melzner et al. 2012). Seawater carbonate chemistry was manipulated by addition of equimolar HCl and $NaHCO_3$ to seawater prior to introduction into tanks (Gattuso 2010, Bignami et al. 2013a). Tank pH was monitored daily using a handheld pH meter (pH11, Oakton) and Ross Electrode (Orion 9102BWNP, Thermo Scientific) calibrated daily with TRIS buffer. Water samples were collected every 5 d in 250 mL PET bottles, fixed with 100 μL of saturated mercuric chloride, and the total

alkalinity (TA) and pH_T were measured using automated Gran titration checked for accuracy with Dickson standards (Scripps Institution of Oceanography; Langdon et al. 2000). CO2SYS was used to solve the carbonate system using the two measured parameters (pH_T and TA; Lewis & Wallace 1998). Temperature and dissolved oxygen were measured with a combination meter (550A, YSI Inc.) twice and once per day, respectively, and salinity was measured once per day using a refractometer (RHS-10 ATC, Premium Aquatics). During EX1 and EX3, ambient pCO_2 of seawater was 50 - 200 μatm higher than expected, likely due to respiration in source seawater (Biscayne Bay) and within facility plumbing, but was well below elevated pCO_2 treatment levels. This did not occur during EX2. See Table 5.1 for a summary of water parameters from each experiment.

All experiments were conducted using adapted methodology for the rearing of larval cobia (Benetti et al. 2008b, Bignami et al. 2013a). Mahi-mahi eggs and larvae for each experiment were produced at the University of Miami Experimental Hatchery (UMEH) from a population of three wild-caught broodstock (two females, one male). We collected eggs within 12 h of evening/nighttime spawning, gently transferred them into an aerated 300 L fiberglass tank, and provided a 1 h formalin sterilization treatment (100 ppm) followed by rapid flushing of seawater. During EX1, eggs were then placed in a 1000 L incubator tank where they hatched within ~36 h and were allowed to develop until 2 days post hatch (dph) prior to being stocking into 6 replicated 400 L flow-through experimental tanks per treatment, at a density of 9-10 eggs L^{-1}. To allow for exposure to elevated pCO_2 during possible early windows of susceptibility (Baumann et al. 2011) during EX2 and EX3, eggs were stocked directly into 4 replicated experimental tanks per

treatment immediately following the sterilization treatment. Experimental tanks received filtered, UV-sterilized seawater at an exchange rate of ~ 400 % d^{-1} and were aerated with a small amount (< 1 L min^{-1}) of bubbled air. Tanks were partially submerged in a large water bath to maintain replicate tank temperatures within ±0.2°C of each other, positioned under 95 % shade cloth to reduce light intensity, and fitted with translucent white polyethylene lids to prevent CO_2 off-gassing, intrusion of rain water, or introduction of contaminants.

Larvae were fed enriched rotifers (*Brachionus plicatilis*) 4 - 5 times d^{-1}, providing total densities of 3 - 8 rotifers mL^{-1} d^{-1} for larvae 2 dph to 10 - 12 dph (depending on larval size), gradually increasing with age (Benetti et al. 2008b). Beginning at 5 - 8 dph (depending on larval size), larvae were also provided enriched *Artemia* nauplii at total densities of 0.25 – 1.5 mL^{-1} d^{-1}, gradually increasing with age (Benetti et al. 2008b). We utilized greenwater rearing techniques to provide supplemental nutrients and increase tank turbidity, which has been shown to improve larval feeding and survival in other species (Naas et al. 1992, Faulk & Holt 2005). Between 1 and 12 dph, we added 15 - 20 ml d^{-1} of a concentrated blend of whole cell suspended microalgae (RotiGreen Omega, Reed Mariculture Inc.) to rearing tanks throughout the day along with rotifer feedings, maintaining a level of turbidity in which visibility of the tank bottom (~75 cm depth) was obscured. During EX1, EX2, and EX3, rearing continued until 17, 13, and 21 dph, respectively.

Hatch rate, size, and development

While stocking experimental tanks during EX2 and EX3, 50 eggs from each replicate tank were reserved and placed into a closed 1 L high density polyethylene jar

with a 300 μm nylon mesh window and flow-through seawater. During EX2, these chambers were suspended in each tank until hatching in experimental tanks was observed to be complete, at which point chambers were retrieved and eggs/larvae preserved for later analysis of hatch rate and size. To determine if embryonic duration varied with CO_2 treatment, during EX3, hatching chambers were collected at a set time (sundown), while hatching in experimental tanks was still in progress. We collected subsamples of 5 - 20 larvae from each replicate tank throughout ontogeny and specifically targeted a period during the transition from pre-flexion through post-flexion. During EX3, fewer sampling days were selected to ensure sufficient sample sizes for swimming ability tests at 20 dph. Larvae were stored in 95% ethanol, a digital image of each larva was captured, and standard length (SL) was measured to the nearest 0.1 mm using the software ImageProPlus (v7.0, MediaCybernetics). We qualitatively assessed progression through flexion according to notochord position (i.e., flexing upwards) and development of caudal fin rays, scoring each larva as "pre-flexion" or "undergoing/post-flexion".

Swimming activity and ability

Swimming activity tests were performed at 8 dph during EX1 and EX2 according to previous techniques (Bignami et al. 2013a). Prior to the first daily feeding, three subsamples of five larvae each were removed from each replicate tank and allowed to acclimatize to a 15 cm diameter observation container for at least 1 h. Following acclimatization, each container of five larvae was placed individually on a clear shelf inside an enclosed PVC observation chamber with a translucent lid and allowed to recover for 2 min. Routine swimming activity was subsequently recorded for 2 min using a low-light video camera (Hi-Res EXvision, Super Circuits) set 40 cm beneath the larvae.

Directly following routine swimming observation, a pipette was used to gently add 1 ml of an olfactory stimulant to the center of the observation dish and swimming activity in response to olfactory stimulation was recorded for an additional 2 min. During EX1, food-scented water (30 μm filtered rotifer culture water) was used as an olfactory stimulant. During EX2, we selected a different olfactory stimulant, chemical alarm cues, which are known to be released by a diversity of fish taxa upon damage to the skin (Mathis et al. 1995, Brown 2003, Holmes & McCormick 2010). Prior to behavioral observations, a single 2.5 cm juvenile mahi-mahi was anesthetized with 10% quinaldine, euthanized by immersion in MS-222, and rinsed with seawater. Both sides of the fish were scored with a blade and it was submerged in 100 ml of seawater which was subsequently used as the olfactory stimulant. Observation videos were converted to digital files, reduced to 2 frames per second, and analyzed with the software ImageJ (v1.46p, National Institutes of Health) using the MTrackJ plug-in (Meijering et al. 2012). Individual larvae were tracked over the entire 2-min routine swimming and subsequent 2-min olfactory stimulation observation periods. Swimming track data were analyzed for mean and maximum swimming speed, average angle change between 0.5-s observation points, and net-to-gross displacement (del Carmen Alvarez & Fuiman 2005). To capture periods of active swimming, and because larvae were sometimes stationary for extended periods of time, analyses of average angle change and net-to-gross displacement were performed on one randomly selected 20-s period of active swimming per larva (defined as a minimum of 5 cm gross distance traveled in 20 s).

We measured U_{crit} during EX3 for 20 dph post-flexion fish using a six lane swimming flume similar to that described by Munday et al. (2009b). Prior to the first

daily feeding, we collected subsamples of six larvae from each replicate tank in 1-L containers and allowed the larvae to acclimatize to flume water conditions for at least 1 h. Subsequently, individual larvae were placed in randomly assigned lanes and allowed to acclimatize to the flume for 5 min at a current speed of 2 cm s^{-1}. Larvae were tested in their respective CO_2 treatment groups by adding equimolar HCl and $NaHCO_3$ to the large, recirculating seawater reservoir connected to the flume. This required larvae to be tested in order of increasing pCO_2 treatment, beginning with ambient seawater and ending with the highest treatment. U_{crit} was measured using the same methodology as Bignami et al. (2013a), modified from Stobutzki & Bellwood (1997). Water current speed was increased by approximately 3 cm s^{-1} at 2-min intervals until larvae failed to maintain their position and were swept against a mesh barrier at the end of each flume lane. Flow calibration was verified using dye immediately before and after the day of testing and U_{crit} speeds were calculated in body lengths s^{-1} using the equation $U_{crit} = V_p + ((t_f / t_i) \times V_i)$, where V_p is the penultimate velocity increment successfully completed for the full time interval (t_i), prior to the velocity increment at which failure occurred (V_i) at a failure time (t_f) less than the full time interval (Hammer 1995).

Otolith analysis

During EX3, left and right sagittal and lapillar otoliths were dissected from four 21 dph larvae per replicate tank, stored in medium viscosity immersion oil, and imaged sulcus side down under a total magnification of 400x. Using ImageProPlus (v7.0), we digitally outlined each otolith according to pixel contrast and collected measurement data for dimensions including area, length, width, rectangularity, and roundness (otolith area divided by the area of the smallest rectangle able to contain it, and otolith perimeter

divided by the circumference of the smallest circle able to contain it, respectively;(Munday et al. 2011a, 2011b). Otolith dimensions were scaled according to the SL of each larva.

Data analysis

All statistical analyses were conducted in R (v2.15.1) using tank mean data and results were considered significant at $P < 0.05$. Standard length, proportion of fish having entered flexion, U_{crit}, swimming activity metrics, left sagittal and left lapillar otolith shape (roundness and rectangularity), and otolith relative density data were analyzed using one-way analysis of variance (ANOVA) with CO_2 as a fixed factor and the respective measurement as the response variable. Otolith size metrics were tested using analysis of covariance (ANCOVA) procedures, with each metric as a response variable, pCO_2 as a fixed factor, and standard length as a continuous covariate. In the event that one treatment exhibited a significantly different regression slope, that treatment was removed from further analysis. Arcsine transformations were applied to the proportion of fish having entered flexion. Normality and homoscedasticity were verified using Shapiro-Wilk and Bartlett's tests prior to all statistical procedures. Although the majority of data met all assumptions for ANOVA, during EX2, tank mean SL data from control treatments were not normally distributed on 13 dph and tank mean data of net-to-gross displacement were heteroscedastic across treatments. However, underlying distributions of subsampled fish were normal and homoscedastic. ANOVA procedures were conducted because of this underlying normality and because F-values are robust to departures from normality and homoscedasticity, with negligible to modest inflation of Type I error (Harwell et al. 1992, Underwood 1997).

Results

Experiment 1

The SL of larvae during EX1 was not significantly affected by elevated-CO_2 treatments for any sampling day (5, 8-12 dph, Fig. 5.1a). Larvae from the termination of the experiment (17 dph) showed a similar trend, but statistical analyses were not conducted due to high mortality in some replicate tanks. Larval progression through flexion was not affected by elevated-CO_2 treatments on any targeted sampling day ranging 9 to 12 dph (Fig. 5.1b). Similarly, no metric of routine swimming activity was affected by CO_2 treatment and larvae did not exhibit any treatment-related change in swimming activity following olfactory stimulation with food-scented water. See Table 5.2 for summary statistics from all EX1 analyses.

Experiment 2

Hatch rate and size-at-hatch were not significantly affected by CO_2 treatment during EX2, but larvae demonstrated significantly increased SL under both elevated-CO_2 treatments (770 & 1460 μatm pCO_2) compared to controls at 5 dph (Fig. 5.2a). A similar result was found at the highest treatment level at 8 dph, but not at 9 or 13 dph. Correspondingly, a significantly higher proportion of larvae were undergoing flexion at 8 dph in the 1460 μatm pCO_2 treatment compared to controls (Figure 5.2b). By the second day of sampling for flexion nearly all larvae had begun to flex and thus no significant differences were evident. Routine swimming activity was significantly impacted by CO_2 treatment for one metric: larvae from the 1460 μatm pCO_2 treatment level exhibited significantly lower maximum swimming velocity compared to controls (Fig. 5.3a). A similar trend was observed for mean swimming velocity, but this pattern was not

significant (Fig. 5.3b). Angle change and net-to-gross-displacement were not significantly affected by CO_2 treatment, and there were no significant treatment effects on swimming activity following olfactory stimulation with conspecific chemical alarm cue. See Table 5.3 for summary statistics from all EX2 analyses.

Experiment 3

Neither proportion of eggs hatched nor larval size-at-hatch were significantly impacted by CO_2 treatment during EX3, although both elevated-CO_2 treatments (1190 & 2170 μatm pCO_2) exhibited a trend of lower proportion hatched compared to controls (Fig. 5.4a). There were no significant differences in the SL of larvae on any sampling day (2, 6, and 21 dph; Fig. 5.4b) and fish were not sampled to analyze progression through flexion. Larvae at 20 dph demonstrated a trend of lower U_{crit} with increasing pCO_2, was this pattern was not significant (Fig. 5.5).

Left sagittal and lapillar otolith length, width, and area were all significantly larger in larvae from the 2170 μatm pCO_2 treatment, compared to controls (Fig. 5.6). 1190 μatm pCO_2 treatment otoliths were of intermediate size and not significant different from either treatment or controls, with the exception of left sagitta being significantly wider compared to controls. The regression of lapillus length and area against SL from the 1190 μatm pCO_2 treatment did not satisfy the assumption of equal slopes compared to ambient and 2170 μatm pCO_2 treatments, and was removed from further analysis. There were no significant differences in otolith roundness or rectangularity. See Table 5.4 for summary statistics from all EX3 analyses.

Discussion

In contrast to our original hypotheses, our results indicate a general pattern of resistance to ocean acidification, with no observed effects on hatch rate, size, development, and swimming ability in larval mahi-mahi. However, this pattern of resistance is disrupted by significantly increased larval size-at-age, altered routine swimming activity, and otolith overgrowth in acidified treatments during two of three experiments. The lack of major impacts of acidification on mahi-mahi larvae is consistent with the assumption that this species has the ability to maintain tight physiological regulation of its internal environment, a general trait of organisms with high metabolic rates (Melzner et al. 2009a). By maintaining tight control over internal pH, mahi-mahi may be able to avoid reduced protein biosynthesis that occurs at reduced pH (Langenbuch & Pörtner 2003), thus preventing negative impacts on size and development. Alternatively, effects of increased pCO_2 may have been too small to be detected during the present study. During Experiment 1 (EX1), it is possible that an effect of acidification on growth and development was masked by temperature-driven depression of metabolic and growth rates across controls and treatments. EX1 was conducted at the lowest average temperature (~24°C) and larvae were observed to grow and develop at the slowest rates of any experiment. If acidification impacted the metabolism of larvae, which could be manifested as either a depression (Pörtner et al. 2005) or increase in metabolic rate (Miller et al. 2012), the magnitude of this effect could have been reduced to the point of non-detection due to the overall lower metabolic rates and slower growth.

Contrasting our results from EX1, mahi-mahi larvae in Experiment 2 (EX2) exhibited an increase in size and developmental rate in high-CO_2 seawater. The growth-mortality hypothesis suggests that such results could be beneficial to larval survival, allowing them to pass through gape-limited predation windows more quickly than smaller larvae (Anderson 1988). We are not the first observe an increase in the size of larval fish under acidified conditions: Munday et al. (2009b) reported up to 18 % longer damselfish larvae at high-pCO_2 levels and suggested that such an effect could be accomplished through either increased energy intake or decreased energy expenditure. In a captive rearing environment, prey are consistently provided at higher quantities than what is expected in nature, thus larvae in the present study may have been able to increase their energy intake to compensate, or overcompensate, for an increase in the metabolic cost of acid-base balance under acidified conditions (Deigweiher et al. 2008, Miller et al. 2012). This has been demonstrated with mussels in both laboratory and natural marine environments, where food availability predominantly influences growth compared to increased pCO_2 (Thomsen et al. 2013). Reduced energy expenditure is also possible through either metabolic or behavioral acclimation. Metabolic depression occurs in some marine organisms in response to environmental hypercapnia (Langenbuch & Pörtner 2003), but this typically has been detected at very low pH (~6.5) and would likely result in reduced growth (Pörtner et al. 2004). Our results indicate that a reduction in energy expenditure is more likely explained via altered behavioral activity. Mahi-mahi larvae exhibited a lower maximum swimming velocity, and a trend of lower mean swimming velocity during the same experiment (EX2) when increased larval size was observed. This contrasts with EX1, during which no change in swimming activity or

larval size was detected. Our observation of altered routine swimming activity is consistent with our original hypothesis and could help compensate for possible CO_2-driven increases in the metabolic cost of acid-base regulation, allowing for re-allocation of metabolic resources to somatic growth. Differences in larval size between treatments decreased to a point of non-significance after 8 dph, suggesting that this behavioral response or its effectiveness may be transient.

In nature, reduced routine swimming velocity has ecological implications for the feeding and survival of larvae. Prey encounter rate is directly related to predator swimming speed (Gerritsen & Strickler 1977), thus larvae under elevated-CO_2 conditions may exhibit reduced feeding success due to lower encounter rates. This would not be evident in a laboratory scenario with abundant prey availability, but could be critical in nature, where starvation is thought to be an important process influencing larval survival and recruitment magnitude (Houde 1989b). Reduced food intake has the further potential to interact with larval susceptibility to elevated pCO_2, for example cobia exhibits reduced starvation resistance under acidified conditions (Bignami et al. in review). This combination of effects could negatively influence cumulative survival through the larval stages and result in lower recruitment magnitude into the adult population.

During Experiment 3 (EX3), we observed no significant change in hatch rate or U_{crit} under elevated-CO_2 treatments. These results are consistent with reports for the U_{crit} of other species at various ontogenetic stages (Melzner et al. 2009b, Munday et al. 2009b, Bignami et al. 2013a, in review). Not surprisingly, otolith growth was significantly higher in larvae raised under acidified conditions. This has been observed in a number of species (Munday et al. 2011b, Bignami et al. 2013a, b) and is thought to be caused by the

establishment of a more favorable calcifying environment through the retention of bicarbonate ions in the extracellular fluid (Checkley et al. 2009), a physiological mechanism used by fishes to compensate for increased blood pCO_2 during hypercapnic events (Esbaugh et al. 2012). The presence, absence, or severity of otolith overgrowth may be directly related to the magnitude of this physiological response as well as the non-carbonate blood buffer capacity of different species. The lowest pCO_2 known to affect otolith devlopment is 800 μatm, reported for 21 dph cobia (Bignami et al. 2013a), while in other species much higher treatments (~4000 μatm) have failed to induce a significant change (Frommel et al. 2012). Otolith size and density influences the function of otoliths as auditory sensory organs: modeling of larval cobia otoliths with up to 58% greater mass indicated increased auditory sensitivity and a ~ 50 % extension of hearing range (Bignami et al. 2013b). In the present study, we found an increase in otolith size, thus it is likely that mass is also increased, which will similarly impact the auditory sensitivity and hearing range of mahi-mahi larvae. Although mahi-mahi do not navigate and recruit to a noisy benthic environment like many reef fishes, auditory sensation likely plays an important role in prey detection, communication, and navigation in the pelagic environment as well.

The experimental results presented here provide perspective on the impact of acidification on larval mahi-mahi as a model for other offshore pelagic species with similar life history strategies and physiological characteristics. Generally, our results are consistent with previous reports for cobia (Bignami et al. 2013a, b, in review), which was unexpected due to the more strictly offshore pelagic life history of mahi-mahi. Together, these studies allow us to conclude that while mahi-mahi and cobia are not immune to

ocean acidification, these pelagic fishes have not demonstrated greater susceptibility to ocean acidification than presumably more resistant nearshore demersal species (Munday et al. 2008a, Pörtner 2008). Although our results offer a potentially optimistic outlook for the future of large pelagic species in the face of future ocean acidification, our study is only the first on the larvae of an offshore pelagic tropical species. Furthermore, our study did not address several important factors: the possibility for subtle impacts of acidification that were not measured, the cumulative effect of long-term exposure to ocean acidification and its potential effects on growth and reproduction (Pörtner et al. 2005), and the combined effect of multiple simultaneous stressors which fishes will experience as a result of climate change.

While it has been demonstrated that ocean acidification has the potential to impact fisheries productivity and the economies that they support (Cooley & Doney 2009, Cheung et al. 2011), the magnitude of such impacts is not always apparent. Even as we improve our understanding of the basic physiological responses of marine organisms to acidification, our ability to scale these responses up to population- and ecosystem-level effects remains limited (Le Quesne & Pinnegar 2011). By focusing on a high-value tropical pelagic species, this study strengthens our foundation of knowledge and contributes to the ultimate goal of reaching sound conclusions which can contribute to informed management and conservation decisions.

Table 5.1 Water chemistry conditions during Experiment 1 (EX1), Experiment 2 (EX2) and Experiment 3 (EX3). Temperature, pH, and total alkalinity (TA) were measured and mean pCO_2 calculated with the software CO2SYS (Lewis & Wallace 1998). Values are means (± SE).

Treatment level	**Temp.** °C	**pH** total scale	**TA** µmol kg^{-1}	***p*CO₂** µatm
EX1				
Ambient/Control	23.9 (± 0.2)	8.00 (± 0.01)	2457 (± 22)	487 (±24)
1600 µatm pCO_2	23.9 (± 0.2)	7.54 (± 0.01)	2462 (± 22)	1595 (± 85)
EX2				
Ambient/Control	25.3 (± 0.2)	8.11 (± 0.005)	2387 (± 24)	348 (± 17)
770 µatm pCO_2	25.3 (± 0.2)	7.82 (± 0.01)	2384 (± 23)	767 (± 52)
1460 µatm pCO_2	25.3 (± 0.2)	7.56 (± 0.01)	2394 (± 23)	1461 (± 56)
EX3				
Ambient/Control	28.1 (± 0.2)	7.99 (± 0.01)	2326 (± 30)	454 (± 17)
1190 µatm pCO_2	28.1 (± 0.2)	7.62 (± 0.01)	2320 (± 28)	1189 (± 65)
2170 µatm pCO_2	28.1 (± 0.2)	7.38 (± 0.02)	2332 (± 43)	2172 (± 159)

Table 5.2 Summary of statistical results from analysis of variance of standard length (SL), flexion, and routine swimming activity of mahi-mahi larvae across multiple days post hatch (dph) during Experiment 1.

Metric	F-value	P-value	df
SL (dph)			
5	0.557	0.473	1,10
8	0.014	0.909	1,10
9	0.058	0.814	1,10
10	0.012	0.915	1,10
11	0.286	0.605	1,10
12	0.089	0.772	1,10
Flexion (dph)			
9	0.061	0.810	1,10
10	0.293	0.601	1,10
11	0.527	0.484	1,10
12	0.966	0.349	1,10
Swimming activity			
Mean velocity	2.535	0.142	1,10
Max velocity	0.117	0.740	1,10
Net-to-gross displacement	0.892	0.367	1,10
Angle change	0.574	0.466	1,10

Table 5.3 Summary of statistical results from analysis of variance of proportion of mahi-mahi larvae hatched (Hatch), standard length (SL), flexion, and routine swimming activity across multiple days post hatch (dph) during Experiment 2. Tukey post-hoc results compare ambient pCO_2 (C), 770 µatm pCO_2 (1), and 1460 µatm pCO_2 (2) treatments.

Metric	F-value	P-value	df	Tukey
Hatch				
	0.685	0.528	2,9	C = 1 = 2
SL (dph)				
0	0.598	0.570	2,9	C = 1 = 2
5	6.635	**0.017**	2,9	C < 1 = 2
8	5.191	**0.032**	2,9	1 = C < 2 = 1
9	1.512	0.272	2,9	C = 1 = 2
13	0.866	0.457	2,8	C = 1 = 2
Flexion (dph)				
8	4.988	0.**035**	2,9	C < 1 = 2
9	2.835	0.112	2,9	C = 1 = 2
Swimming activity				
Mean velocity	3.122	0.093	2,9	C = 1 = 2
Max velocity	9.454	**0.006**	2,9	C < 1 = 2
Net-to-gross displacement	0.321	0.734	2,9	C = 1 = 2
Angle change	0.021	0.980	2,9	C = 1 = 2

Table 5.4 Summary of statistical results from analysis of variance (ANOVA) of proportion of mahi-mahi larvae hatched (Hatch), standard length (SL), critical swimming speed (U_{crit}), and otolith size/shape across multiple days post hatch (dph) during Experiment 3. Analysis of covariance (ANCOVA) results presented for otolith length, width, and area are from left lapillus (LL) and left sagitta (LS). Regression slopes for LL length and area of 1190 µatm pCO_2 treatment otoliths differed from ambient and 2170 µatm pCO_2 treatments, and were therefore removed from analysis. Tukey post-hoc results compare ambient pCO_2 (C), 1190 µatm pCO_2 (1), and 2170 µatm pCO_2 (2) treatments.

Metric	F-value	P-value	df	Tukey
Hatch				
	3.475	0.076	2,9	C = 1 = 2
SL (dph)				
0	0.817	0.472	2,9	C = 1 = 2
2	1.546	0.265	2,9	C = 1 = 2
6	0.909	0.437	2,9	C = 1 = 2
21	0.802	0.482	2,9	C = 1 = 2
U_{crit}				
20	2.091	0.179	2,9	C = 1 = 2
Otolith size/shape				
LS length	6.797	**0.023**	2,7	1 = C < 2 = 1
LS width	28.444	**<0.001**	2,7	C < 1 = 2
LS area	11.321	**0.006**	2,7	1 = C < 2 = 1
LS Rectangularity	0.321	0.735	2,8	C = 1 = 2
LS Roundness	1.795	0.227	2,8	C = 1 = 2
LL length	8.529	**0.043**	1,4	C < 2
LL width	5.806	**0.033**	2,7	1 = C < 2 = 1
LL area	13.948	**0.020**	1,4	C < 2
LL Rectangularity	0.381	0.695	2,8	C = 1 = 2
LL Roundness	0.939	0.430	2,8	C = 1 = 2

Figure 5.1 Effects of ocean acidification on (**a**) size-at-age and (**b**) proportion of larval mahi-mahi undergoing flexion during Experiment 1. White and black bars represent ambient (490 μatm) and high (1600 μatm) pCO_2 treatments. Within each day post hatch, bars that share a letter are statistically similar ($P > 0.05$). Values are tank means ±SEM ($N = 6$ per treatment).

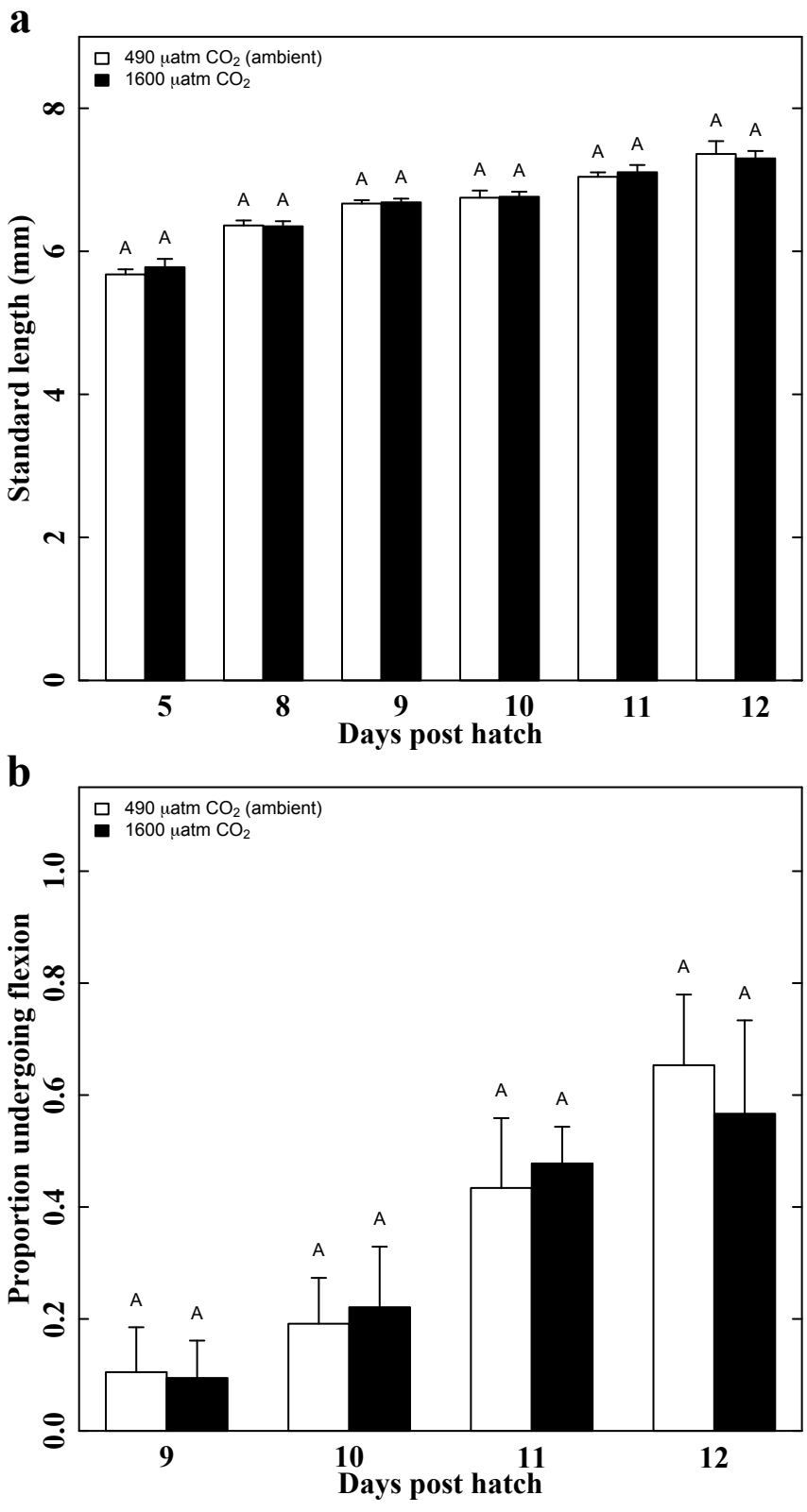

a
490 μatm CO2 (ambient)
1600 μatm CO2
Standard length (mm)
0
2
4
6
8
A
5
8
9
10
11
12
Days post hatch
b
490 μatm CO2 (ambient)
1600 μatm CO2
Proportion undergoing flexion
0.0
0.2
0.4
0.6
0.8
1.0
A
9
10
11
12
Days post hatch

Figure 5.2 Effects of ocean acidification on (**a**) size-at-age and (**b**) proportion of larval mahi-mahi undergoing flexion during Experiment 2. White, gray, and black bars represent ambient (350 μatm), 770, and 1460 μatm pCO_2 treatments, respectively. Within each day post hatch, bars not sharing a letter are significantly different ($P < 0.05$). Values are tank means ±SEM ($N = 4$ per treatment).

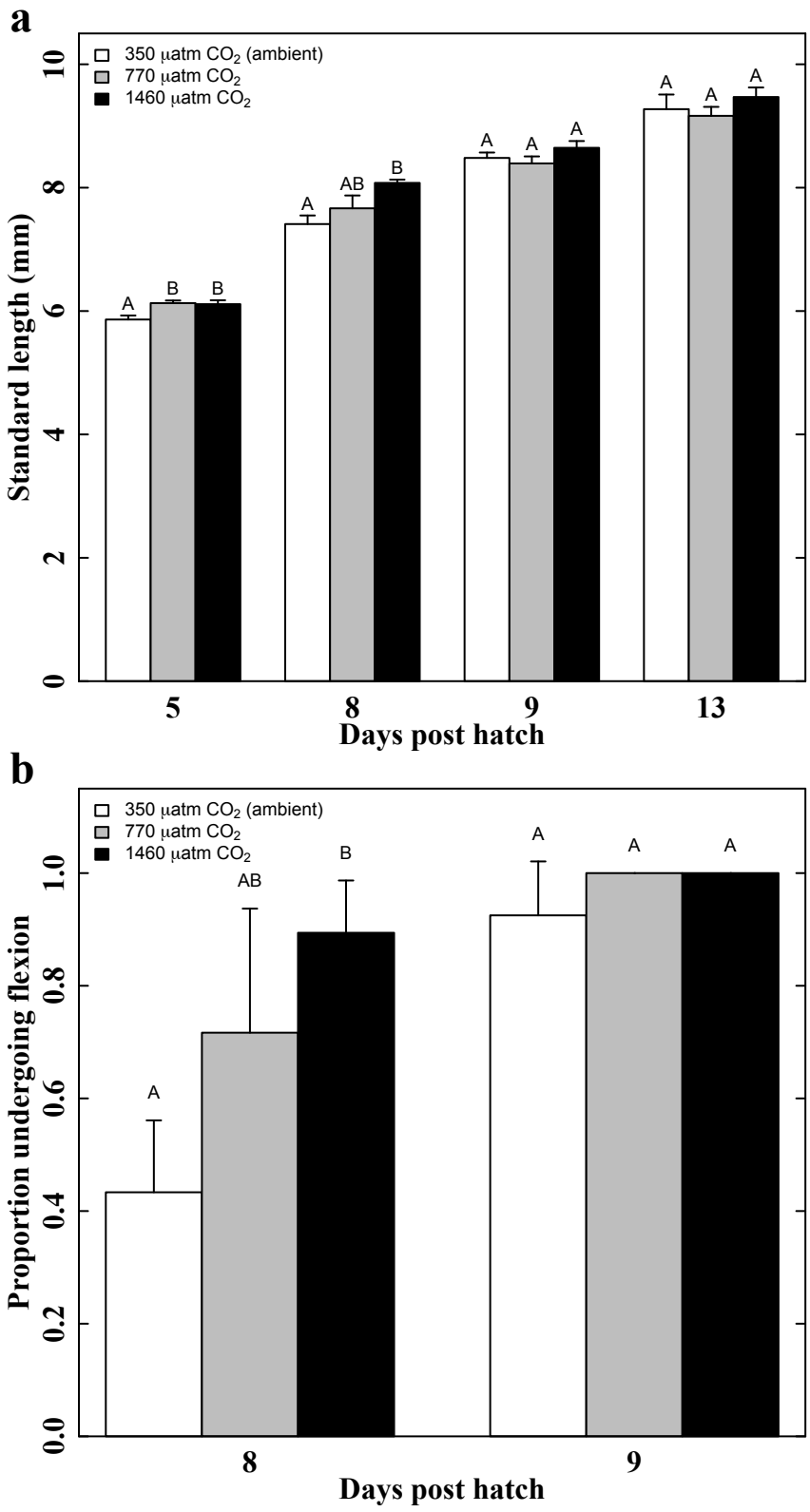

a
350 μatm CO2 (ambient)
770 μatm CO2
1460 μatm CO2
Standard length (mm)
0
2
4
6
8
10
A
B
B
A
AB
B
A
A
A
A
A
A
5
8
9
13
Days post hatch
b
350 μatm CO2 (ambient)
770 μatm CO2
1460 μatm CO2
Proportion undergoing flexion
0.0
0.2
0.4
0.6
0.8
1.0
A
AB
B
A
A
A
8
9
Days post hatch

Figure 5.3 Results of routine swimming activity observations and analysis of variance of (**a**) maximum swimming velocity and (**b**) mean swimming velocity for larval mahi-mahi during Experiment 2. White, gray, and black bars represent ambient (350 μatm), 770, and 1460 μatm pCO_2 treatments, respectively. Bars not sharing a letter are significantly different ($P < 0.05$). Values are tank means ±SEM ($N = 4$ per treatment).

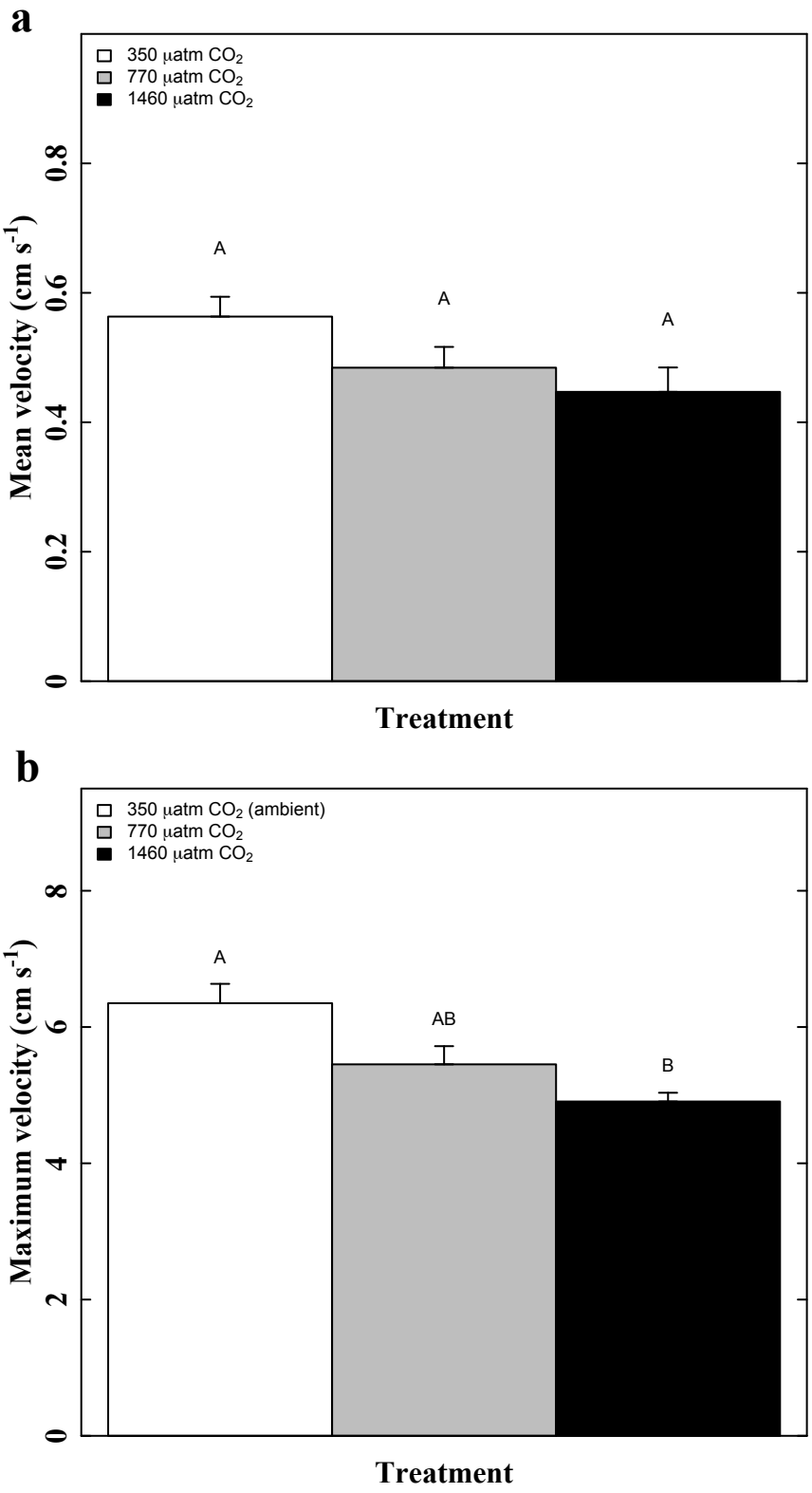
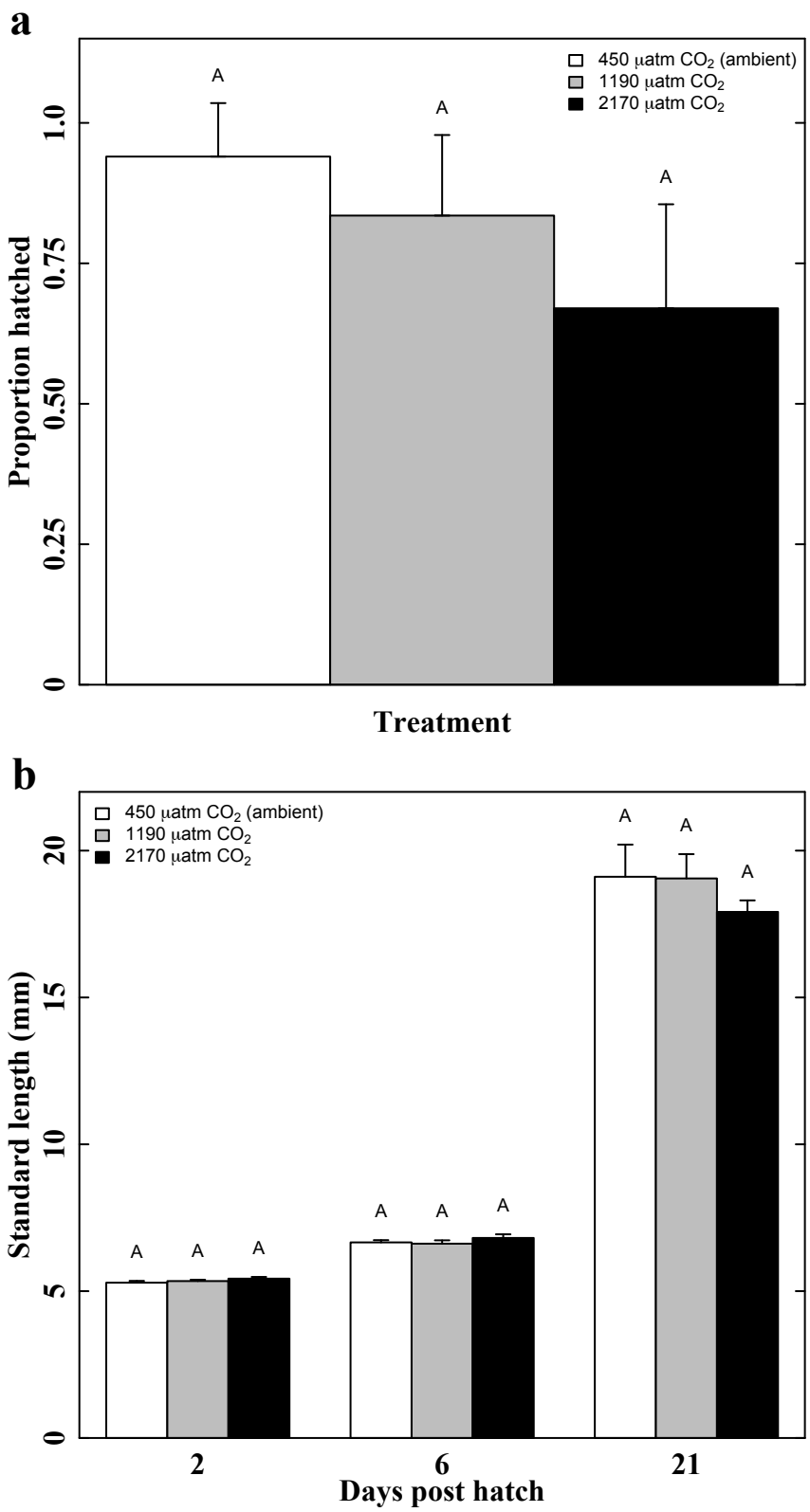

a
350 μatm CO2
770 μatm CO2
1460 μatm CO2
Mean velocity (cm s-1)
0
0.2
0.4
0.6
0.8
A
A
A
Treatment
b
350 μatm CO2 (ambient)
770 μatm CO2
1460 μatm CO2
Maximum velocity (cm s-1)
0
2
4
6
8
A
AB
B
Treatment

Figure 5.4 Effects of ocean acidification on (**a**) the proportion of eggs hatched, (**b**) size-at-age, and (**c**) proportion of larval mahi-mahi undergoing flexion during Experiment 3. White, gray, and black bars represent ambient (450 μatm), 1190, and 2170 μatm pCO_2 treatments, respectively. Bars not sharing a letter (within each day post hatch in **b** and **c**) are significantly different ($P < 0.05$). Values are tank means ±SEM ($N = 4$ per treatment).


a
450 μatm CO2 (ambient)
1190 μatm CO2
2170 μatm CO2
A
A
A
Proportion hatched
0
0.25
0.50
0.75
1.0
Treatment
b
450 μatm CO2 (ambient)
1190 μatm CO2
2170 μatm CO2
A
A
A
A
A
A
A
A
A
Standard length (mm)
0
5
10
15
20
2
6
21
Days post hatch

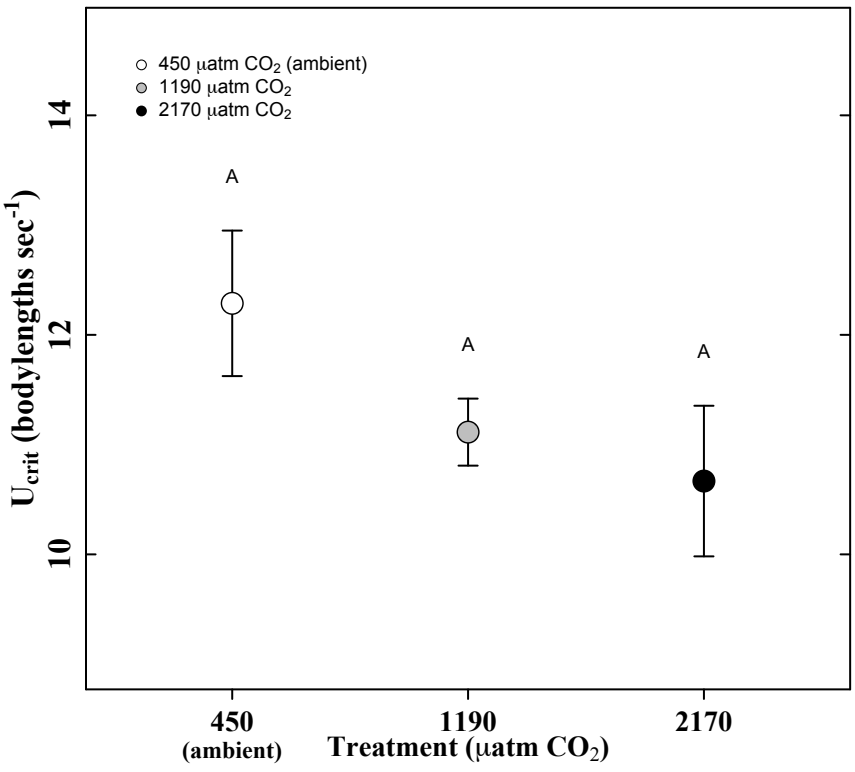
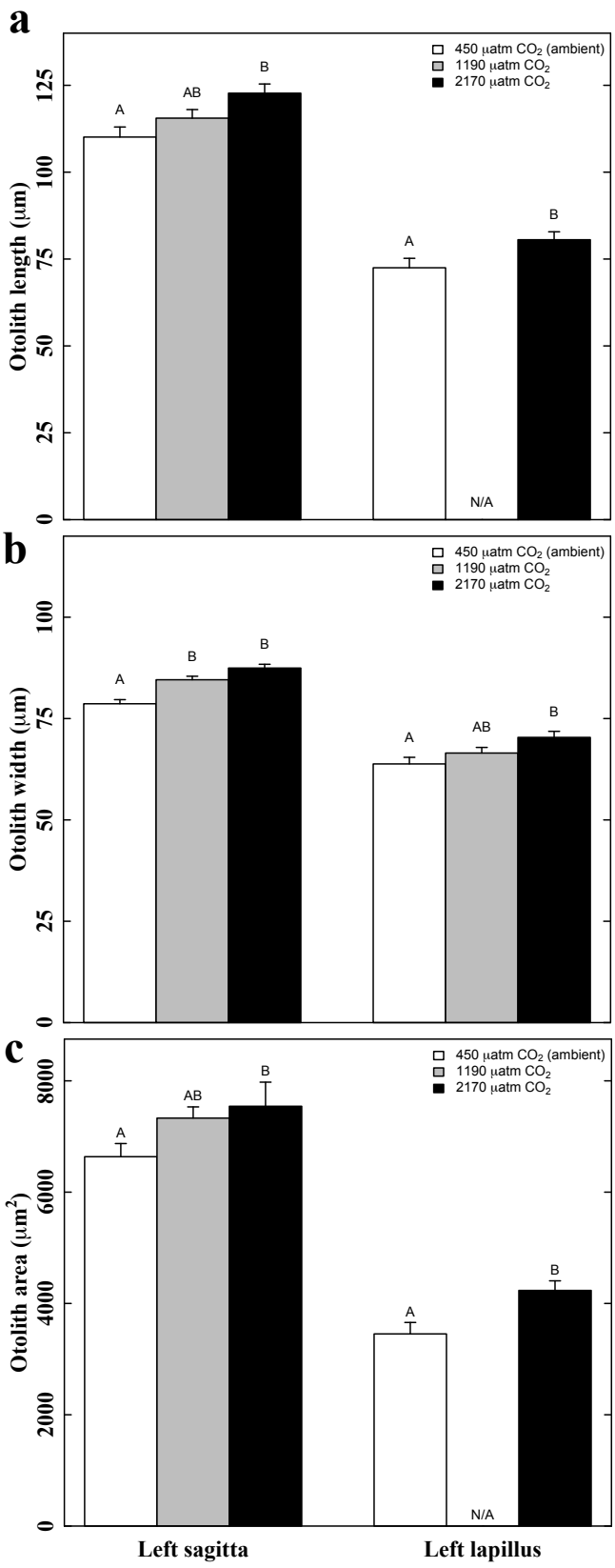


Figure 5.5 Critical swimming speed (U_{crit}) of 20 day post hatch larval mahi-mahi during Experiment 3. Data points sharing a letter are statistically similar ($P > 0.05$). Values are tank means ±SEM ($N = 4$ per treatment).

Figure 5.6 Results from analysis of covariance (ANCOVA) of sagittal and lapillar otolith (**a**) length, (**b**) width, and (**c**) area for 20 day post hatch larval mahi-mahi during Experiment 3. White, gray, and black bars represent ambient (450 μatm), 1190, and 2170 μatm pCO_2 treatments, respectively. ANCOVA results are from left lapillus and left sagitta. In **(a)** and **(c)**, the slope of the regression of left lapillus length and area against fish standard length differed other treatments, therefore it was removed from analysis. Within each otolith type, bars not sharing a letter are significantly different ($P < 0.05$). Values are adjusted tank means ±SEM ($N = 4$ per treatment).


a
b
c
450 μatm CO2 (ambient)
1190 μatm CO2
2170 μatm CO2
Otolith length (μm)
Otolith width (μm)
Otolith area (μm2)
0
25
50
75
100
125
2000
4000
6000
8000
A
AB
B
N/A
Left sagitta
Left lapillus

CHAPTER 6. Conclusions

Summary

This dissertation contributes a number of novel advances and improvements to our understanding of the impact of ocean acidification on larval fishes. Experiments demonstrated that larval cobia, the largest tropical pelagic species studied to date, is resistant to major impacts on growth, development, swimming activity, and swimming ability due to acidification levels projected for the next several centuries. The exception to this pattern was our report of reduced larval growth rate, size-at-age, and development due to elevated CO_2 during the experiment crossing acidification and temperature. Additionally, more extreme acidification clearly resulted in smaller sizes-at-age and delayed development. When more discreet metrics were evaluated, it became evident that cobia is not entirely resistant to acidification. Otoliths were signifcantly larger at pCO_2 levels as low as 800 μatm, with higher levels also producing larger and more dense otoliths of up to 50% greater mass. Using mathematical modeling, we demonstrated that this effect on otoliths is likely to result in increased auditory sensitivity and up to a 50% increase in hearing range. Although larval cobia also appeared to be resistant to impacts of acidification under conditions of reduced food ration, when subjected to complete feeding cessation, they demonstrated vulnerability to acidification in the form of decreased time to 50% mortality.

Our study of larval mahi-mahi is the first on a high-value study species that strictly inhabits offshore pelagic waters, and produced similar findings as those for cobia. Mahi-mahi were resistant to acidification as measured by larval size, development, and swimming ability, and even exhibited a transient increase in size-at-age and

developmental progression under elevated CO_2 conditions during one experiment. Those growth effects corresponded to a significant reduction in maximum swimming velocity, as well as a non-significant trend of decreased mean swimming velocity, during routine swimming observations. This observation may reflect a behavioral modification of metabolic demand to compensate for the acid-base challenge of acidification. Our evaluation of mahi-mahi otolith development once again revealed significantly larger otolith size with increased pCO_2, further demonstrating that metrics for inconspicuous effects of ocean acidification can identify susceptibility when more gross metrics do not. Together, these results allow us to conclude that both species, while resistant to ocean acidification as measured by many of our chosen metrics, exhibit some susceptibility, the consequences of which are not yet clear.

Susceptibility of pelagic species to ocean acidification

Our study is the first report of the impact of acidification on large, tropical, pelagic fish species. Based on the results presented here, we are able to conclude that while cobia and mahi-mahi are not entirely resistant to ocean acidification, these pelagic fishes have not demonstrated greater susceptibility than previously studied demersal species which are generally expected to exhibit greater resistance (Munday et al. 2008a, Pörtner 2008). Instead, this resistance is consistent with the concept that tight physiological regulation of the internal environment may result in some resistance to acidification for species with high metabolic rates (Melzner et al. 2009a). The general lack of major impacts on larval growth and development during the limited duration of our experiments, suggests that neither study species will experience reduced larval survival as according to the growth-mortality hypothesis (Anderson 1988). However,

more work is necessary before it will be possible to reach firm conclusions about long term impacts of ocean acidification on tropical pelagic species. Our identification of effects on otolith development and starvation resistance provide an important illustration of the discreet effects that could compound to cause a more critical overall effect of ocean acidification. Additionally, based on the results of our relatively short duration experiments (up to 21 d), it cannot be assumed that cobia and mahi-mahi will be unaffected over their entire ontogeny or that populations will ultimately tolerate the rapid, sustained acidification of the marine environment. Thus, despite a definite improvement in our understanding of the impacts of ocean acidification on larval fishes, the management implications for topical pelagic species remain unclear. Our ability to provide useful conclusions for managers will require a more complete understanding of the impact of ocean acidification on fishes and will only be possible with the continued advancement of this field of research by expanding upon current information and filling critical knowledge gaps.

Experimental design and statistical power

During these experiments, the occurrence of significant treatment effects was variable and dependent on the chosen metric, highlighting the importance of including both gross (i.e., whole organism) and discreet (i.e., inconspicuous or sub-organismal) measures of treatment effect into the design of experimental studies. This is evident in the literature, where few studies have reported direct negative impacts on gross metrics such as growth, survival, or swimming ability (except see Baumann et al. 2011, Miller et al. 2012), yet many more have found significant effects of acidification on more discreet metrics such as behavior, sensory function, and otolith development. Experimental

designs are often limited by the availability of resources, consequently, it is not uncommon for studies to have limited replication, which reduces statistical power and constrains the ability to detect treatment effects. This can be further exaggerated by relative small magnitude treatment effects and high variance among replicates.

Our results for mahi-mahi mean swimming velocity reflect the problems with analyzing data of low statistical power. This analysis did not produce significant results despite a 20% reduction in mean swimming velocity between control and high-CO_2 treatments. When calculated using treatment means and standard deviation (G*Power v3.1.5.1; Erdfelder et al. 1996), our data had a effect size of 0.72 and a resulting power of 0.46. This is well below 0.80, the recommended minimum level of statistical power needed to adequately detect treatment effects (Cohen 1988). To have achieved sufficient power, we needed to have doubled the number of total replicate tanks from 12 to 24 (Figure 6.1), or increased our effect size to 1.07 via greater magnitude treatment effects or reduced variance. In contrast, our analysis of maximum swimming velocity had a power of 0.93 due to an overall greater effect size of 1.31. Although the magnitude of our treatment effect on maximum swimming speed was similar to mean velocity (23% versus 20%), the standard deviation was nearly half that of mean velocity (8% versus 14%) and likely caused the increased effect size and higher statistical power.

As demonstrated in Chapter One, the magnitude (or presence) of treatment effects on metrics such as size or survival may correspond to treatment severity. However, in contrast to early studies which utilized extremely high pCO_2 (e.g. >50000 μatm; Hayashi et al. 2004), it is presently recommended that most ocean acidification studies apply more realistic pCO_2 treatments (up to ~1000ppm) according to projected scenarios (Barry et al.

2010). These “best practices” constrain the use of treatments exaggerated beyond what is projected to occur over the next century. As an alternative to extreme treatments, one way to increase the power of statistical analyses is to minimize variability among replicates. Although this fundamental aspect of experimental design is often desired, variability is not always under the investigator’s control, especially when measuring intrinsically variable metrics. Increased replication is therefore the most tractable solution to low statistical power. This has been achieved in studies utilizing well-designed (and well-funded) experimental systems capable of providing high replication. Maximizing replication should be a high priority when designing future studies and experimental research systems, especially when using gross metrics to evaluate treatment effects.

Future Directions

Within the past five years, our understanding of the impacts of projected ocean acidification on fishes has rapidly expanded beyond a body of literature solely focused on the physiological responses of fishes to severe hypercapnia. Amidst rare reports of direct impacts on size or survival (Baumann et al. 2011), some of the highlights during this period have included the discovery that exposure to elevated pCO_2 interferes with olfactory detection and homing ability in damselfish (Munday et al. 2009a). This subsequently led to reports of numerous altered behavioral traits (e.g., Simpson et al. 2011, Ferrari et al. 2012b, and others) and the finding that these effects are likely driven by a disruption in neurological function due to the physiological response of fishes to elevated pCO_2 (Nilsson et al. 2012). Other unexpected results included impacts on otolith size and density across multiple species (Checkley et al. 2009, Munday et al. 2011b, Bignami et al. 2013a, b), as well as tissue damage in Atlantic cod (Frommel et al. 2011).

The addition of our studies on large tropical pelagic fishes has improved the scope of study species and provided additional perspective on species with life history characteristics contrasting those of many other study species. Collectively, these advances have allowed us to better understand the potential impacts of ocean acidification on fishes, however, we are still greatly limited by the nascency of this field.

While the initial development of this research necessitated simple experimental designs to provide a basic understanding of the impacts of ocean acidification on larval fishes, continuing along this path for too long puts us a risk for developing a misguided understanding of the true impacts of ocean acidification as it will be experienced in nature. Many acidification studies on fishes have aimed to describe gross effects that can be easily translated into potential implications for impact at the population level. These types of metrics are valuable when detected, but when no effect is observed, they can hinder our ability to determine true susceptibility to ocean acidification. Additional approaches are necessary if we hope to detect sublethal effects that may not be observed during short experimental durations, identify underlying mechanisms that can help explain broader patterns, or simply create a more accurate representation of a high-CO_2 marine environment in our experiments. These approaches include the lengthening of experimental durations, the use of more sensitive and informative metrics, the application of multiple stressors, and the inclusion of variable pH during acidification experiments.

Experimental duration

Subtle effects of ocean acidification may not manifest during the relatively short duration of early ontogeny, but can accumulate over the long term to impact endpoints such as growth and reproduction (Pörtner et al. 2005). When logistically possible, the lengthening of experimental duration to include full ontogeny, or even multiple generations, will improve the likelihood of observing these impacts. This was recently achieved by Miller et al. (2012), who reported trans-generational effects wherein offspring of parents living in elevated pCO_2 conditions were less susceptible to ocean acidification. However, long multigenerational experiments are not feasible for some study species, for example, pelagic species that require extensive aquaculture facilities. Therefore there is also a need for the use of more sensitive and informative metrics that can detect less conspicuous effects in the short term.

Sensitive and informative metrics

Our report of acidification impacts on otolith development is an example of how some metrics provide more sensitive identification of treatment effects while gross metrics may indicate no effect. Another way to detect discreet effects and determine underlying mechanisms is to utilize more physiological metrics at the organism, organ, cellular, or subcellular level, including the study of genomic, transcriptomic, and proteomic processes (Somero 2012). The study of these functionally informative metrics can help explain the reason for higher-level effects, as was illustrated by Nilsson et al. (2012) through their explanation of the cellular processes driving the various behavioral effects of acidification on fishes. They can also help to elucidate impacts that are otherwise not observable at the organism level. Stress-induced changes in gene

expression or other physiological metrics such as cardiac function can indicate when stress thresholds are surpassed and can help determine if metabolic resources are being reallocated within an organism (Somero 2012, Pörtner 2012). Genetic techniques have been well applied to the study of acidification effects on marine invertebrates, identifying key changes in the expression of genes controlling calcification, cellular stress response, metabolism, and acid-base balance (Todgham & Hofmann 2009, O'Donell et al. 2010). These techniques have been less widely used to study fish, although some studies have reported significant changes in gene expression while measuring acid-base balance (e.g., Deigweiher et al. 2008, Esbaugh et al. 2012) and metabolism (Tseng et al. 2013). These metrics also allow for the comparison between congeneric species that endure different levels of acidification depending on their local environment, potentially illustrating phenotypic plasticity or adaptability, as well as the comparison between responses to individual and multiple stressors (Somero 2012). Although it can be difficult to interpret how discreet effects may influence important processes at the organism-, population-, or ecosystem-level, their addition will surely improve the quality and depth of information we obtain from our studies.

Multiple stressors

Although discreet effects may seem less important than gross effects on growth or survival, when multiple small effects are compounded they have the potential to result in more significant impacts. The potential for compounded discreet effects is high due to the presence of multiple stressors in the marine environment. As discussed in Chapter Four, ocean acidification will occur along with other stressors associated with global climate change, such as increased temperature, altered food availability, and more widespread

hypoxia (Pörtner et al. 2005, Fabry et al. 2008, Guinotte & Fabry 2008, Pörtner 2012). Multi-stressor treatments are often utilized in ocean acidification research on invertebrates (Byrne 2011) and have been applied to studies of adult fish (Munday et al. 2009c), but have not been addressed in the study of larval fishes. Our study addressed the influence of increased temperature and starvation stress on the response to acidification by larval cobia. Unexpectedly, increased temperature and reduced ration did not result in increased susceptibility to acidification. However, the influence of multiple stressors was evident in cobia larvae undergoing complete feeding cessation in high-CO_2 treatments: larvae exhibited a significantly shorter time to 50% mortality. This more rapid mortality likely reflected the presence of subtle increases in metabolic demand under acidified conditions, an effect that was not evident from our analyses of more conspicuous metrics (e.g., size). Furthermore, these results indicate that under future environmental conditions, larvae may face increased risk of starvation, which is already thought to be a natural stressor that influences larval survival and recruitment magnitude (Houde 1989a). While our study provides just one example, there is clear potential for the occurrence of compounding effects in multi-stressor scenarios (Pörtner 2012) and more accurate simulation of the natural marine environment must be considered as a critical factor during the investigation of acidification impacts on any marine organism.

Variable pH

Although the pelagic environment has a relatively stable pH (Hofmann et al. 2011), many nearshore marine environments demonstrate natural variability in pH. High diurnal variation in pCO_2 and pH has been documented in a number of benthic environments, driving pH changes of > 0.5 units in seagrass beds (Invers et al. 1997,

Perez-Dominguez et al. 2006), 0.3 units in kelp forests (Frieder et al. 2012), and as much as 0.7 units on coral reefs (Ohde & van Woesik 1999). Consequently, pH levels can be as low as those projected to occur in the open ocean when atmospheric CO_2 reaches ~700 ppm (~7.8), and exhibit a diurnal swing equivalent to the total change in pH expected over the next three centuries if atmospheric CO_2 reaches nearly 2000ppm (Caldeira & Wickett 2005). In upwelling areas, low pH ranges can be further exacerbated during seasonal upwelling of low pH water into coastal environments (Feely et al. 2008). Also, as more CO_2 is absorbed by the oceans, an increase in the Revelle factor (reduction in the buffer capacity of seawater) will cause a non-linear amplification of pCO_2 as a result of respiration and calcification, producing wider diurnal cycles that are simultaneously declining in pH (Figure 6.2; Shaw et al. 2013). In other words, as ocean acidification worsens, more of the CO_2 in seawater will be stored as dissolved CO_2 (higher pCO_2) and less as carbonic acid, bicarbonate, or carbonate. This will inflict greater impacts on fishes because CO_2 is more bio-permeable and produces more drastic effects than pH change alone (Hayashi et al. 2004, Kikkawa et al. 2004). Although not all species live in variable benthic environments (i.e. pelagic species), these areas represent of some of the most productive and biologically diverse habitats in the world (Lalli & Parsons 1997).

Variable pCO_2/pH has been considered as a potential driver of pre-adaptation and thus resistance to ocean acidification by demersal fishes (Munday et al. 2008a), although this has received limited empirical support because many studies on demersal fishes have reported significant treatment effects. Given the propensity of fishes to fully compensate for CO_2-driven decreases in blood pH as quickly as 2 - 4 h after exposure (Esbaugh et al. 2012), it is possible that diurnal cycles in pCO_2/pH trigger a similar diurnal variation in

this physiological response. This assumes that high pCO_2/low pH values exceed the non-carbonate buffering capacity of the blood and require HCO_3^- accumulation (Melzner et al. 2009a). Conversely, diurnal pCO_2 minimums/pH maximums would "release" this stress, allowing or even requiring the reversal of this physiological response to maintain proper blood pH (Figure 6.3). Even as ocean acidification drives mean pH declines below this threshold, a physiological "release" will still be attained during diurnal pCO_2 minimums/pH maximums until acidification shifts the entire range beyond the non-bicarbonate blood buffering threshold. This has the potential to fundamentally challenge our understanding of the most widely reported impact of acidification, the alteration of fish behavior.

Behavioral impacts on larval and juvenile fish are thought to be caused by the disruption of neurological function as a side effect of physiological balancing of blood pH during hypercapnia (Nilsson et al. 2012). However, behavioral effects have been shown to be inducible only following a minimum 2 - 4 d exposure to elevated-pCO_2 conditions, depending on treatment severity, and were retained for over 24 h after transfer into control seawater conditions (Munday et al. 2010). This implies that a rapid increase in blood $[HCO_3^-]$ did not result in the measurable disruption of neurological function until it was sustained for at least 2 d. We hypothesize that if blood $[HCO_3^-]$ undergoes a similarly rapid decrease upon exposure to diurnal low pCO_2/high pH conditions, then the fundamental driver of neurological disruption and thus behavioral impacts may not be sustained long enough to actually produce these effects. To date, there has been no investigation of the physiological or behavioral response of fishes to diurnally variable pH, both of which are necessary to empirically verify this hypothesis.

Cleary, the study of ocean acidification and fishes has room for significant expansion and advancement. Improvements to experimental duration and the sensitivity of assessed metrics, as well as the incorporation of multi-stressor scenarios and variable pH treatments, will help to build a better understanding of acidification impacts on individual fishes, their populations, and the ecosystems in which they exist. If achieved, this will enable more informed and effective adaptive management that may help mitigate the impacts that a rapidly changing world has on the marine environment.

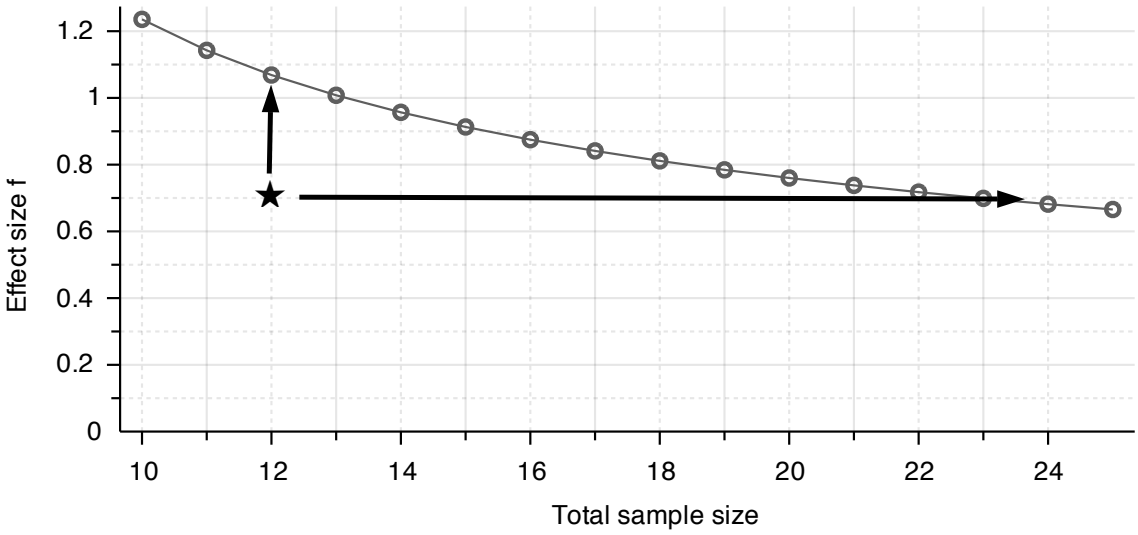
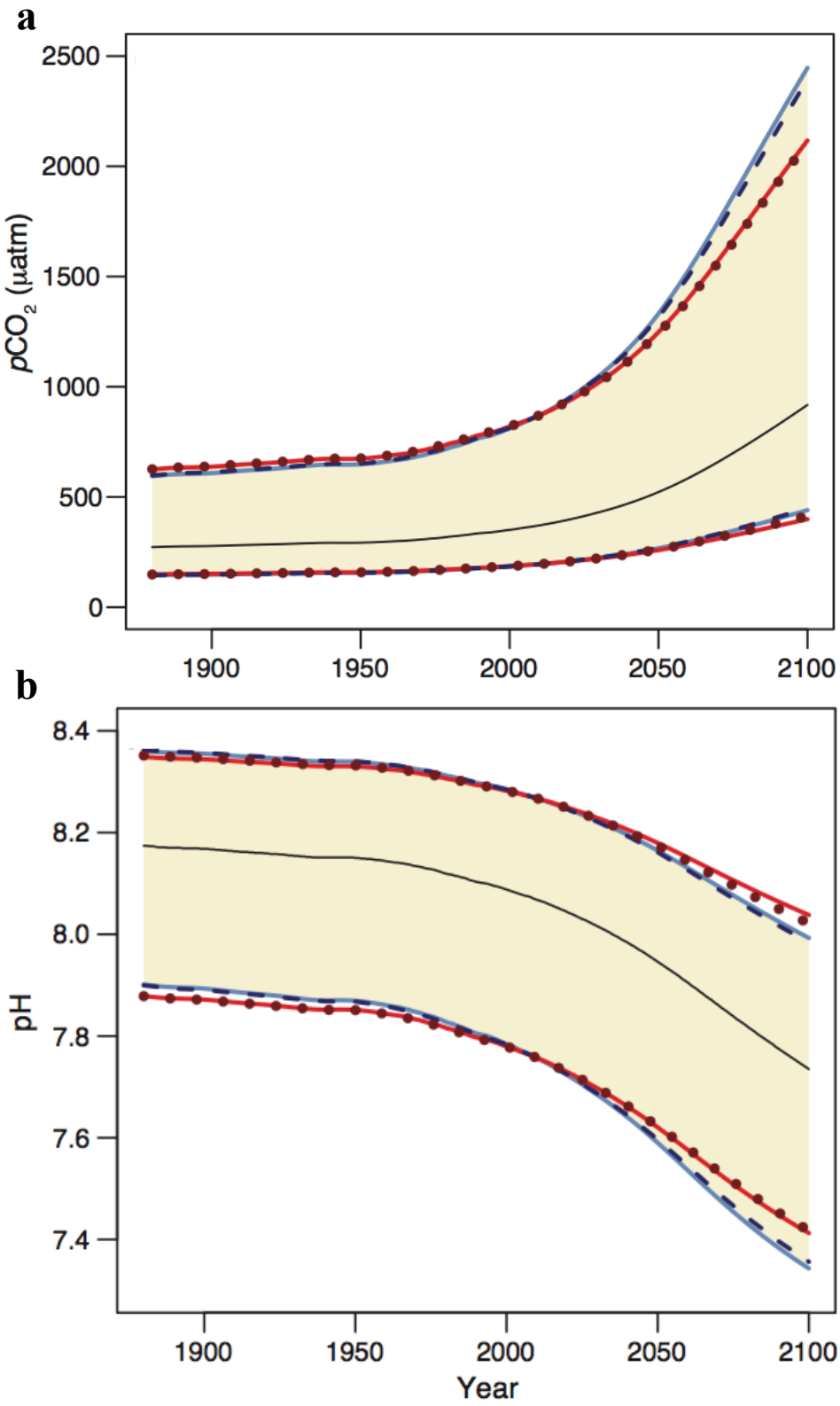


Figure 6.1 Sample sizes required to achieve a power of 0.8 or more depending on effect size. During Experiment 2, mean swimming velocity data (star) produced low statistical power, which could be improved by increasing effect size or replication (arrows). Calculations are based on fixed factor one way analysis of variance.

Figure 6.2 Illustration of a widening diurnal (**a**) CO_2 and (**b**) pH range as the result of ocean acidification and the reduced buffer capacity of seawater. Note the nonlinear increase in upper pCO_2 values relative to lower pCO_2 values. Ranges are based on RCP8.5 emission scenarios and different color lines represent various modeling specifications by the original author. Adapted from Shaw et al. (2013).


a
2500
2000
1500
1000
500
0
pCO2 (µatm)
1900
1950
2000
2050
2100
b
8.4
8.2
8.0
7.8
7.6
7.4
pH
1900
1950
2000
2050
2100
Year

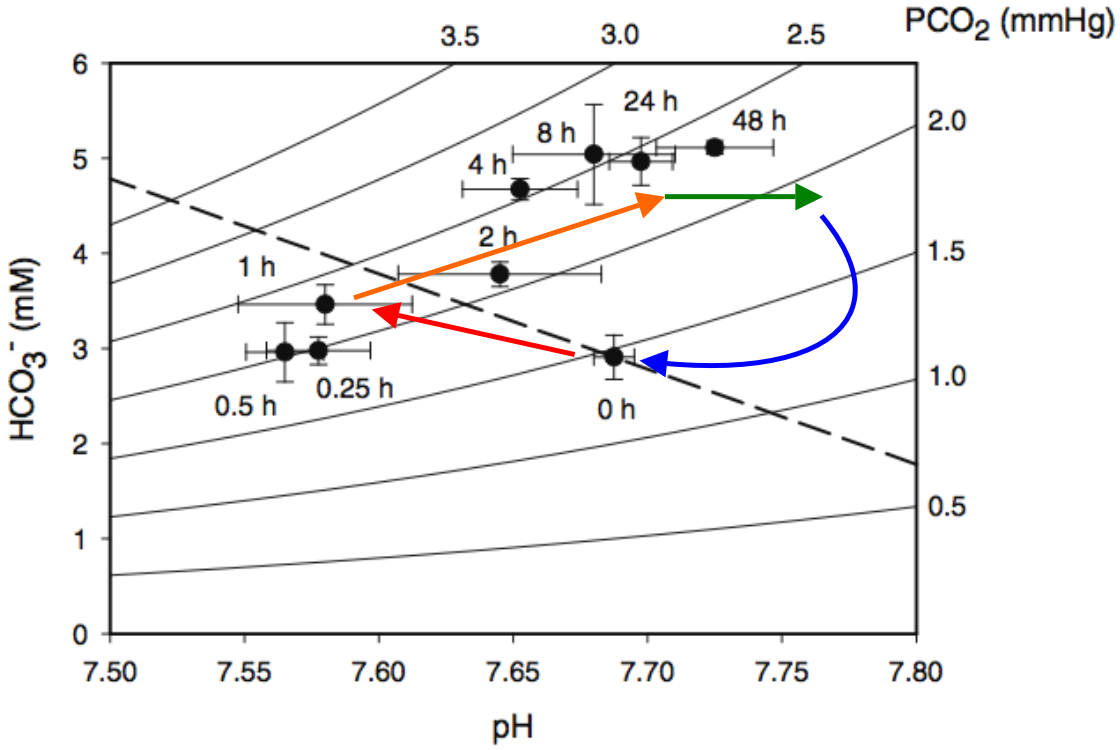


Figure 6.3 Davenport diagram illustrating the hypothesized physiological response to diurnal variation in seawater pCO_2. Adapted from an original diagram of gulf toadfish (*Opsanus beta*) response to 1900 µatm pCO_2 exposure over a 48 h period (Esbaugh et al. 2012). As blood pCO_2 (curved black lines) increases, blood pH initially declines and $[HCO_3^-]$ increases slightly according to the non-bicarbonate buffer capacity of blood (red arrow) before active retention and uptake of HCO_3^- (orange arrow) restores and maintains blood pH near original levels. Subsequent exposure to low pCO_2/High pH during a diurnal cycle would initially result in blood alkalosis (green arrow) until HCO_3^- is excreted to restore proper blood pH (blue arrow).

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APPENDIX 1. Detailed methods from Chapter 4

Materials and methods

Study Species

Cobia (*Rachycentron canadum)* is a highly mobile marine fish that attains sizes > 1.5 m and over 60 kg (Shaffer & Nakamura 1989, Fry & Griffiths 2010). It has a nearly circumglobal distribution in tropical to warm temperate regions and is primarily found in coastal and continental shelf waters (Shaffer & Nakamura 1989). Pelagic spawning occurs during warmer months (25-30°C) and planktonic larvae hatch, undergo flexion, and develop via a gradual transition into the juvenile stage within 30 d (Shaffer & Nakamura 1989). Cobia is a highly predatory fish targeted by recreational and commercial fisheries throughout its distribution (Shaffer & Nakamura 1989, Fry & Griffiths 2010). Global fishery landings of cobia were approx. 11,000 tons in 2000 and aquaculture production had a global value of over USD 36 million in 2004 (Kaiser & Holt 2007).

Experimental design and rearing

Seawater carbonate chemistry was manipulated by addition of equimolar HCl and $NaHCO_3$ prior to introduction into tanks (Gattuso 2010, Bignami et al. 2013a), and tank pH was monitored daily using a handheld pH meter (pH11, Oakton) and Ross Electrode (Orion 9102BWNP, Thermo Scientific) calibrated daily with TRIS buffer. Water samples were collected every 5 d in 250 mL PET bottles, fixed with 100 μL of saturated mercuric chloride, and the total alkalinity (TA) and pH_T were measured using automated Gran titration checked for accuracy with Dickson standards (Scripps Institution of Oceanography; Langdon et al. 2000). CO2SYS was used to solve the carbonate system

using the two measured parameters (pH_T and TA; Lewis & Wallace 1998). Temperature and dissolved oxygen were measured with a combination meter (550A, YSI Inc.) twice and once d^{-1}, respectively, and salinity was measured once d^{-1} using a refractometer (RHS-10 ATC, Premium Aquatics).

Cobia eggs and larvae for each experiment were produced at the University of Miami Experimental Hatchery (UMEH) from a population of 10 F1-generation broodstock (six females, four males). We collected eggs within 12 h of spawning and allowed them to hatch and develop until 2 d post hatch (dph) prior to stocking larvae into 12 replicated (3 replicates per unique treatment combination) 400 L flow-through experimental tanks at a density of 9-10 larvae L^{-1}. Temperature and CO_2 treatments were applied upon stocking, reaching full effect within 24 h. Experimental tanks received filtered, UV-sterilized seawater at a daily exchange rate of 250 % and 350 % during CO_2/Temp and CO_2/Ration, respectively, and were aerated with a small amount (< 1 L min^{-1}) of bubbled air. Tanks were positioned under 95 % shade cloth and fitted with translucent white polyethylene lids to prevent CO_2 off-gassing, intrusion of rain water, or introduction of contaminants. During CO_2/Temp, we fed larvae enriched rotifers (*Brachionus plicatilis*) four to five times d^{-1}, providing total densities of 5 - 10 mL^{-1} d^{-1} between 2 dph and 12 dph, gradually increasing with age (Benetti et al. 2008b). Beginning at 8 dph, larvae were also provided enriched *Artemia* nauplii at total densities of 0.25 - 2 mL^{-1} d^{-1}, gradually increasing with age (Benetti et al. 2008b).

Swimming activity and ability

To evaluate swimming activity, three subsamples of five fish each were removed from each replicate tank prior to the first daily feeding and allowed to acclimatize to a 15 cm diameter observation container for at least 1 h. Following acclimatization, each container of five larvae was placed individually on a clear shelf inside an enclosed PVC observation chamber with a translucent lid and allowed to recover for 2 min. Routine swimming activity was subsequently recorded for 2 min using a low-light video camera (Hi-Res EXvision, Super Circuits) set 40 cm beneath the larvae. Directly following routine swimming observation, a pipette was used to gently add 1 ml of food-scented water (30 mm filtered rotifer culture water) to the center of the observation dish as an olfactory stimulant. Swimming activity in response to olfactory stimulation was then recorded for an additional 2 min. Video observations were converted to digital files, reduced to 2 frames per second, and analyzed with the software ImageJ (v1.46p, National Institutes of Health) using the MTrackJ plug-in (Meijering et al. 2012). Individual larvae were tracked over the entire 2-min routine swimming and subsequent 2-min olfactory stimulation observation periods. Analyses of average angle change and net-to-gross displacement were performed on one randomly selected 20-s period of active swimming per larva (defined as a minimum of 5 cm gross distance traveled in 20 s), to capture periods of active swimming, and because larvae were sometimes stationary for extended periods of time.

To test swimming ability, we collected subsamples of five larvae from each replicate tank in 1-L containers prior to the first daily feeding and allowed the larvae to acclimatize to flume water conditions for at least 1 h. Individual larvae were then placed

in a randomly assigned lane and allowed to acclimatize to the flume for 5 min at a current speed of 2 cm s^{-1}. In a previous study with cobia, logistical constraints required that all fish be tested in water at control conditions (Bignami et al. 2013a), however, during this study we tested larvae in their respective CO_2 treatment conditions at a common intermediate temperature (30.5 ±0.5 °C). Water current speed was increased by approximately 3 cm s^{-1} at 2-min intervals until larvae failed to maintain their position and were swept against a mesh barrier at the end of each flume lane. Flow calibration was verified using dye immediately before and after the day of testing and U_{crit} speeds were calculated in body lengths s^{-1} using the equation $U_{crit} = V_p + ((t_f / t_i) \times V_i)$, where V_p is the penultimate velocity increment successfully completed for the full time interval (t_i), prior to the velocity increment at which failure occurred (V_i) at a failure time (t_f) less than the full time interval (Hammer 1995).

APPENDIX 2. Micro-computed tomography data from mahi-mahi otoliths

Materials and methods

Micro-CT scans

During EX3, three 20-day post hatch larvae per tank were individually scanned in a micro-computed tomography (micro-CT) scanner (Skyscan 1174v2, 7 μm resolution 0.3° step, 180° total rotation). X-ray attenuation was standardized across scans using hydroxy-apetite bone mineral density (BMD) standards. Two-dimensional x-ray images were reconstructed into three-dimensional image stacks using NRecon (v1.6.6.0, Skyscan) and analyzed using CTan (v1.12.4.3, Skyscan). Gray-scale x-ray images were thresholded to isolate regions of interest (ROIs) containing otoliths. Mean BMD was recorded from within these regions, three-dimensional volumes were interpolated across image stacks using a "shrink-wrap" function, and both volume and surface area were measured. For each larva, data from left or right sagittal and lapillar otoliths were haphazardly selected for analysis of volume and surface area, while density was measured as one mean value across all otoliths (sagitta, lapilli, and asterisci). Relative densities of otoliths were calculated using the grand mean of control larvae otolith densities as a reference.

Data analysis

All statistical analyses were conducted in R (v2.15.1) using tank mean data and results were considered significant at $P < 0.05$. Otolith relative density data were analyzed using one-way analysis of variance (ANOVA) with CO_2 as a fixed factor and the respective measurement as the response variable. Three-dimensional metrics of otolith size (volume, surface area) were tested using analysis of covariance (ANCOVA)

procedures, with each metric as a response variable, pCO_2 as a fixed factor, and standardlength as a continuous covariate. Normality and homoscedasticity were verified using Shapiro-Wilk and Bartlett's tests prior to all statistical procedures.

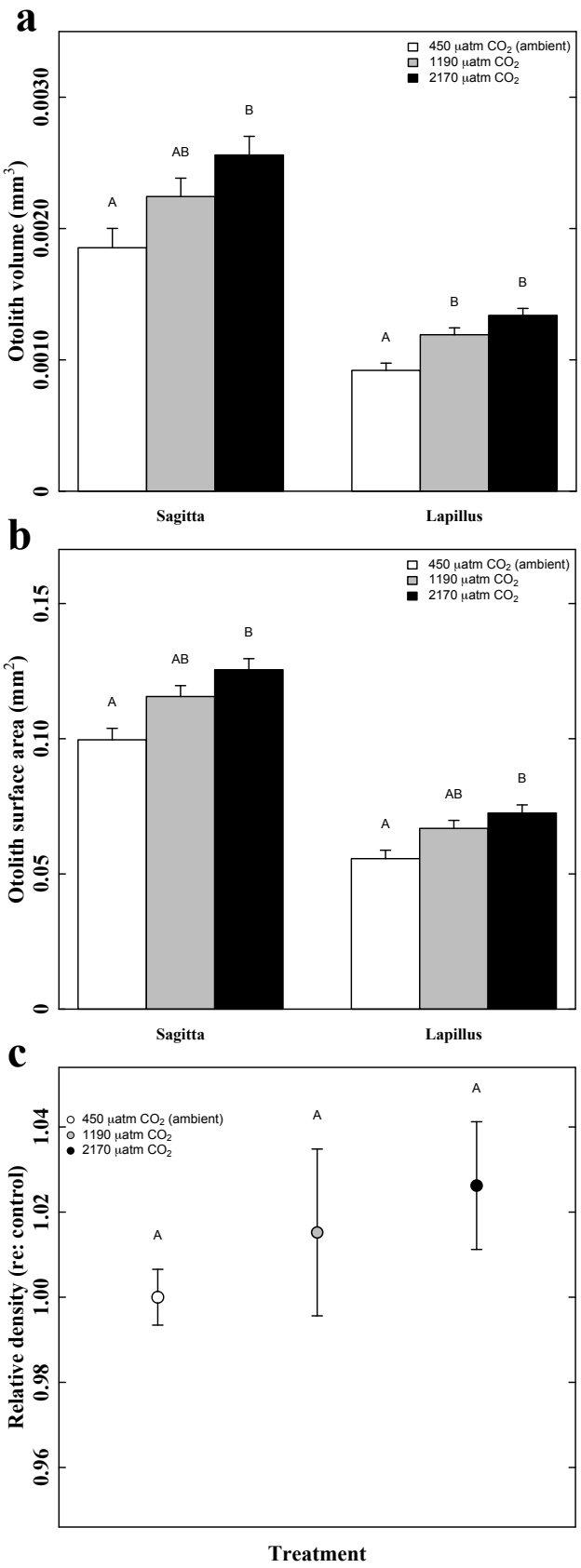
Results

Three-dimensional measures of otolith size were significantly affected by increased pCO_2. Sagittal otoliths from the 2170 μatm pCO_2 treatment had significantly greater volume than controls, with an average increase of 38% ($P = 0.014$, Fig. A2.1a). Lapillar otoliths from both high-CO_2 treatments were also significantly more voluminous than controls, with an average increase of 30 and 46 % ($P = 0.001$). Similarly, otolith surface area was significantly greater for sagittal and lapillar otoliths from the 2170 μatm pCO_2 treatment, compared to controls (both $P < 0.05$, Fig. A2.1b). Micro-CT data did not indicate a significant treatment effect on relative otolith density; otoliths from larvae in the 2170 μatm pCO_2 treatment only exhibited ~ 2.5 % higher density than controls ($P = 0.508$, Fig. A2.1c). See Table A2.1 for summary statistical results.

Table A2.1 Summary of statistical results from analysis of covariance (ANCOVA) of three-dimensional measures of larval mahi-mahi otolith size and analysis of variance (ANOVA) of relative otolith density during Experiment 3. ANCOVA results for otolith volume and surface area (SA) are from a haphazardly selected otolith. ANOVA testing of relative density utilized one combined otolith density measurement per fish. Tukey post-hoc results compare ambient pCO_2 (C), 1190 µatm pCO_2 (1), and 2170 µatm pCO_2 (2) treatments.

Metric	F-value	P-value	df	Tukey
Otolith size/shape				
Sagitta volume	7.668	**0.014**	2,8	1 = C < 2 = 1
Lapillus volume	19.480	**0.001**	2,8	C < 1 = 2
Sagitta SA	12.505	**0.003**	2,8	1 = C < 2 = 1
Lapillus SA	9.955	**0.007**	2,8	1 = C < 2 = 1
Relative density	0.7301	0.508	2,9	C = 1 = 2

Figure A2.1 Results from analysis of covariance (ANCOVA) of sagittal and lapillar otolith (**a**) volume, (**b**) surface area, and (**c**) analysis of variance of relative density for 20 day post hatch larval mahi-mahi during Experiment 3. White, gray, and black bars represent ambient (450 μatm), 1190, and 2170 μatm pCO_2 treatments, respectively. Relative otolith density was calculated in reference to the grand mean of control otolith density. Within each otolith type, bars/points not sharing a letter are significantly different ($P < 0.05$). Values are adjusted tank means ±SEM ($N = 4$ per treatment).


a
450 μatm CO2 (ambient)
1190 μatm CO2
2170 μatm CO2
Otolith volume (mm3)
0
0.0010
0.0020
0.0030
A
AB
B
A
B
B
Sagitta
Lapillus
b
450 μatm CO2 (ambient)
1190 μatm CO2
2170 μatm CO2
Otolith surface area (mm2)
0
0.05
0.10
0.15
A
AB
B
A
AB
B
Sagitta
Lapillus
c
450 μatm CO2 (ambient)
1190 μatm CO2
2170 μatm CO2
Relative density (re: control)
0.96
0.98
1.00
1.02
1.04
A
A
A
Treatment