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The Influence of Increased CO2 on Early Developmental Stages of Cobia, Rachycentron canadum

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THE INFLUENCE OF INCREASED CO_2 ON EARLY DEVELOPMENTAL STAGES OF COBIA, *RACHYCENTRON CANADUM*

By

Dwight A. Ebanks

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THE INFLUENCE OF INCREASED CO_2 ON EARLY DEVELOPMENTAL STAGES OF COBIA, *RACHYCENTRON CANADUM*

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Projected increases in atmospheric CO_2 and the resulting decrease in oceanic pH over the course of the 21st century warrant studies on the effects of these abiotic environmental changes on marine teleosts. The goal of this dissertation was to assess the impact of projected changes in CO_2 on the early physiological dynamics and development of cobia, *Rachycentron canadum*. Cobia are widely distributed marine teleosts, which typically hatch in ca. 24 h. Respiration rates of cobia spermatozoa displayed a non significant decrease from 1.0 $\pm$ 0.2 nmol O_2/mL semen·sec $\pm$ S.E. at 380 ppm CO_2 to 0.7 $\pm$ 0.1 nmol O_2/mL semen·sec $\pm$ S.E. at 800 ppm CO_2. Spermatozoa mean velocity decreased from 22.1 $\pm$ 0.5 to 14.9 $\pm$ 0.4 μm/sec $\pm$ S.E. for one male and from 16.9 $\pm$ 0.4 to 15.5 $\pm$ 0.5 μm/sec $\pm$ S.E. for the second male. Both decreases in velocity were statistically significant. Respiration rates of cobia embryos for the year 2100 CO_2 level (800 - 870 ppm) and beyond (~ 2900 ppm) were assessed. Utilizing pooled data for the respirometer methodology, the embryo respiration rate decreased from 3.9 $\pm$ 1.1 to 3.2 $\pm$ 0.9 nmol O_2/embryo/hr $\pm$ S.E. at the year 2100 projection but it was not significant. Regression analysis did not show a statistically significant difference across the CO_2 levels examined (400, 480, 500, 580, 1020, 2920 ppm) for the biological oxygen demand bottle method. Embryonic development after 9 and 19 hrs exposure to

elevated levels of CO_2 (876, 949, 957 ppm) were not significantly different. The mean rate of oxygen consumption of cobia yolk-sac larvae decreased from 10.7 $\pm$ 3.4 nmol O_2/larvae/hr $\pm$ S.E. at 380 ppm to 6.6 $\pm$ 2.3 nmol O_2/larvae/hr $\pm$ S.E. at 800 ppm. Yolk-sac larvae mean total length decreased from 2.59 $\pm$ 0.34 to 2.29 $\pm$ 0.28 mm $\pm$ S.D. at 560 ppm but increased to 2.89 $\pm$ 0.28 mm $\pm$ S.D. at 800 ppm CO_2 in the first trial. These differences were significant. Mean total length of yolk-sac larvae increased at high CO_2 levels (800, 876, and 957 ppm) for three of the four trials but only the first and last were statistically significant. These results on pH effects suggest early life history stages of cobia may be resistant to near future CO_2 levels.

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Chapter 1: Introduction

1.1 Background

Since the end of the pre-industrial period in the mid-to-late 19th century, atmospheric carbon dioxide (CO_2) levels have increased from 280 ppm to 379 ppm as of 2005, which based on ice core analyses, exceeds the natural range of 180 to 300 ppm over the last 650, 000 years (IPCC 2007). Anthropogenic activity such as fossil fuel combustion, cement production and gas flaring have contributed significantly to the observed increase (Le Quéré et al. 2009). Recently, at the NOAA Mauna Loa Observatory a daily mean that surpassed 400 ppm for the first time since recordings began in 1958 has been recorded (NOAA 2013). The Intergovernmental Panel on Climate Change (IPCC) has predicted that the increased atmospheric CO_2 levels have caused a decrease in oceanic pH of 0.1 with a continued projected decrease of 0.14 to 0.35 over the course of the 21st century (IPCC 2007). In a recent study on temporal coastal ocean pH variation over an 8 year period at temperate latitudes in the Northeast Pacific, Wootton et al. (2008) found that model simulations underestimated the rate of pH decrease when compared to empirical observations. This indicated that ocean acidification may be worse in some areas than previously predicted. During the course of the study, pH fluctuated by a unit or more during any given year and by 1.5 units over the course of the study (Wootton et al. 2008).

An increase in atmospheric CO_2 poses a two-fold problem for organisms in an aqueous environment as the atmospheric CO_2 will dissolve in water. This dissolution will potentially lead to aqueous hypercapnia (higher than average aqueous CO_2 levels)

and simultaneously acidify the aqueous environment. Both hypercapnia and decreasing pH are problems for many aquatic organisms, which include but are not limited to the commercially important species such as red sea bream *Pagrus majo*, Japanese whiting *Sillago japonica*, Japanese flounder *Paralichthys olivaceus*, and eastern little tuna *Euthynnus affinis* (Kikkawa et al. 2003).

Other marine organisms, such as corals and sea urchins can also be greatly affected. Coral calcification, the process by which corals produce $CaCO_3$ for their endoskeleton, and thus their reef building ability are negatively affected by increased CO_2, which enhances $CaCO_3$ dissolution (Kleypas et al. 1999, Langdon et al. 2000, Leclercq et al. 2000, Marubini et al. 2001, Leclercq et al. 2002). The relatively stable environments in which corals are found are highly recreationally utilized and provide a refuge from predators for many organisms. Additionally, they serve as an ontogenetic habitat for commercially important juvenile and adult fish species such as but not exclusively the Nassau grouper (Eggleston et al. 1998), gray snapper (Luo et al. 2009), and lemon damselfish (Beukers & Jones 1998), and contribute to species abundance and richness (Walter & Haynes 2006). Sea urchins, *Hemicentrotus pulcherrimus* and *Echinometra mathaei*, exposed to increased CO_2 levels have shown decreased fertilization rates as pCO_2 concentrations ranged from 1000 ppm, pH 7.7 to 10,000 ppm, pH 6.8 (Kurihara & Shirayama 2004). The negative impact on fertilization rates for both species was consistently greater in water that was treated with CO_2 versus a reduction in pH by HCl alone. The percentage of non-cleaved *H. pulcherrimus* eggs increased with increasing CO_2 concentration and was significantly high at 10,000 ppm (pH 6.8); for HCl-acidified water the percentage was significantly high at pH 7.0 and 6.8.

The percentage of embryos that completed their first cleavage decreased as pH decreased both for CO_2- and acid-treated water. As with the eggs that had not cleaved, significant differences occurred at 10,000 ppm for CO_2 treated water and at pH 7.0 and 6.8 for HCl-treated water.

Contrary to these findings Havenhand et al. (2008) found a significant impact on the sea urchin, *Heliocidaris erythrogramma*, at a CO_2 concentration of 1000 ppm, pH 7.7, which is the upper limit projection for the year 2100. They were able to show that at this concentration there was a 25% decrease in fertilization success, a 20.4% decrease in cleaving embryos, and 25.9% decrease in embryos that developed into swimming larvae.

1.2 Statement of the Problem

An increase in atmospheric CO_2 will impact all major bodies of water causing aqueous hypercapnia and ocean acidity, both of which are a problem for many marine organisms. Thus fish tolerance of these perturbations will be limited by their ability to either physiologically compensate or survive at depths that are unaffected or less affected by the changes near the surface. Physiologically (as reviewed by Pörtner et al. 2004, 2005, Pörtner 2008) CO_2 enters the body through diffusion decreasing the extracellular and intracellular fluid thereby causing cellular acidosis. Hypercapnia (higher than normal CO_2 levels in blood) is not compensated for through ventilation in water breathing organisms due to the small CO_2 diffusive gradient between the organism and the water. Instead marine fish address this disturbance by ion regulation primarily across their gills but also incorporate to a lesser extent the intestine and kidney. The exchange of ions for acid-base control can lead to an increase in NaCl uptake, causing an increased need for

osmoregulation and further energetic demands. Increased CO_2 levels can also lead to a reduction in thermal tolerance as an alteration to pH balance can cause oxygen levels in the body to fall and depress metabolism causing an organism to reach limiting levels earlier than it would have under normal oxygen levels. Conversely, thermal extremes will cause increased sensitivity to increased levels of CO_2 or hypoxia. Many of the studies demonstrating depressed metabolism have been done with invertebrates but Franke & Clemmesen (2011) have shown a significant negative relationship between CO_2 and RNA/DNA ratios in Atlantic herring *Clupea harengus* implying decreased protein biosynthesis, which could impact somatic growth in the future.

Physiological impacts due to increased CO_2 may not be limited to the consequence of the resulting decrease in pH but more directly by the resulting hypercapnia. Kikkawa et al. (2004) observed significantly higher mortalities for red sea bream eggs (21 h post fertilization) and pre-flexion larva (10-12 days post hatch) under increased CO_2 vs. HCl-acidified seawater. Crocker and Cech (1996) showed that there was a significant decrease in growth for juvenile white sturgeon only under aqueous hypercapnic conditions but not in HCl-acidified seawater. Hayashi et al. (2004), who used Japanese flounder to examine acid-base responses of fish exposed to CO_2-acidified water, observed fish death starting 8 h after the experiments began and that all fish were dead within 48 h. Fish began to die in H_2SO_4-acidified water after 72 hours of exposure. Hypercapnia has also been shown to affect the central nervous system (Söderström & Nilsson 2000), behavior (Ross et al. 2001), respiration (Graham et al. 1990, Cruz-Neto & Steffensen 1997), blood circulation (Lee et al. 2003), metabolism (Perry et al. 1988), and olfactory discrimination and homing ability (Munday et al. 2009a) of fish (Table 1.1).

These combined effects undoubtedly affect the physiology of fish and consequently may have larger scale implications for the fitness of the fish populations.

Considering the broad socioeconomic importance of fisheries to many cultures and industries (Brinson 2008) and the ecological significance of these aquatic organisms, it is essential to expand our understanding of the impacts of increased atmospheric CO_2 on pelagic marine teleost fish. The high metabolism of marine pelagic fish such as tunas, billfishes, dolphin fish (Brill 1996) and cobia (Feeley et al. 2007, pers. obs.) implies that they could be quickly affected by elevated CO_2 levels. While much work has been done to address the impacts of increased concentrations of CO_2 on marine invertebrates (Kleypas et al. 1999, Langdon et al. 2000, Leclercq et al. 2000, Marubini et al. 2001, Leclercq et al. 2002, Kurihara & Shirayama 2004, Adam et al. 2011, Rodolfo-Metalpa et al. 2011, Ross et al. 2011, Doropoulos et al. 2012, Edmunds et al. 2012) and fish in freshwater environments (Perry et al. 1988, Crocker & Cech 1996, Ingermann et al. 2002), there is a dearth of information for the early life stages of marine fish examined in this dissertation. In a review by Ishimatsu et al. (2008) on high CO_2 and aqueous acidification, they reported that the physiological effects could not be conclusively correlated to the effects of ocean acidification on marine fish because the pCO_2 levels used in 92% of the 116 articles published from1969-2008, were at levels >50,000 µatm, which greatly exceeded the projected future concentration of 1,900 µatm for the year 2300 (Caldeira & Wickett 2003). Additionally, 79% of *in vivo* studies were under exposures less than 4 d in duration, only 25% of the studies included marine fish, 58% of the research emphasis had been on acid-base regulation and cardio-respiratory control, and only two papers focused on the effects in early developmental stages

(Ishimatsu et al. 2008). In the years since Ishimatsu et al. (2008) there has been an expansion in the number of studies addressing the impact of ocean acidification on marine fish (Bignami 2013) however, few studies have examined the spermatozoa, embryo, and yolk-sac larvae stages addressed in the current study. The lack of information for how marine teleost may respond during their early life stages to near future CO_2 scenarios prevents researchers from determining if the effects are cumulative between stages and will negatively impact fish populations.

1.3 Goal

Cobia have a large geographic distribution, spanning tropical, subtropical, and warm temperate waters in every ocean basin (Figure 1.1, Shaffer & Nakamura 1989) including a scarce presence off of Iquique and Isla Mas Afuera, in the Eastern Pacific off Chile (Fowler 1944) thereby ensuring their exposure to any likely near-future perturbations. Cobia have fast growth rates, whereby they hatch in ca. 24 h (Feeley et al. 2007, pers. obs.), begin exogenous feeding 3 days post hatch (dph; Benetti et al. 2008a) and become juveniles ca. 20 dph (based upon stomach development; Holt et al. 2007). As juveniles, they increase body mass by 2.46% day $^{-1}$ in sub-optimal conditions (Feeley et al. 2007), mean total lengths can range from 18.85-27.48 mm at 21 days post hatch (Benetti et al. 2008a), and they can weigh up to 6 kg by the end of their first year (Benetti et al. 2006, Benetti et al. 2010). Such growth rates make cobia comparable to pelagic marine teleost such as skipjack tuna *Katsuwonus pelamis*, yellowfin tuna *Thunnus albacares*, dolphin fish *Coryphaena hippurus*, and Pacific blue marlin *Makaira nigricans*, which also have high growth rates (Brill 1996). Additionally, the fast growth

rate of cobia makes them an ideal physiological model because an applied treatment should be quickly assimilated.

The goal of this dissertation was to determine the impact that aqueous hypercapnia, expected as a result of global climate change, may have on the reproductive success of pelagic marine teleosts by using cobia, *Rachycentron canadum*, as an experimental species. Projected levels of hypercapnia were based on IPCC CO_2 forecasts for the year 2065 (560 ppm) and the year 2100 (800 ppm) levels with ambient concentrations (380 ppm) used for controls. To address the goal of this dissertation, there were three objectives:

1. Determine whether the different levels of aqueous hypercapnia caused by IPCC projected changes in atmospheric CO_2 concentration significantly impacted cobia sperm respiration rates and motility.

2. Determine whether the respiration rate and rate of development of cobia embryos were significantly suppressed by exposure to forecasted aqueous hypercapnia.

3. Evaluate whether cobia yolk-sac larval respiration rate and development were significantly suppressed by the exposure to different intensities of anticipated aqueous hypercapnia.

1.4 Carbon Dioxide Gas System

This section is included here because it only addresses the system used for achieving the treated water in each science chapter (Ch 2 – 4) and not any of the experiments.

Experimental waters at the CO_2 levels of interest were generated in an experimental seawater facility for culturing corals under controlled temperature (~ 27ºC) at the University of Miami Experimental Hatchery (UMEH). Target CO_2 levels for seawater were either control (pCO_2 ~ 380 ppm; pH ~ 8.0) or experimental [CO_2] (years 2065 (pCO_2 ~ 560 ppm; pH ~ 7.9) and 2100 (pCO_2 ~ 800 ppm; pH ~ 7.8) based on the IPCC (2007) forecast. Seawater from Bear Cut, Virginia Key, FL was pumped into a holding tank then through a sand filter. This water was retained in 200 l header tanks where it was manipulated by bubbling with either ambient air for control conditions or a mix of ambient air and CO_2 gas regulated by mass flow controllers for experimental [CO_2]. Each header tank was connected to a 60 l aquarium into which treated water was pumped and returned to the header tank via overflow. A fresh supply of seawater was added to each header tank at a continuous slow flow; however, the flow rate could not be determined due to fluctuations in the system. Temperature was regulated by heating coils in the header tank or water chilled (26ºC) coils that lined the header tank.

Table 1.1 Physiology impacted by hypercapnia with the CO_2 concentration(s) examined in the studies

Author	Physiological Impact	CO_2 (ppm)
Söderström & Nilsson 2000	central nervous system	10226
		30679
		102264
Ross et al. 2001	behavior	13363
		26725
		48678
Graham et al. 1990	respiration	10226
Cruz-Neto & Steffensen 1997		13635
		27271
		40906
		54541
Lee et al. 2003	blood circulation	47723
Perry et al. 1988	metabolism	8181
Munday et al. 2009	olfactory discrimination and homing ability	550
		750
		1030

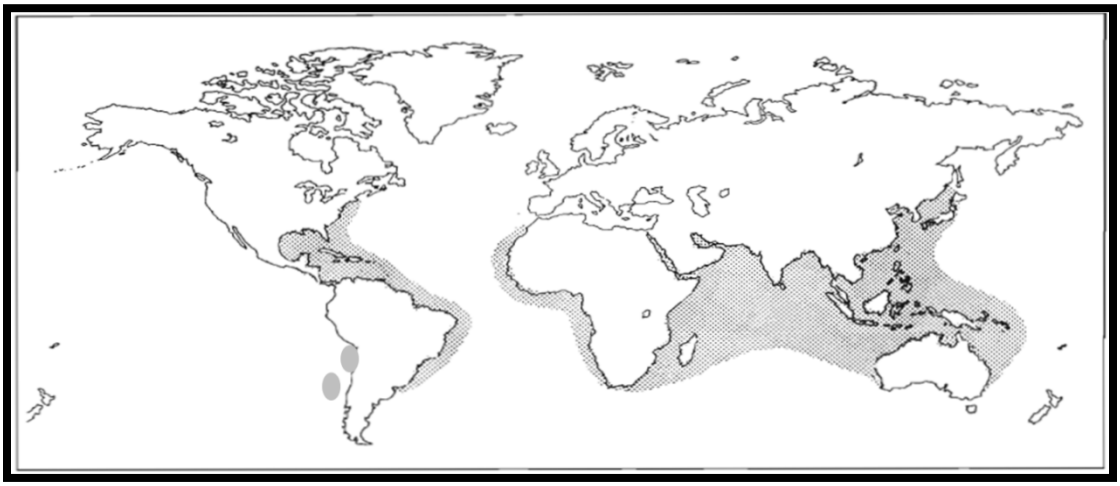

Figure 1.1 Range of the cobia, *Rachycentron canadum*. Modified from Shaffer and Nakamura 1989

Chapter 2: Influence of Increased CO_2 on Cobia Spermatozoa Respiration and Motility

2.1 Background

Anthropogenic activities such as fossil fuel combustion, cement production and gas flaring have caused an increase in atmospheric carbon dioxide (Le Quéré et al. 2009), which has already led to a decrease in oceanic pH of 0.1 units as the oceans have absorbed this gas in an attempt to reach equilibrium with the overlying atmosphere (IPCC 2007). Oceanic acidity will increase as projected pH levels decrease an additional 0.14 – 0.35 units over the course of the 21st century (IPCC 2007) to concentrations that have been shown to deleteriously impact marine organisms. While not all encompassing, some impacts that have been demonstrated include decreased coral calcification (Kleypas et al. 1999, Langdon et al. 2000, Leclercq et al. 2000, Marubini et al. 2001, Leclercq et al. 2002), adverse impacts to both the development of sea uchins (Kurihara & Shirayama 2004, O'Donnell et al. 2009) and the physiology of fish (Perry et al. 1988, Graham et al. 1990, Cruz-Neto & Steffensen 1997, Söderström & Nilsson 2000, Lee et al. 2003, Munday et al. 2009a). Even though adult fish possess the physiological mechanisms for ion exchange across the gills and bicarbonate accumulation, both of which enable acid base regulation (Claiborne et al. 2002, Evans et al. 2005, Melzner et al. 2009) to help cope with projected changes to ocean acidity, the early life-history stages of fish may be susceptible (reviewed by Pörtner 2005). This susceptibility makes early life stages a potential pitfall to a successful reproductive process under this perturbation.

The metabolism and motility of spermatozoa are important factors for successful fertilization (Ingermann 2008). While sperm are capable of generating ATP to maintain

basal metabolism, the rate of production may not be sufficient to meet its rate of utilization once the sperm has been activated as has been shown with rainbow trout *Salmo gairdneri* (Christen et al. 1987), carp *Cyprinus carpio* (Perchec et al. 1995) and turbot *Psetta maxima* (Dreanno et al. 1999, Dreanno et al. 2000). Additionally, the metabolism of fish spermatozoa has been shown to be impacted by changes in pH. Steelhead trout *Oncorhynchus mykiss* sperm metabolism, measured via respiration, have been shown to have a linear relationship with pH (~ 7.25 – 8.75) which was achieved by altering pCO_2 levels (~ 0–5 kPa) (Ingermann et al. 2003). Rainbow trout *Salmo gairdneri* spermatozoa displayed a curvilinear decrease in oxygen consumption with increasing CO_2 levels (~0–50 mM CO_2; pH 6.0) (Inoda et al. 1988).

Sperm motility can be influenced by both abiotic and biotic factors. Carbon dioxide has been shown to have inhibitory effects on freshwater fish sperm (Bencic et al. 2000a, Bencic et al. 2000c, Bencic et al. 2001, Ingermann et al. 2002). In the specific case of marine flat fish such as Atlantic halibut *Hippoglossus hippoglossus* and turbot *Psetta maximus*, sperm activation is actually dependent upon decreased CO_2 concentrations (Inaba et al. 2003). The effects of pH have been more variable than those of CO_2. Studies have shown that both fresh and saltwater fish sperm may remain motile in an acidic environment but that performance was better in an alkaline environment and that marine species have shown a larger range of tolerance than freshwater fish (Stoss 1983). However, not all fish studied have been raised in a consistent environment. One study used two year old grey mullet *Mugil capito* transferred to salt water 20 days before the experiments after being raised in fresh water (Hines & Yashouv 1971). Biologically, spermatozoa motility is limited by the amount of ATP stored in the midpiece of the

gamete (Christen et al. 1987, Perchec et al. 1995, Bencic et al. 1999, Dreanno et al. 1999, Bencic et al. 2000b, Dreanno et al. 2000) and the ability to deliver ATP rapidly enough for the flagellum (Dreanno et al. 2000). Additionally, ovarian fluid can have an important role in the response of sperm to possible ambient pH shifts. Ovarian fluid has a greater pH buffering capacity than seminal fluid (Ingermann et al. 2002), its concentration increases as the sperm approaches the egg and it can influence swimming speed, longevity, and path trajectory as a form of cryptic female choice (mate choice after spawning) as was shown with chinook salmon by Rosengrave et al. (2008). Even though these studies were on anadromous fish it is potentially relevant in pelagic waters due to the proximity of the fish when spawning occurs.

Due to the potential impact that increasing levels of CO_2 may have on the early life-history stages of fish, the purpose of the current study was to determine if the metabolism and motility of cobia *Rachycentron canadum* spermatozoa would be significantly impacted by near future CO_2 concentrations based on IPCC projections (IPCC 2007).

2.2 Materials and methods

Sperm Respiration Assessment

Milt (both spermatozoa and seminal fluid) samples for respiration experiment trials were obtained by sedating three brood stock cobia males from UMEH with 14 ppm clove oil. The males were cannulated with a 2 mm (internal diameter) polyethylene catheter through the urogenital opening. Extracted samples were pooled together in microcentrifuge tubes and maintained in their seminal fluid for approximately an hour,

which is when the trial began. On the day of the trial, seawater samples at pCO_2 ~ 380 ppm (control-CO_2) and ~ 800 ppm (high-CO_2) were obtained and filtered through a 0.2 µm filter to eliminate background respiration that may have been a result of biological activity. The experimental water was obtained from the previously described experimental seawater facility at UMEH. Each respirometer received 4 ml of either control water or high-CO_2 seawater so that there were two respirometers at each CO_2 concentration (Table 2.1). Seawater treated with CO_2 was added just prior to the addition of milt. A 6 or 12 µl milt aliquot was deposited in each respirometer so that there were two respirometers with a 6 µl and two with a 12 µl milt addition. Two volumes of milt were used to regress oxygen concentration against time for the removal of sensor oxygen consumption. Respiration rates were determined by simultaneously measuring change in dissolved oxygen concentration in four syringe respirometers for 5 hrs. using an ENDECO 1125 four-channel Pulsed Oxygen Monitor. Respirometer temperature was maintained at 27ºC $\pm$ 0.8ºC via a water bath. Oxygen concentration and temperature were measured every 10 min through the ENDECO system connected to a personal computer. Each respirometer was rotated by hand at 30-minute intervals for the duration of the trial. The rate of oxygen consumption per sperm was calculated for each respirometer by multiplying the change in oxygen concentration by the volume used in the respirometer and dividing by the length of the trial and mean number of spermatozoa. The mean number of spermatozoa (10236 for 6 µl) was determined by using 1 µl milt aliquots from three different males and standard hemacytometer procedures.

Sperm Motility Assessment

Milt samples for motility experiment trials were obtained by removing the gonads from two cobia euthanized with Tricaine mesylate, MS222. The seminal ducts were notched and the gonad massaged toward the notch to release milt (Figure 2.1). Care was taken not to make the notch too deep to prevent contamination from blood and premature activation of the spermatozoa. A 0.5 µl milt aliquot was taken and added to a microscope slide previously prepared with F-127 to prevent spermatozoa from adhering to the slide (Cosson et al. 2008). Due to fluctuation in the CO_2 system carbon dioxide levels higher than IPCC end of the century projections, ~ 2920 ppm, were used in these trials (Table 2.2). The high $[CO_2]$ ~ 2920 ppm is still biologically relevant as it has been shown that CO_2 levels can exceed 2300 µatm (1 µatm ~ 1 ppm) in the Kiel Fjord in the Baltic Sea (Franke & Clemmesen 2011). The appropriate activation solution (50 µl of 0.2 µm filtered seawater at either control ~ 577 ppm or experimental $[CO_2]$ ~ 2920 ppm) and F-127-laced cover slip were added. The large dilution ratio of 50 µl of activation solution to 0.5 µl milt was used to reduce the alteration of $[CO_2]$ due to spermatozoa respiration. The slide was placed under a Leica CME compound microscope at 40X magnification. Video files were made using a Spot Idea 3MP digital camera with Spot Advance software. Three 40-sec control videos and three experimental $[CO_2]$ videos were made for each male. MetaMorph tracking software was used for spermatozoa velocity determination. Twenty spermatozoa per video were randomly selected by using the random number generator in Microsoft® Excel to first select a frame between 1 and 10, then an x and y coordinate on the selected frame (Albright 2011). The spermatozoan closest to the coordinates was tracked for as long as possible. A single velocity

observation was randomly selected for each spermatozoa and 30 sequential observations, including the random observation, taken to obtain an even number of observations for statistical analysis. Each independent video was used as the level of replication for statistical analysis so than n = 1800 per [CO_2].

Statistical Procedures

Due to some of the literature not providing the number of spermatozoa per unit of volume the respiration rates were converted and reported in nmol O_2/mL semen·sec $\pm$ S.E. The rank-based Mann-Whitney statistical test was used to determine significant differences between control (~ 380 and ~ 577 ppm CO_2) and treatment levels (~ 800 and ~ 2920 ppm CO_2) because data did not exhibit equal variance. Spermatozoa velocities were reported in μm/sec $\pm$ S.E. and also analyzed with the Mann-Whitney statistical test because the data did not exhibit a normal distribution (Kolmogorov-Smirnov, $P < 0.05$). Attempts at data transformations were unsuccessful. All analyses were conducted using SigmaStat for Windows version 3.5 and considered significantly different at $P < 0.05$.

2.3 Results

The mean rate of oxygen consumption of semen at control CO_2 levels (380 ppm) was 1.0 $\pm$ 0.2 nmol O_2/mL semen·sec. Milt respiration decreased to 0.7 $\pm$ 0.1 nmol O_2/mL semen·sec at 800 ppm CO_2. However, this 30% decrease was not statistically significant (Mann-Whitney U(3) = 6, p = 0.667) (Figure 2.2).

Spermatozoa from both males showed a decrease in velocity at the experimental CO_2 level ~ 2920 ppm. The mean velocity of milt for one male decreased from 22.1 $\pm$ 0.5 to 14.9 $\pm$ 0.4 μm/sec $\pm$ S.E. A 32% decrease that was significantly different (Mann-Whitney U(3719) = 3874640, p < .001) (Figure 2.3). The mean velocity for spermatozoa

from the second male decreased from 16.9 $\pm$ 0.4 to 15.5 $\pm$ 0.5 µm/sec $\pm$ S.E.; a 8.5% decrease that was also significantly different (Mann-Whitney U(3719) = 3685821, $p < .001$) (Figure 2.4). Data for the motility trials were not pooled due to significant differences between both the control groups and the experimental groups.

2.4 Conclusion

Cobia sperm respiration displayed a 30% decrease at elevated levels of CO_2. This was not a statistically significant decrease and may suggest that cobia spermatozoa metabolism is moderately impacted by this perturbation. The decrease in respiration was consistent with the findings of both Inoda et al. (1988) and Ingermann et al. (2003). The following respiration rates have been converted to similar units and adjusted for 27ºC assuming the temperature coefficient (Q10) = 2.0. The respiration of rainbow trout, *Salmo gairdneri*, spermatozoa decreased ~ 40% from 69.0 nmol O_2/mL semen · sec at 6 mM CO_2 (~ 264 ppm CO_2) to 41.4 nmol O_2/mL semen·sec at 18 mM CO_2 (~ 792 ppm CO_2; Inoda et al. 1988). Steelhead trout, *Oncorhynchus mykiss*, sperm respiration displayed ~ 77% decrease (1.7 to 0.4 nmol O_2/mL semen·sec) over a pH range of 8.5 – 7.5 in experiments conducted by Ingermann et al. (2003); pH ranged ~ 8.0 to 7.8 for the present study. It should be noted that the fishes used in Inoda et al. (1988) and Ingermann et al. (2003) are now classified as the same species, *O. mykiss* (Smith & Stearley 1989). Cobia sperm respiration was 1.0 $\pm$ 0.2 nmol O_2/mL semen·sec at 380 ppm and 0.7 $\pm$ 0.1 nmol O_2/mL semen·sec at 800 ppm. The cobia respiration values are between the values for the trout in the two previous studies. The differences among respiration rates could be attributed to species differentiation and differences in life

histories. Cobia are a pelagic species that release their milt in a marine environment while steelhead trout are anadromous (Staley & Mueller 2000) and release their milt in fresh water. Additional sources of differences could also come from the different equipment and methodologies utilized among the different studies. For additional comparison, other teleost spermatozoa respiration rates under non-experimental conditions have been converted to similar units, adjusted for 27ºC assuming the temperature coefficient (Q10) = 2.0 and included: salmon *Salmo salar* 0.7 nmol O_2/mL semen·sec and cod *Gadus morhua* 0.8 nmol O_2/mL semen·sec (Mounib 1967) (Table 2.3). The lack of statistical significance associated with the 30% decrease in respiration for cobia may be due to the variability of the data. A large degree of variability is possible even within the same species as has been shown in Inoda et al. (1988) and Ingermann et al. (2003). The respiration experiment in this study could not be repeated beyond what was conducted as there were insufficient sperm samples available.

Sperm motility displayed a 32% and 8.5% decrease in velocity relative to controls in this study, both of which were statistically significant. There was a large difference between the decreases in sperm velocity for the two males, potentially indicating different levels of resistance to a CO_2 perturbation. Stoss and Holtz (1983) have indicated a large amount of variability in the quality of sperm from different individuals for rainbow trout *S. gairdneri*. A statistically significant decrease in spermatozoa velocity was not consistent with the findings of Frommel et al. (2010) for Baltic cod *Gadus morhua*. Baltic cod spermatozoa velocity decreased from a mean of 58.61 µm/sec at 390.8 µatm to56.60 µm/sec at 1364.8 µatm, a 3% decrease (Frommel et al. 2010). The lack of a significant decrease in velocity for Baltic cod sperm may indicate that the males

from which they came have acclimated to the high CO_2 levels already found in their habitat and provided some level of resistance to their sperm. Miller et al. (2012) found that the negative effects of hypercapnia on standard length, mass, survival and metabolism were reversed or absent in juvenile anemonefish *Amphiprion melanopus* when both parents and juveniles were exposed to hypercapnia. The mechanisms responsible have not been identified but Miller et al. (2012) believed one possibility to be transgenerational epigenetic inheritance whereby changes for gene expression are passed between generations allowing offspring to improve their performance, potentially over a period as short as weeks.

Sperm motility can be initiated by hyposmotic conditions in freshwater teleost and hyperosmotic conditions in marine teleosts (Morisawa & Suzuki 1980, Morisawa et al. 1983, Billard et al. 1993, Suquet et al. 1994, Linhart et al. 1999). However, Oda and Morisawa (1993) have also shown that elevated internal Ca^{2+} and pH levels initiated by external hyperosmotic conditions are important for spermatozoa motility; increased internal pH levels also occur in bovine (Babcock et al. 1983) and sea urchin (Johnson et al. 1983, Lee et al. 1983) spermatozoa. Conversely, a decrease in internal pH can suppress motility as Oda and Morisawa (1993) demonstrated with *Takifugu niphobles* and *Kareius bicoloratus* sperm. The internal pH of spermatozoa can be changed by external pH according to reports in Alavi et al. (2004) and performance is influenced by a range of pH that is species specific (Chauvaud et al. 1995, Alavi et al. 2004). Decreased motility with increased levels of CO_2 has been demonstrated among a variety of animals (Bencic et al. 2000a, Bencic et al. 2000c, Bencic et al. 2001, Ingermann et al. 2002, Inaba et al. 2003a, Havenhand et al. 2008). The velocity decrease within the pH range (7.3 –

8.0) used in this study indicates a threshold has been crossed. Even though motility has been identified as important for successful reproduction (Ingermann 2008) a velocity threshold leading to unsuccessful reproduction has not been identified. Whether the velocity decrease observed in the current study would be sufficient to impact fertilization could be determined experimentally. However, such experiments were beyond the logistical constraints of this study. Nevertheless, this study has demonstrated that the spermatozoa of a marine pelagic species are impacted at an environmentally relevant CO_2 concentration. CO_2 levels in the Kiel Fjord already exceed 2300 µatm and are projected to exceed 4000 µatm in the future (Franke and Clemmesen 2011). The finding from this study could also be used in future studies to determine where the threshold occurs for this species.

In conclusion, cobia spermatozoa respiration decreased at the elevated level of CO_2 examined but not significantly. However, cobia sperm velocity did decrease significantly. This was a sub-lethal impact, which has the potential to impact successful fertilization.

Table 2.1 Conditions for cobia spermatozoa respiration experiment utilizing the ENDECO 1125 respirometer. Mean ± SD, C = control, T = treatment

Milt volume (µl) /respirometer	Respirometer volume (ml)	CO_2 (ppm)		pH
6	4	C	380 ± 3	7.99 ± 0.01
12		C		
6		T	800 ± 10	7.84 ± 0.01
12		T		

Table 2.2 Conditions for cobia spermatozoa motility experiment trials. Mean ± SD, C = control, T = treatment.

Trial		CO_2 (ppm)	pH
1	C	577 ± 3	7.96 ± 0.00
	T	2920 ± 549	7.33 ± 0.07
2	C	577 ± 3	7.96 ± 0.00
	T	2920 ± 549	7.33 ± 0.07

Table 2.3 Mean respiration rates of fish spermatozoa available in literature and this study. Rates were adjusted for 27ºC assuming the temperature coefficient (Q10) = 2.0. NR = not reported

Author	Species	Respiration (nmol O_2/mL semen ·sec)	CO_2 (ppm)	pH
Mounib 1967	Salmon	0.7	Ambient	
	Cod	0.8	(NR)	
Inoda et al. 1988	Rainbow trout	69.0	~ 264	6
		41.4	~ 792	6
Ingermann et al. 2003	Steelhead trout (now rainbow trout)	1.7	~ 511	8.5
		0.4	~ 22500	7.5
Current study	cobia	1.0	380	8.0
		0.7	800	7.8

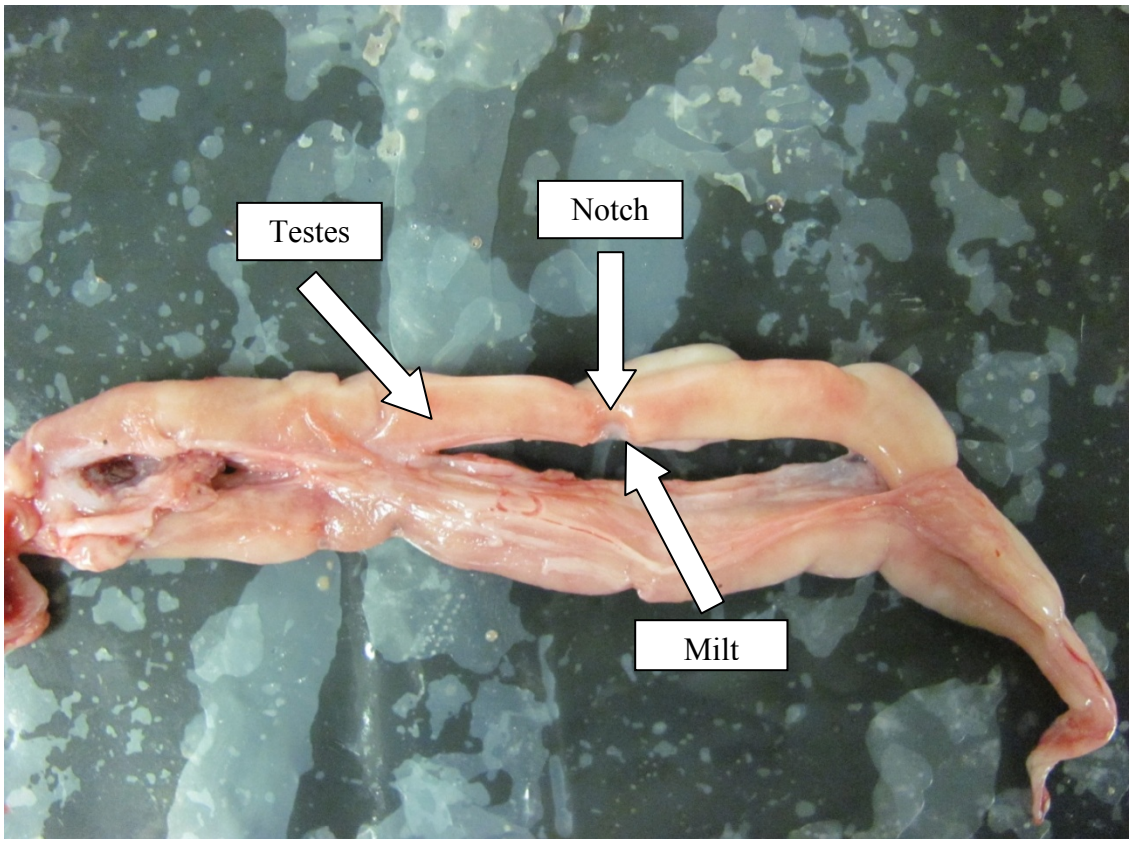

Figure 2.1 Cobia testes with the seminal duct notched and milt emerging.

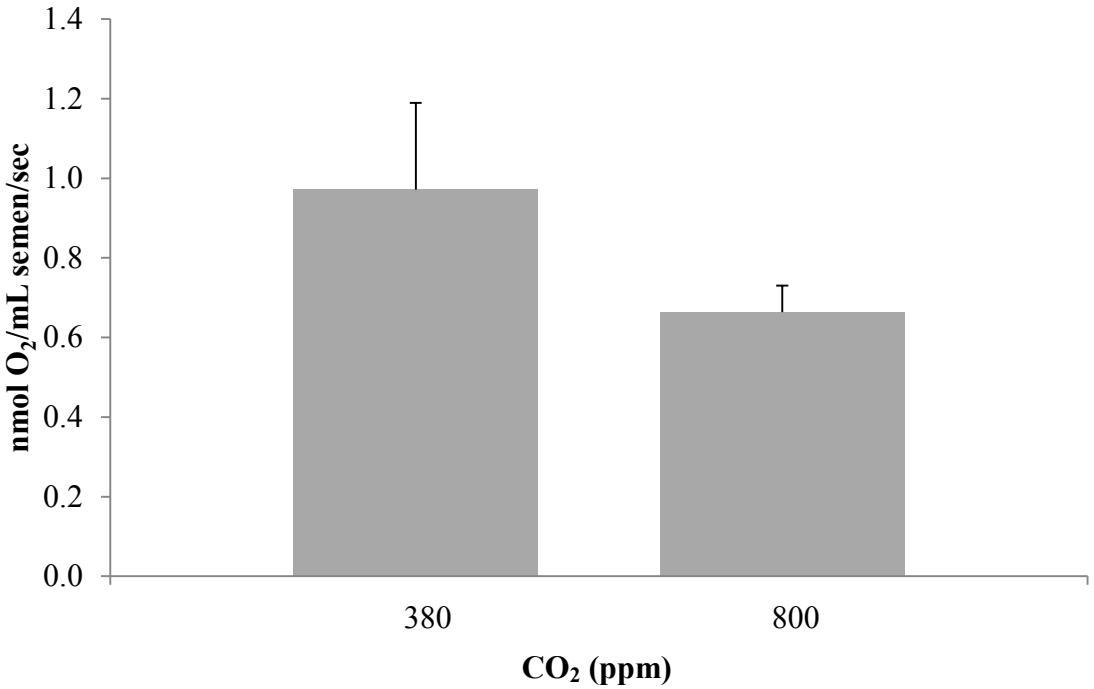

Figure 2.2 Mean respiration rates (nmol O_2/mL semen·sec $\pm$ S.E.) of cobia spermatozoa. Spermatozoa respiration was 1.0 $\pm$ 0.2 at 380 ppm CO_2 and 0.7 $\pm$ 0.1 at 800 ppm CO_2. Respiration rates were not significantly different. n = two replicates per [CO_2]

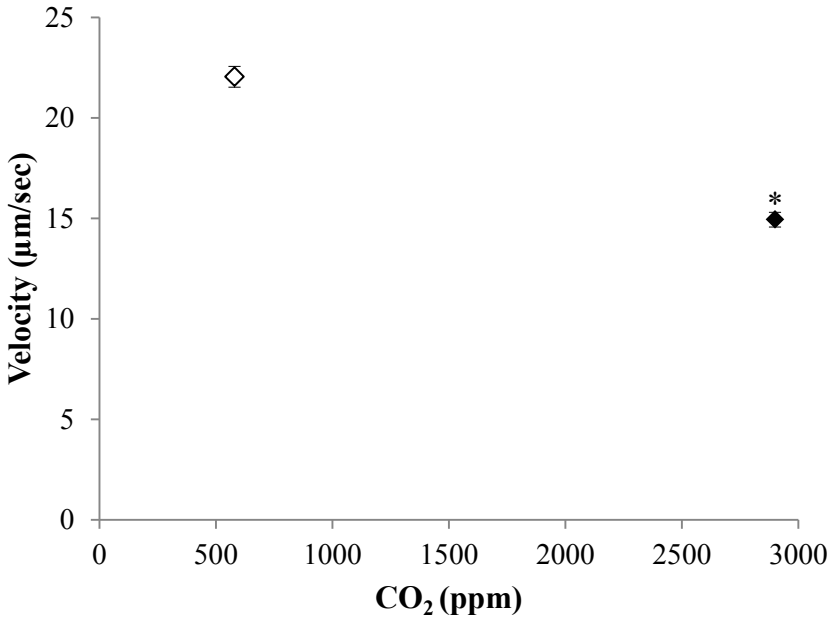

Figure 2.3 Mean velocities (µm/sec ± S.E.) of cobia spermatozoa from the first male; 22.1 ± 0.5 at ~ 577 ppm CO_2 and 14.9 ± 0.4 at ~ 2920 ppm CO_2. Each data point represents 30 observations per 20 spermatozoa per video. n = 1800 observations per [CO_2]. Asterisk indicates velocity is significantly lower than control velocity.

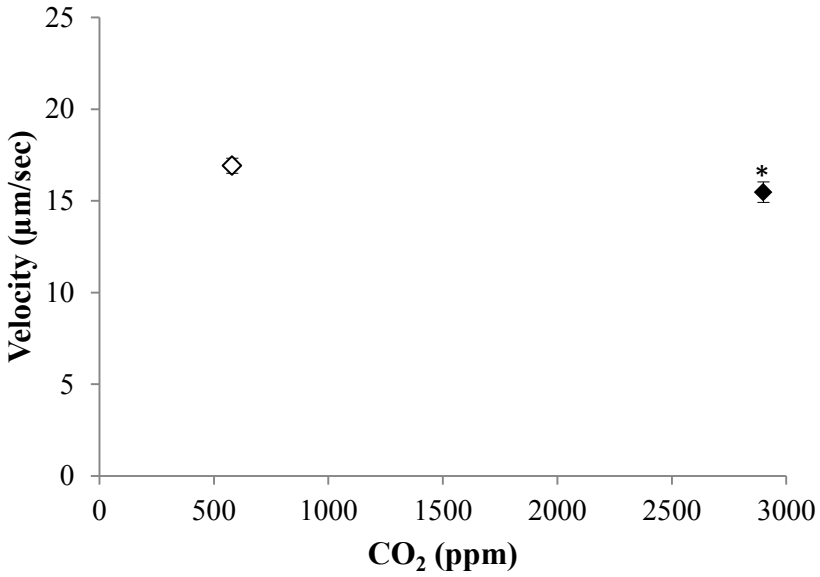

Figure 2.4 Mean velocities (µm/sec ± S.E.) of cobia spermatozoa from the second male; 16.9 ± 0.4 at ~ 577 ppm CO_2 and 15.5 ± 0.5 at ~ 2920 ppm CO_2. Each data point represents 30 observations per 20 spermatozoa per video. n = 1800 observations per [CO_2]. Asterisk indicates velocity is significantly lower than control velocity.

Chapter 3: Influence of Increased CO_2 on Cobia Embryo Respiration and Development

3.1 Background

Atmospheric CO_2, corrected for the average seasonal cycling, was approximately 397 ppm as of May 2013 (Tans & Keeling 2013) with a daily mean that surpassed 400 ppm for the first time since recordings began in 1958 (NOAA 2013) These data, from the NOAA Mauna Loa Observatory, highlight the relatively rapid rise in atmospheric CO_2, which was approximately 315 ppm in 1958 and has been increasing at a rate of no less than 1.56 ppm/yr since 2000 (Tans & Keeling 2013) Anthropogenic sources such as fossil fuel combustion, cement production and gas flaring (Le Quéré et al. 2009) contribute ~ 10 billion tons of carbon annually (Doney et al. 2009). These contributions have led to atmospheric concentrations that exceed levels over the last 800, 000 years (Luthi et al. 2008) with approximately 50% of the concentration mitigated by terrestrial (Schimel et al. 2001) and oceanic sinks (Sabine et al. 2004). However, for the oceans this absorption has already resulted in a pH decline of 0.1 units with a continued projected decrease of 0.14 – 0.35 units over the course of the 21st century (IPCC 2007) in a process that is termed ocean acidification.

Projected year 2100 levels for CO_2 (~ 1000 ppm) have been shown to physiologically impact many marine organisms. Some examples include decreased coral calcification with increased acidity (Kleypas et al. 1999, Langdon et al. 2000, Leclercq et al. 2000, Marubini et al. 2001, Leclercq et al. 2002); declining sea urchin fertilization success and embryo cleavage (Havenhand et al. 2008) and altered fish behavior (Munday et al. 2013). Adult fish have the physiological means, through ion exchange across the

gills and bicarbonate accumulation, to partially compensate for pH disturbances (Claiborne et al. 2002, Evans et al. 2005, Melzner et al. 2009) but earlier life stages are potentially more susceptible (reviewed by Pörtner et al. 2005). This vulnerability poses a potential problem for successful recruitment to later life stages.

Fish embryos have been shown to be susceptible to elevated levels of carbon dioxide. In a study that addressed CO_2 sequestration, which necessitated very high CO_2 concentrations, embryos in the cleavage stage were the most vulnerable, but the concentration was species dependent (Kikkawa et al. 2003). For a 360 min exposure, median lethal pCO_2 was ~ 13820 ppm for red sea bream *Pagrus major*, ~ 23690 ppm for Japanese whiting *Sillago japonica*, ≈ 27600 ppm for Japanese flounder *Paralicthys olivaceus* and ~ 116490 for eastern little tuna *Euthynnus affinis* (Kikkawa et al. 2003). In another study red sea bream *P. major* embryos suffered 85.8% mortality at ~ 48690 ppm CO_2 and 97.4% at ~ 97380 ppm CO_2 (Kikkawa et al. 2004). Munday et al. (2009b) recently addressed the impact of near future CO_2 levels (550, 750, and 1030 ppm CO_2) on the early life stages of the orange clownfish *Amphiprion percula*. They were not able to detect any impacts based on embryonic duration, embryo survival or size at hatching but the growth rate of larvae increased. Franke and Clemmesen (2011) conducted a study addressing the impact of high CO_2 levels on the early life stages of Atlantic herring *Clupea haarengus* in the Kiel Fjord in the Baltic Sea where pCO_2 levels already exceed 2300 µatm in 2011 and are projected to exceed 4000 µatm (1 µatm ~ 1 ppm). Franke and Clemmesen (2011) did not find an impact to the development of Atlantic herring *Clupea haarengus* embryos over a range of CO_2 concentrations (1260, 1859, 2626, 2903, 4635 µatm). However, there was an impact to the deoxyribonucleic acid (DNA)/ribonucleic

acid (RNA) ratio at the larval stage, which indicated an impact to the protein biosynthesis capability of the larva and thereby its metabolism (Franke & Clemmesen 2011). The amount of DNA is relatively constant but the amount of RNA changes with the physiological status, requirement for protein synthesis and growth (Buckley et al. 1999). Even though there were no impacts during the embryonic stages for (Munday et al. 2009b) and Franke and Clemmesen (2011), there was an impact to the larval stage when the embryo was exposed, potentially indicating that exposure at an early life stage may influence a later life stage (Franke & Clemmesen 2011).

Due to the varied responses and potential effect that increasing levels of CO_2 may have on the early life-history stages of fish, the purpose of the current study was to determine if the metabolism and 9- and 19-hour development post spawn of cobia *Rachycentron canadum* embryos would be significantly impacted by near-future CO_2 concentrations based on IPCC projections (IPCC 2007).

3.2 Materials and Methods

Embryo respiration

On the nights trials were conducted, seawater samples at pCO_2 ~ 380 ppm (control-CO_2) and ~ 800 ppm (high-CO_2) were obtained and filtered through a 0.2 µm filter to eliminate background respiration that may have been a result of biological activity. Embryos were collected by dip net within 1 hr of spawning from the egg collector at UMEH and placed in a dedicated container with aeration. Subsamples were taken from the container and counted in a petri dish for placement in the respirometers, which utilized the ENDECO 1125 previously described in section 2.2. Each respirometer

received the same amount of either control water or high-CO_2 seawater just prior to the addition of the embryos so that there were two respirometers at each CO_2 concentration. Respiration rates were determined for embryos within 2 hrs of spawning through the gastrula stage (~ 7 hrs post fertilization). The first three trials were conducted with a water volume of 12.5 ml per respirometer and 10 or 20 embryos so that there were two respirometers with 10 embryos and two with 20 embryos (Table 3.1). In an effort to increase the signal strength and reduce the amount of observed variance in the results, the trials were conducted two more times with a water volume of 5 ml per respirometer. The number of embryos was also reduced to eliminate the potential of overcrowding in the respirometer due to the reduced volume. These two trials used 5 or 10 embryos. The respirometers were turned by hand every half hour for all of the five trials and temperature was maintained via a water bath at 27ºC $\pm$ 0.8ºC. The rate of oxygen consumption per embryo was determined by multiplying the change in oxygen concentration over time, corrected for background respiration, by the volume and dividing by the number of embryos used in the respirometer.

In an attempt to corroborate results from the respirometer, a second methodology was used. Three more respiration trials were conducted on the same embryonic stages using 60 ml biological oxygen demand (BOD) bottles and a custom designed Winkler titrator to determine oxygen concentrations. Technical difficulties with the CO_2 system resulted in higher than intended control and experimental CO_2 levels. Control CO_2 levels were ~ 480, 400, and 580 ppm; respective high CO_2 levels were ~ 500, 1020, and 2920 ppm (Table 3.2). The first two trials had three replicates per CO_2 level. The third trial had 15 replicates per CO_2 level. Respiration rates were calculated by multiplying

the change in oxygen concentration by the volume used in the BOD bottle and dividing by the duration of the trial and number of embryos. Embryos within each bottle were counted following the titration.

Nine- and 19-hr development

Prior to the trials, hourly visual observations of cobia embryo development from within 1 hr of being spawned through hatching were completed to establish a development timeline guide. The guide was used to grade embryo development during the trials. Embryos for the trials were collected by dip net from the egg collector at UMEH within 1 hr of spawning. The embryos were accumulated in a graduated cylinder and 10 ml subsamples of floating embryos were taken and deposited into each of two aquaria (one for each treatment). Due to equipment being stolen the number of aquaria per treatment level was reduced to one. The trialss were repeated three times to address the reduction in replicates. Sub samples were collected from the aquaria at 9 and 19 hrs and placed in a 0.5 ml bullet tube. Seawater was removed and replaced with 10% formalin for ~ 20 min to arrest further development. After the fixing period, the 10% formalin was replaced with 1% PBS and the embryos stored until examination. These time points were chosen to allow for exposure to the experimental conditions that equaled approximately 30% and 80% of the time it would take for hatching to occur (i.e., ~ 10 and 20 hours post-spawn respectively). Additionally, at these time points there were easily identifiable visual markers of developmental stage. The embryo can be seen at 9 hrs (Figure 3.1) and at 19 hrs (Figure 3.2) the finfold has formed. Embryos in the 0.5 ml bullet tube were categorized and counted based on the presence or absence of the appropriate visual marker for the time point. Control CO_2 levels were ~ 440, 390, and

420 ppm; high CO_2 levels were ~ 950, 880, and 960 ppm. Temperatures were ~ 27.3ºC $\pm$ 0.3ºC, 28.0ºC $\pm$ 0.8ºC, and 28.0ºC $\pm$ 0.2ºC, respectively.

Statistical Procedures

The Mann-Whitney statistical test was used to detect significant differences between the control (~ 380 ppm CO_2) and treatment level (~ 800 ppm CO_2) for respirometer trials because data did not exhibit equal variance. Replicates for the respirometer method were pooled for the five trials. After determining there was not a significant difference among data for the 400, 480, and 500 ppm CO_2 treatment levels with the BOD methodology, the replicates within each treatment were pooled together, which increased the number of replicates for 400 ppm CO_2 to 9. A separate linear regression on the non-pooled data was used to detect differences among the BOD bottle trials. Results for the respiration rates with the respirometer were reported in nmol O_2/embryo/hr $\pm$ (S.E.), and BOD bottle rates were reported in nmol O_2/embryo/hr $\pm$ (S.D.). The 9 and 19 hr development data were analyzed with a Chi-Square Test. All analyses were conducted using SigmaStat for Windows version 3.5 and considered significantly different at $P < 0.05$.

3.3 Results

Embryo respiration

The mean rate of oxygen consumption of cobia embryos at control CO_2 levels (380 ppm) for the 5 trials using the respirometer method was 3.9 $\pm$ 1.1 nmol O_2/embryo/hr. Mean respiration rates decreased to 3.2 $\pm$ 0.9 nmol O_2/embryo/hr at the elevated CO_2 level of 800 ppm. This 17% decrease in respiration rates between the two

CO_2 levels was not statistically significant (Mann-Whitney U(19) = 114, p = 0.520) (Figure 3.3).

Utilizing the BOD bottle method, the mean rate of oxygen consumption of cobia embryos at control CO_2 levels (~ 400 ppm) was 2.3 $\pm$ 0.1 nmol O_2/embryo/hr (Figure 3.4). The mean rate of oxygen consumption displayed an inconsistent trend at higher levels of CO_2 concentrations. At a CO_2 level of ~ 580 ppm, respiration increased to 2.7 $\pm$ 0.1 nmol O_2/embryo/hr followed by a decrease to 2.2 $\pm$ 0.1 nmol O_2/embryo/hr at 1000 ppm with a final increase to 2.6 $\pm$ 0.1 nmol O_2/embryo/hr at ~ 2920 ppm. Based on regression analysis, 2% (R^2 = 0.0249) of the change in oxygen consumption could be explained by the CO_2 concentration (Figure 3.5). These differences were not statistically significant ($F_{1,2}$ = 0.298, p = 0.640).

Nine and 19 hr development

In the first trial embryos in the control group for the nine hour sampling were delayed approximately 1 hr in development from the high CO_2 treatment group. This assessment was based on personal hourly visual observations of cobia development from within 1 hr of being spawned through hatching carried out prior to the trials. This difference was statistically significant (Chi-square (1) = 258.668, p < 0.001) (Table 3.3). The temperature in the control tank suddenly decreased approximately 1-2 hrs after the trial began and ranged from 26.67 – 27.41 °C (Mean 27.0 °C) prior to the first sampling. The temperature in the high CO_2 tank ranged from 27.28 -27.51 °C (mean 27.4 °C) by the time these samples were taken. All of the embryos for both groups at the 19- hr sampling were at their correct stage of development (Table 3.4) and as result the Chi-square test could not be completed by SigmaStat.

In the second set of trials only 1 embryo for both groups was not at the correct stage of development for the 9-hr sampling. Thus, there was not a significant difference (Chi-square (1) = 0.427, p = 0.513) (Table 3.5). One embryo in each group was not at the correct stage of development at the 19-hr sampling and no statistical difference was found (Chi-square (1) = 0.00872, p = 0.926) (Table 3.6).

In the third set of trials there was a statistical difference in the 9-hr sampling (Chi-square (1) = 6.290, p = 0.012) (Table 3.7). However, this difference was due to more embryos not being at the correct stage of development in the control group than in the high CO_2 group. This pattern was reversed at the 19-hr sampling with more embryos in the high CO_2 group not being at the correct stage of development. This difference was statistically significant (Chi-square (1) = 1.516, p = 0.218) (Table 3.8).

3.4 Conclusion

The respiration rates for cobia embryos were different between the two methods used. This may have been the result of the time between the use of two methods (~ 1.5 yrs) and/or a change in the broodstock. Control rates with the respirometer method had a mean of 3.9 $\pm$ 1.1 nmol O_2/embryo/hr $\pm$ S.E. and mean of 2.3 $\pm$ 0.1 nmol O_2/embryo/hr $\pm$ S.D. with the BOD method. At 800 ppm CO_2 respiration was 3.2 $\pm$ 0.9 nmol O_2/embryo/hr $\pm$ S.E. with the respirometer and 2.5 nmol O_2/embryo/hr based on the regression model for BOD method. There was a significant difference between the rates from each of the methods that prevented them from being pooled together. Regardless of the method used, there was not a significant difference in respiration rates examined among the CO_2 concentrations used. Embryo respiration rates under similar

conditions as this study were not found in the literature. Respiration rates for embryos of other species under ambient conditions have been converted to similar units, adjusted for 27ºC assuming the temperature coefficient (Q10) = 2.0 and included in Table 3.9. Cobia embryo respiration under control conditions (380 & 400 ppm CO_2) were 3.9 $\pm$ 1.1 nmol O_2/embryo/hr $\pm$ S.E. and 2.3 $\pm$ 0.1 nmol O_2/embryo ·hr $\pm$ S.D., which were similar to bay anchovy 1.4 nmol O_2/embryo ·hr, lined sole 2.5 nmol O_2/embryo ·hr and sea bream 3.9 nmol O_2/embryo ·hr. Cobia embryo respiration rates in this study were less than those recorded for dolphin fish 11.9, 12.5, and 16.5 nmol O_2/embryo ·hr and cobia 11.8 nmol O_2/embryo ·hr.

The finding that embryo development was not impacted by high CO_2 levels was in agreement with the findings of Munday et al. (2009b), Franke and Clemmesen (2011), and (Frommel et al. 2013) but not with Kikkawa et al. (2003, 2004) or Baumann et al. (2012). One reason for the different findings between this study and Kikkawa et al. (2003, 2004) were the CO_2 levels used in the trials. In an effort to address ocean sequestration of CO_2 to mitigate rising atmospheric concentrations both studies (Kikkawa et al. 2003, 2004) used very high CO_2 concentrations. The highest CO_2 level used in the present study for the development trials was 960 ppm but for Kikkawa et al. (2003) it was ~ 116,490 ppm, ~ 97,380 ppm for Kikkawa et al. (2004) and 780 ppm for the embryo experiments conducted by Baumann et al. (2012). Additionally, there were different species used among the studies, which would contribute to different responses. For example, Baumann et al. (2012) used the inland silverside *Menidia beryllina*, which are found schooling in estuaries. Physiologically, Franke and Clemmesen (2011) proposed that the chloride cells used in ion regulation for helping to maintain internal acid/base

control may not have developed during the cleavage stage that Kikkawa et al. (2003) observed as being one of the most sensitive stages. It has been shown that chloride cells are present at later developmental stages in the yolk-sac membrane and body skin of embryos and larvae (Shiraishi et al. 1997, Hiroi et al. 1998, Katoh et al. 2000).

In conclusion, cobia embryo respiration rates did decrease with the respirometer method and fluctuated with the BOD method, but none of the differences were statistically significant. Differences in embryo development at 9- and 19-hrs were not consistent and [CO_2] did not appear to have an impact. Cobia embryos appear to be resistant to the range of CO_2 levels (800 – 2920 ppm) used in this study.

Table 3.1 Conditions for cobia embryo respiration trials utilizing the ENDECO 1125 respirometer. Mean ± SD, Emb = embryos, Res = respirometer, C = control, T = treatment

Trial	Emb/Res	Res volume (ml)	CO_2 (ppm)		pH	T (ºC)
1	10	12.5	C	380 ± 10	8.0 ± 0.01	27 ± 0.8
	20		C			
	10		T	800 ± 5	7.84 ± 0.01	
	20		T			
2	10	12.5	C	380 ± 9	7.99 ± 0.01	27 ± 0.8
	20		C			
	10		T	800 ± 6	7.84 ± 0.01	
	20		T			
3	10	12.5	C	380 ± 10	7.99 ± 0.01	27 ± 0.8
	20		C			
	10		T	800 ± 5	7.84 ± 0.01	
	20		T			
4	5	5	C	380 ± 12	7.98 ± 0.01	27 ± 0.8
	10		C			
	5		T	800 ± 15	7.83 ± 0.01	
	10		T			
5	5	5	C	380 ± 15	7.99 ± 0.01	27 ± 0.8
	10		C			
	5		T	800 ± 20	7.83 ± 0.01	
	10		T			

Table 3.2 Conditions for cobia embryo respiration trials utilizing biological oxygen demand bottles. Mean ± SD, C = control, T = treatment

Trial		CO_2 (ppm)	pH	Temperature (ºC)
1	C	480 ± 33	7.98 ± 0.02	27 ± 0.8
	T	500 ± 7	7.97 ± 0.00	
2	C	400 ± 2	8.05 ± 0.00	27 ± 0.8
	T	1020 ± 14	7.70 ± 0.00	
3	C	580 ± 4	7.96 ± 0.00	27 ± 0.8
	T	2920 ± 549	7.34 ± 0.07	

Table 3.3 Nine hour sampling of cobia embryos for the first trial. A significant number of embryos in the control group were not at the correct stage of development. C = control

CO_2 Level (ppm)	PRESENT (at correct stage of development)	NOT PRESENT (not at correct stage of development)	TOTAL
440 C	0	174 (at time 8 hr development)	174
950	107	6	113

Table 3.4 Nineteen hour sampling of cobia embryos for the first trial. All of the embryos for both groups were at the correct stage of development and the Chi-square test could not be completed. C = control

CO_2 Level (ppm)	PRESENT (at correct stage of development)	NOT PRESENT (not at correct stage of development)	TOTAL
440 C	178	0	178
950	193	0	193

Table 3.5 Nine hour sampling of cobia embryos for the second trial. C = control

CO_2 Level (ppm)	PRESENT (at correct stage of development)	NOT PRESENT (not at correct stage of development)	TOTAL
390	115	1	116
880	106	1	107

Table 3.6 Nineteen hour sampling of cobia embryos for the second trial. C = control

CO_2 Level (ppm)	PRESENT (at correct stage of development)	NOT PRESENT (not at correct stage of development)	TOTAL
390 C	210	2	212
880	138	2	140

Table 3.7 Nine hour sampling of cobia embryos for the third trial. More embryos in the control group were not at the correct stage of development than in the high CO_2 group. C = control

CO_2 Level (ppm)	PRESENT (at correct stage of development)	NOT PRESENT (not at correct stage of development)	TOTAL
420 C	199	12	211
960	216	2	218

Table 3.8 Nineteen hour sampling of cobia embryos for the third trial. C = control

CO_2 Level (ppm)	PRESENT (at correct stage of development)	NOT PRESENT (not at correct stage of development)	TOTAL
420 C	182	0	182
960	239	4	243

Table 3.9 Mean respiration rates of fish embryos available in literature and this study. Rates were adjusted for 27ºC assuming the temperature coefficient (Q10) = 2.0. NR = Not reported, Pers. Com = Personal communications, BOD = Biological oxygen demand bottles

Author	Species	Respiration (nmol O_2/embryo ·hr)	CO_2 (ppm)
Houde & Schekter, 1983	Bay anchovy	1.4	NR
	Lined Sole	2.5	NR
	Sea bream	3.9	NR
Benetti, 1992	Dolphin Fish	12.5 & 11.9 (mean)	NR
Pers. Com. (Benetti 2012)	Mahi/Dolphin Fish	16.5	NR
Pers. Com. (Benetti 2012)	Cobia	11.8	NR
Current study	Cobia		
	(respirometer)	3.9/3.2	~ 380/800
	(BOD)	2.3	~ 400
		2.7	~ 580
		2.2	~ 1000
		2.6	~ 2920

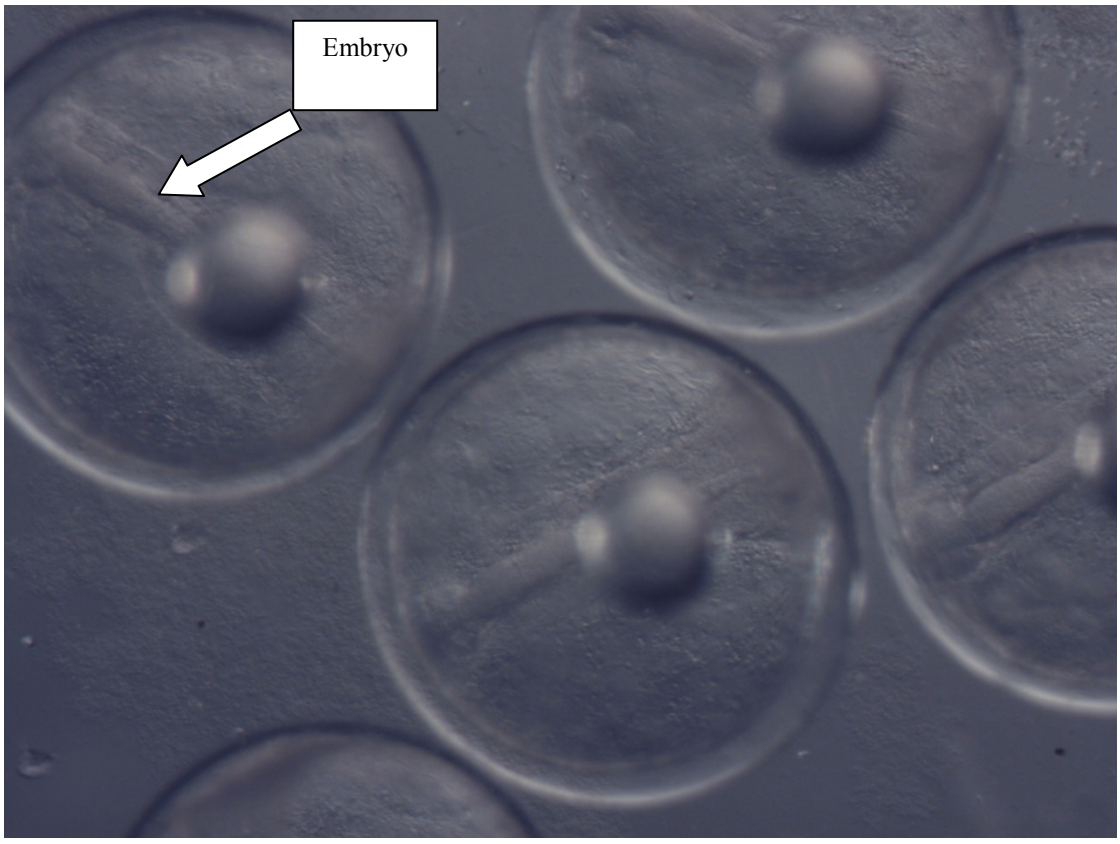

Figure 3.1 Cobia embryos at 9 hrs post fertilization.

Figure 3.2 Finfold present on cobia embryo at 19 hrs post fertilization.

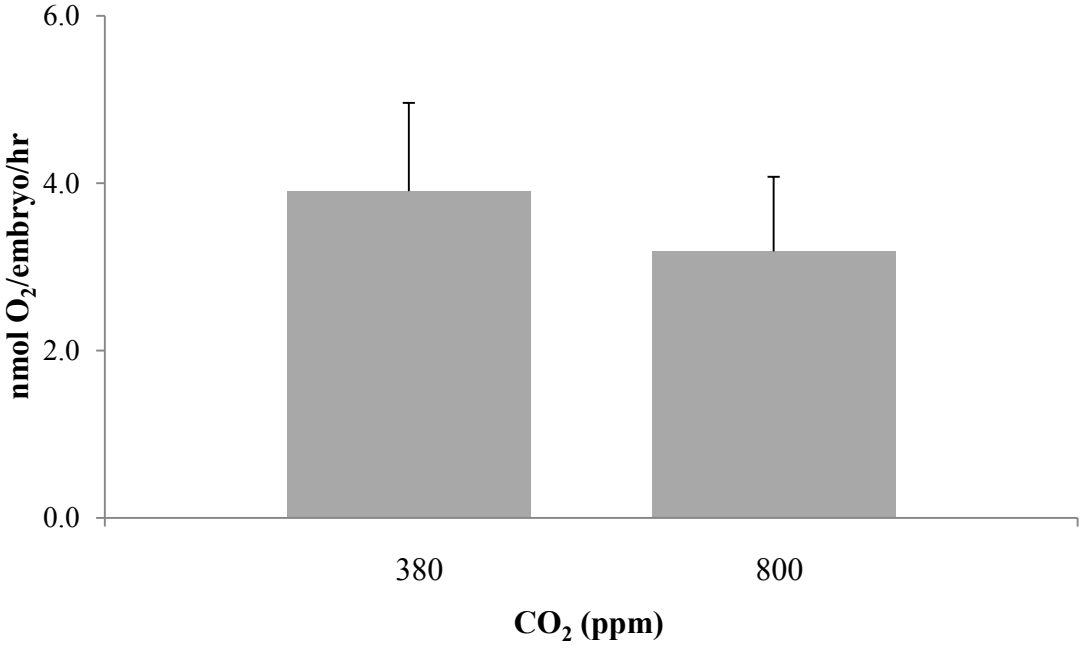

Figure 3.3 Mean respiration rate (nmol O_2/embryo/hr $\pm$ S.E.) of cobia embryos from 5 trials utilizing the respirometer methodology (Table 3.1). n = 10 replicates/CO_2 level.

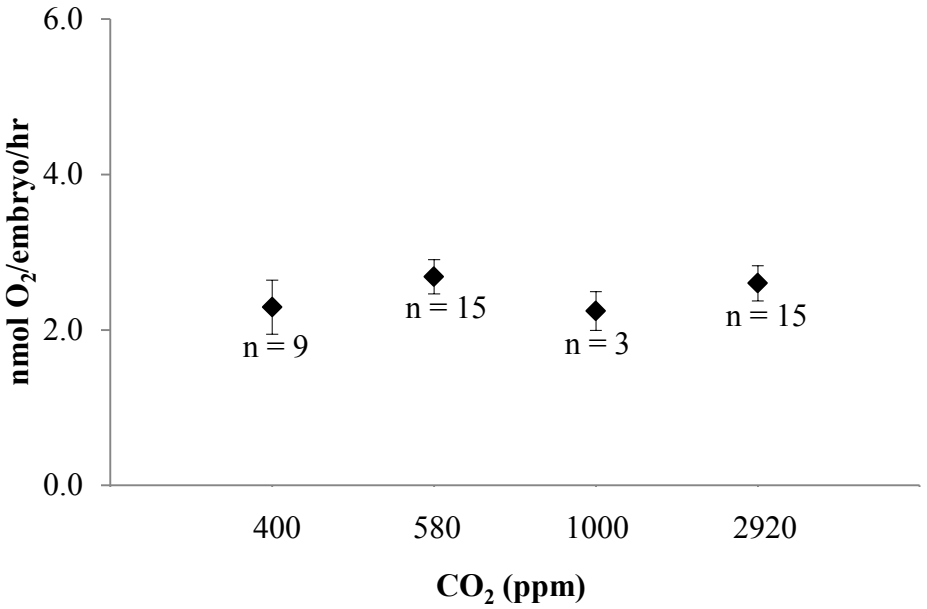

Figure 3.4 Mean respiration rate (nmol O_2/embryo/hr $\pm$ S.D.) from pooled data of cobia embryos from 3 trials utilizing the BOD bottle methodology (Table 3.2). n = number of replicates that were pooled for each data point.

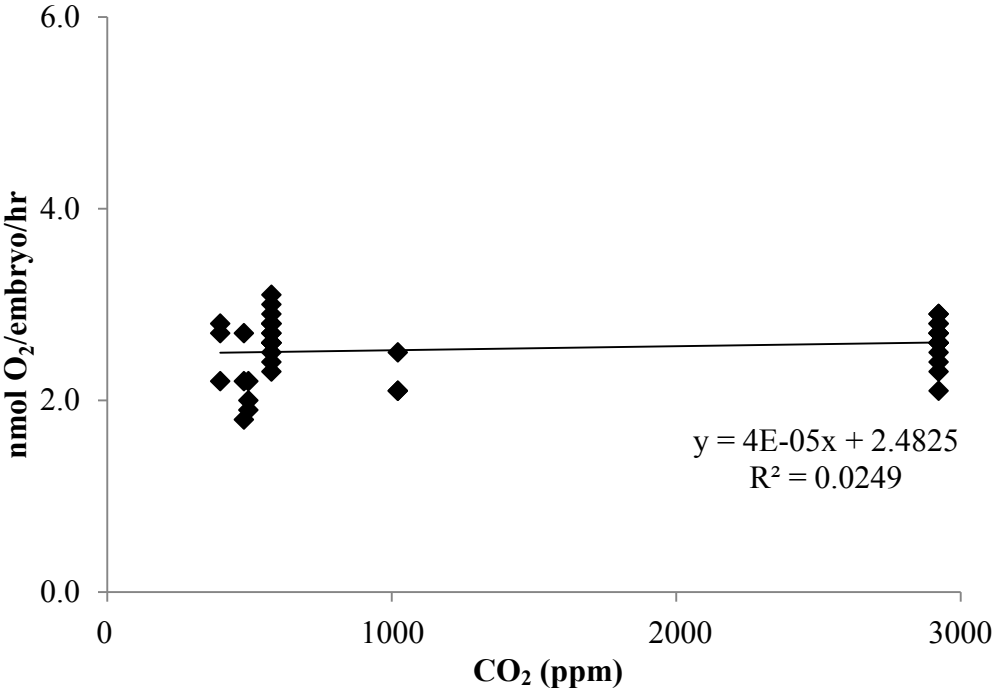

Figure 3.5 Respiration rates (nmol O_2/embryo/hr) of cobia embryos from 3 trials utilizing the BOD bottle methodology (Table 3.2).

Chapter 4: Influence of Increased CO_2 on Cobia Yolk-Sac Larvae Respiration and Total Length

4.1 Background

The already observed decline in oceanic pH of 0.1 since the pre-industrial period (IPCC 2007) is attributable to increased atmospheric carbon dioxide resulting from anthropogenic activity such as fossil fuel combustion, cement production and gas flaring (Le Quéré et al. 2009). The continued absorption of atmospheric carbon dioxide by the oceans will lead to an intensification of oceanic acidity that is forecasted to lead to an additional pH decline of 0.14 – 0.35 over the course of the 21st century (IPCC 2007). The resulting altered environmental conditions have been shown to negatively impact the physiology of numerous marine organisms. Impacts range from decreased coral calcification (Kleypas et al. 1999, Langdon et al. 2000, Leclercq et al. 2000, Marubini et al. 2001, Leclercq et al. 2002) to reduced sea urchin fertilization success and embryo cleavage (Havenhand et al. 2008) as well as sea urchin development (Kurihara & Shirayama 2004, O'Donnell et al. 2009).

The responses in fish to hypercapnic conditions have been variable. Larval reef fish have displayed increased growth at 550, 750, and 1030 ppm CO_2 relative to the control of 390 ppm (Munday et al. 2009b). The ability to detect olfaction cues for suitable habitats was disrupted at ~1000 ppm and completely impaired at ~1700 ppm (Munday et al. 2009a). Yet in another study larval fish, termed juvenile, were not impacted by elevated CO_2 levels (600, 725, 850 μatm) (Munday et al. 2011). At CO_2 levels beyond end of the century projections such as at 1800 and 4200 μatm, which is

realistic in the the Kiel Fjord in the Baltic Sea where it can get up to 2300 µatm, severe to lethal tissue damage to Atlantic cod larvae has been observed (Frommel et al. 2011).

Due to the varied responses that increasing levels of CO_2 may have on the early life-history stages of fish, the purpose of the current study was to determine if the metabolism and total length of cobia *Rachycentron canadum* yolk-sac larvae would be significantly impacted by near-future CO_2 concentrations based on IPCC projections (IPCC 2007).

4.2 Materials and Methods

Yolk-sac larvae respiration

In all instances, each trial of the experiment used embryos collected from a different spawning. Cobia are broadcast spawners, as a result there are potentially multiple parent combinations for each spawning. A typical ratio of broodstock are 6 males to 3 females at UMEH. Prior to the trials being conducted, cobia embryos were collected via dip net at UMEH within 1 hr of spawning. A 10 ml aliquot of floating embryos were deposited into a control tank (pCO_2 ~ 380 ppm), and a high CO_2 tank (pCO_2 ~ 800 ppm) both maintained at 27ºC $\pm$ 0.8ºC. Embryos were incubated in their respective tanks until they hatched. Subsamples of newly hatched yolk-sac larvae were taken from the tanks and counted in a petri dish for placement in the four respirometers, which utilized the ENDECO 1125 as described in section 2.2. Each respirometer received the same amount of either control water or high-CO_2 seawater just prior to the addition of the yolk-sac larvae so that there were two respirometers at each CO_2 concentration (Table 4.1). Water used in the respirometers was obtained and filtered

through a 0.2 μm filter to eliminate background respiration that may have been a result of biological activity. The experiment were conducted with a water volume of 17 ml per respirometer and 5 or 10 yolk-sac larvae so that there were two respirometers with 5 larvae and two with 10 larvae. There were two trials for the experiment. The respirometers were turned by hand every half hour and temperature was maintained via a water bath at 27ºC $\pm$ 0.8ºC for both of the trials. The rate of oxygen consumption per yolk-sac larva was determined by multiplying the change in oxygen concentration over time, corrected for sensor oxygen consumption, by the volume and dividing by the number of yolk-sac larvae used in the respirometer.

Yolk-sac larvae total length

Yolk-sac larvae were obtained in a similar manner as the yolk-sac larvae respiration section, whereby the embryos were obtained from different spawnings on the previous night and maintained in tanks with control or experimental water until hatching occurred. Yolk-sac larvae were placed in a 0.5 ml bullet tube within 1 hr of hatching. Seawater was removed and replaced with 10% formalin for ~ 10 min to euthanize the larvae. After the fixing period, the 10% formalin was replaced with 1% PBS and yolk-sac larvae were stored until pictures were taken. Pictures were taken with a dissecting microscope mounted with a Nikon Coolpix digital camera using Q-Capture software. Yolk-sac larvae in pictures were measured for total length to the nearest millimeter using Coral Point Count. This trial was conducted four times; the first trial included two treatment levels and the remaining three trials had one treatment level (Table 4.2).

Statistical Procedures

A t-test was used to detect significant differences between the control (~ 380 ppm CO_2) and treatment level (~ 800 ppm CO_2) for the pooled data of the two separate respirometer trials so that n = 4 per CO_2 level. Results for the respirometer trials were reported in nmol O_2/larvae/hr $\pm$ (S.E.).

Data for the first yolk-sac larvae total length trial were analyzed with an ANOVA; the Holm-Sidak method was for post hoc analysis. The second and third total length trials were analyzed with the Mann-Whitney statistical test. The final total length trial was analyzed with a t-test. Results for the total length trials were reported in mm $\pm$ (S.D.). All analyses were conducted using SigmaStat for Windows version 3.5 and considered significantly different at $P < 0.05$.

4.3 Results

Yolk-sac larvae respiration

The mean rate of oxygen consumption of cobia yolk-sac larvae at control CO_2 levels (380 ppm) for the 2 trials was 10.7 $\pm$ 3.4 nmol O_2/larvae/hr. Mean respiration rates decreased to 6.6 $\pm$ 2.3 nmol O_2/larvae/hr at the elevated CO_2 level of 800 ppm. This 38% decrease in respiration rates between the two CO_2 levels was not statistically significant ($t(6) = 0.994$, $p = 0.359$) (Figure 4.1).

Yolk-sac larvae total length

The mean total length of control (380 ppm CO_2) yolk-sac larvae in the first trial was 2.59 $\pm$ 0.34 mm. Mean total length decreased to 2.29 $\pm$ 0.28 mm at 560 ppm CO_2

but increased to 2.89 $\pm$ 0.28 mm at 800 ppm CO_2. The differences between the control and both treatment levels, as well as between the treatment levels were statistically significant (F (2, 90) = 14.62, p = < 0.001) (Figure 4.2). The mean total length of control yolk-sac larvae for the second and third trials were 2.10 $\pm$ 0.13 mm at 443 ppm and 4.18 $\pm$ 0.23 mm at 393 ppm respectively. The mean total length in the second trial decreased to 2.05 $\pm$ 0.27 mm at 949 ppm CO_2; total length increased to 4.21 $\pm$ 0.20 mm at 876 ppm CO_2 in the third trial. The differences between the control and treatment level were not statistically significant for both the second (Mann-Whitney U(119) = 1627.5, p = 0.601) (Figure 4.3) and third (Mann-Whitney U(249) = 8445.5, p = 0.211) (Figure 4.4) trials. Yolk-sac larvae in the final trial had a mean total length of 3.48 $\pm$ 0.40 mm for the control CO_2 level of 420 ppm. Mean total length increased to 3.69 $\pm$ 0.33 mm at 957 ppm, which was statistically significant (t(198) = -4.108, p < 0.001) (Figure 4.5). Data for the total length trials were not pooled due to significant differences between both the control groups and the experimental groups. Such differences are potentially the result of different parent combinations among the different spawnings.

4.4 Conclusion

The 38% decrease in yolk-sac larvae respiration at 800 ppm CO_2 indicates that the larvae were impacted but not at a statistically significant level. It is not known if this decrease is large enough to impact the larvae or a later stage of development. Not finding the decrease in respiration to be significant may have been due to the variability of data. A large amount of variability in yolk-sac larvae respiration has been shown in

experiments conducted by Collins and Nelson (1993). Rabbitfish *Siganus randalli* yolk-sac larvae respiration ranged from ~ 5.6 – 12.3 nmol O_2/larvae/hr at 27ºC within 43 hrs post-spawning (Collins & Nelson 1993). Rabbit fish respiration was ~ 4.0 – 8.9 nmol O_2/larvae/hr at 32ºC within the same time frame.

The results for the total length trials were variable. The observed trend of decreased length at mid-level CO_2 with increased total length at high CO_2 levels were consistent with the finding of (Munday et al. 2009b) for the orange clownfish *Amphiprion percula*. Munday et al. (2009b) conducted the experiment with different parental groupings and found that one group was not impacted regardless of the CO_2 level (390-control, 550, 750, 1030 ppm) used. The standard length (SL) from the second group was significantly greater from the control group (390 ppm) by ~ 12% at 750 ppm and by ~ 14% at 1030 ppm CO_2. The SL from the third group decreased ~ 3.3% at 750 ppm but significantly increased by ~ 8.2% at 1030 ppm. The SL of the fourth and final group significantly increased at every CO_2 concentration; ~ 20% at 550 ppm, ~ 15% at 750 ppm, and ~ 18% at 1030 ppm. In the current study, total length in two of the four trials conducted displayed significant differences from the control groups but the total length increased in three of the four trials. Total length decreased 11.6% between 380 ppm and 560 ppm but increased by 12% between the control group (380 ppm $CO_{2)}$ and the high treatment group (800 ppm $CO_{2)}$ in the first trial. This trend remained consistent in the third trial with a 0.7% increase and the fourth trial with a 6% increase. The second trial had a 2.4% decrease in total length between the control of 443 ppm CO_2 and high CO_2 of 949 ppm. Hurst et al. (2013) also had a variable trend finding with walleye pollock *Theragra chalcogramma*, however none of the results were statistically significant. The

reasons for such variability in the responses to hypercapnia among the three studies maybe the result of non-genetic parental effects reviewed with regards to maternal effects by Green and David (2008) and demonstrated with both parents by Miller et al. (2012). In essence, the impact to the offspring can be mitigated by exposing the parents. In this study it was not possible to determine the parentage of the cohorts because cobia are broadcast spawners. It is possible that such variability in the total lengths of the yolk-sac larvae could have been the result of genetic variation from different parent combinations. This could be the reason the maximum mean length of yolk-sac larvae under control conditions ranged from 2.10 $\pm$ 0.13 to 4.18 $\pm$ 0.23 mm ($\pm$ S.D). The possibility of it being a spawn quality issue based on when it occurred in the spawning season (~ April-October) is reduced due to this range occurring from spawns during July within two weeks of one another.

The findings in this study were not consistent with Franke and Clemmesen (2011), Frommel et al. (2013), Munday et al. (2013) or Baumann et al. (2012). For Franke and Clemmesen (2011) and Frommel et al. (2013) the differences could be the result of how rearing occurred. Franke and Clemmesen (2011) used Atlantic herring *Clupea harengus* from the Kiel Fjord which has peak CO_2 levels that exceed 2300 µatm. There may not be an impact to the cod embryos from hypercapnia because of the parents being exposed to such conditions as was shown by Miller et al. (2012) with juvenile anemonefish. Frommel et al. (2013) maintained the Baltic cod embryos used in ambient CO_2 until they hatched then transferred the newly hatched larvae to experimental conditions. Additionally, the Baltic cod were obtained from the Bornholm Basin; they are spawned below the halocline where CO_2 levels can be elevated due to microbial

activity and depleted levels of oxygen (Frommel et al. 2013). Munday et al. (2013) utilized juvenile coral trout *Plectropomus leopardus*, which were further along in development than the life-history stage examined in the current study. Baumann et al. (2012) observed a decrease in SL for inland silversides at all CO_2 concentrations (396-control, 650, 814, and 1068 ppm) examined. Differences between this study and Baumann et al. (2012) may be the result of different species being used, and that inland silversides school in estuaries.

In conclusion, cobia yolk-sac larvae respiration displayed a 38% decrease, which was not statistically significant. However, variable respiration for yolk-sac larvae was consistent with the literature (Collins & Nelson 1993). Variable total length results were consistent with some literature and potentially imply a resistance to hypercapnia based on parental genetic variation. A rapid increase in size would potentially be useful to larvae because it would aid in avoiding predation at an early life stage (reviewed in Anderson (1988). That advantage could be negated by effects that are sub-lethal or undetected at the life stages examined in this study but are magnified at later stages. For example, Franke and Clemmesen (2011) did not observe an impact to total length from hypercapnia but there was a significant negative relationship between CO_2 and RNA/DNA ratios implying decreased protein biosynthesis, which could impact somatic growth in the future. The potential of such an impact and the varied responses observed both in this study and the literature warrants continued investigation.

Table 4.1 Conditions for cobia yolk-sac larvae respiration trials utilizing the ENDECO 1125 respirometer. Mean ± SD, Ysl = Yolk-sac larvae, Res = respirometer, C = control, T = treatment

Trial	Ysl/Res	Res volume (ml)		CO_2 (ppm)	pH	Temp (ºC)
1	5	17	C	380 ± 10	7.99 ± 0.01	27 ± 0.8
	10		C			
	5		T	800 ± 5	7.84 ± 0.01	
	10		T			
2	5	17	C	380 ± 9	7.99 ± 0.01	27 ± 0.8
	10		C			
	5		T	800 ± 6	7.84 ± 0.01	
	10		T			

Table 4.2 Conditions for cobia yolk-sac larvae total length experiment trials. Each trial was conducted using embryos from a different spawning event that were reared under the conditions listed until being removed for sampling. The CO_2 levels for the third and fourth trials were determined with an equilibrator instead of titration and the pH levels could not be calculated. C = control, T = treatment NA = not available

Trial	CO_2 (ppm)		pH	Temperature (ºC)
1	C	380	8.05 $\pm$ 0.01	27.5 $\pm$ 0.5
	T	560	7.96 $\pm$ 0.01	
	T	800	7.80 $\pm$ 0.02	
2	C	440	8.04 $\pm$ 0.01	27.3 $\pm$ 0.5
	T	950	7.76 $\pm$ 0.01	
3	C	390	NA	28.0 $\pm$ 0.8
	T	880	NA	
4	C	420	NA	28.0 $\pm$ 0.2
	T	960	NA	

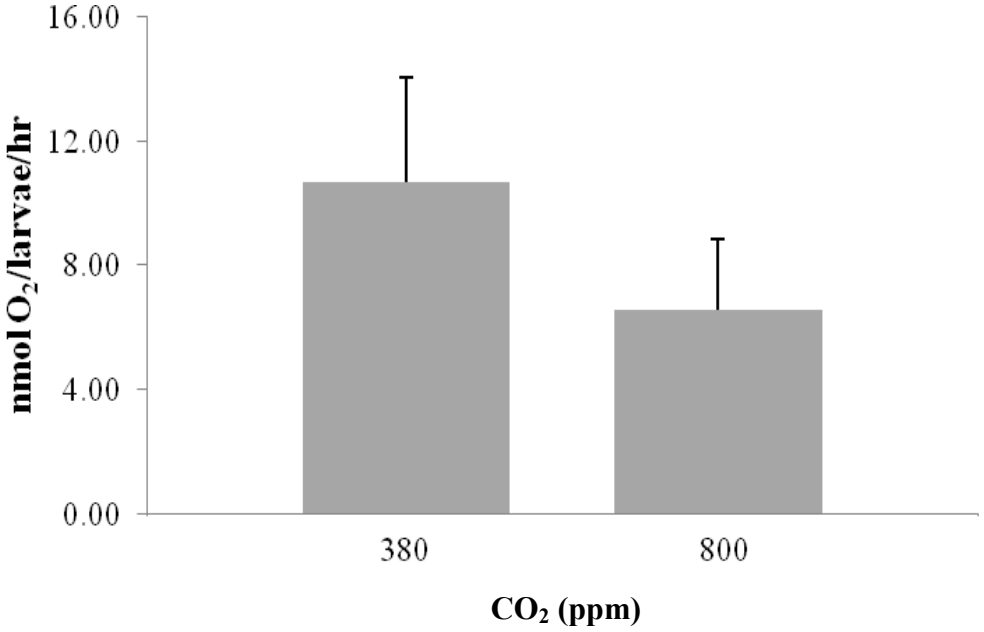

Figure 4.1 Mean respiration rate (nmol O_2/larvae/hr $\pm$ S.E.) of cobia yolk-sac larvae. n = 4 replicates per CO_2 level.

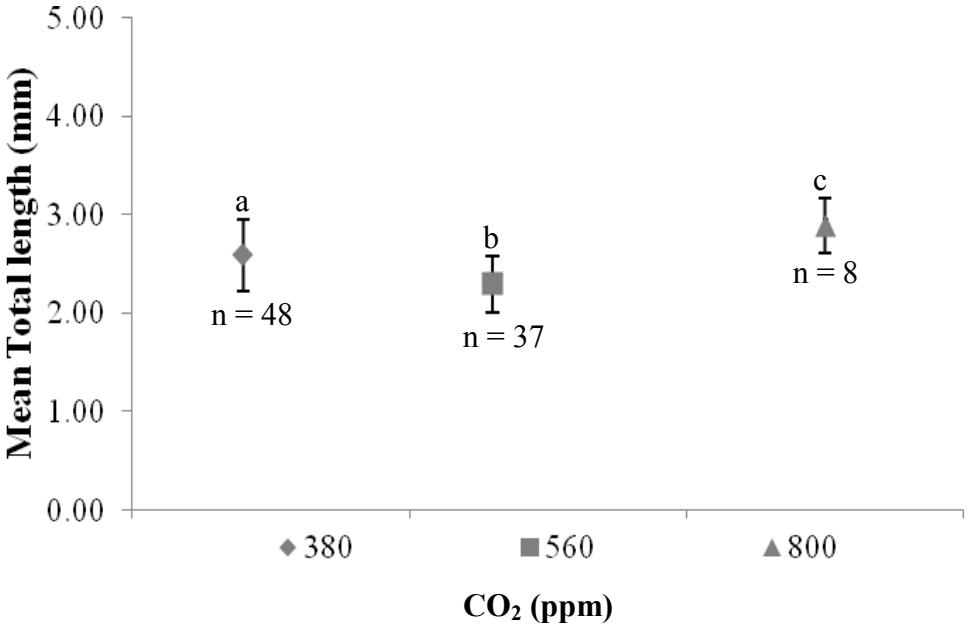

Figure 4.2 Mean total length (mm ± S.D.) of cobia yolk-sac larvae from the first trial. Different letters indicate values are significantly different, n = number of yolk-sac larvae measured.

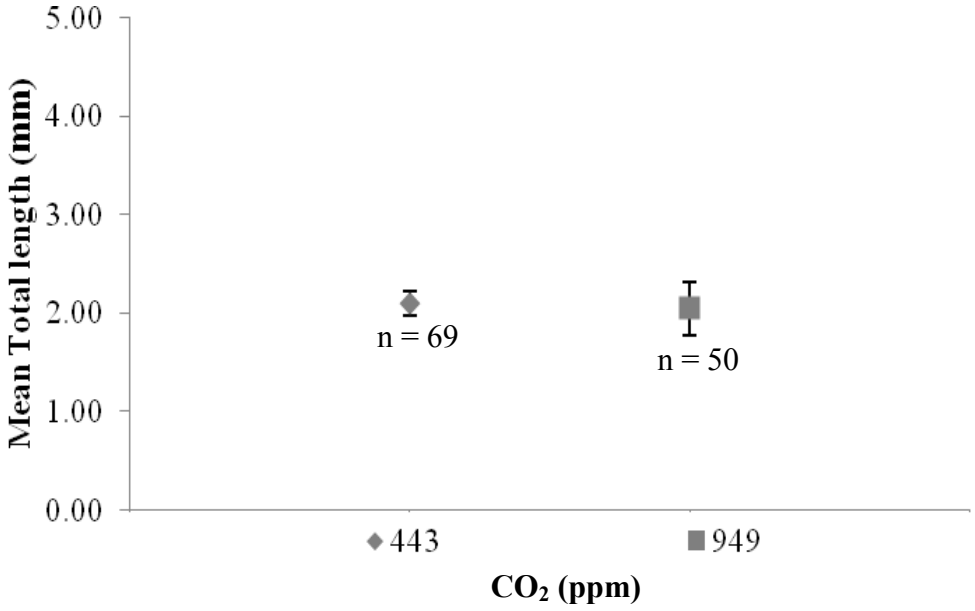

Figure 4.3 Mean total length (mm ± S.D.) of cobia yolk-sac larvae from the second trial. n = number of yolk-sac larvae measured.

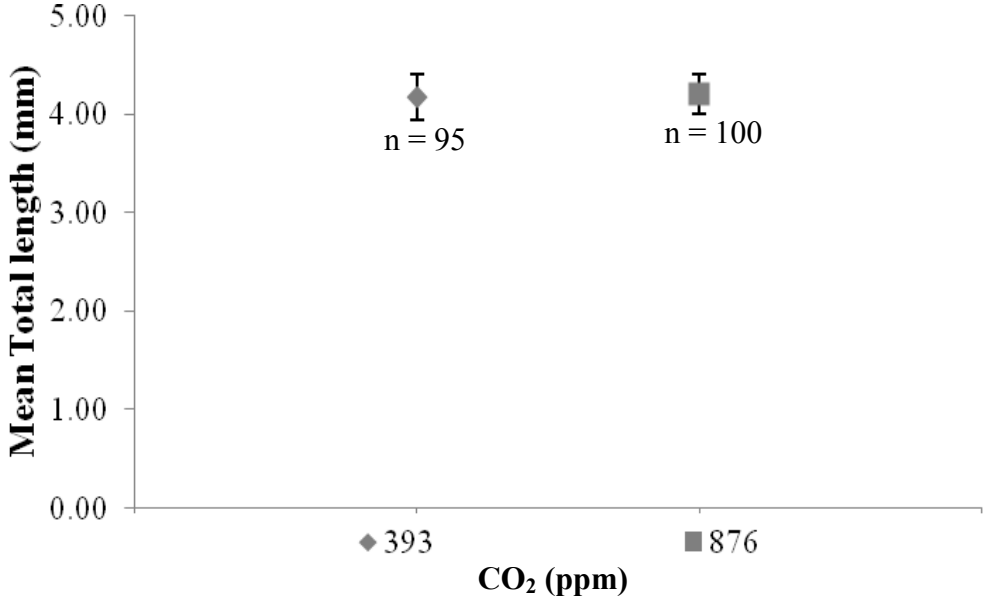

Figure 4.4 Mean total length (mm ± S.D.) of cobia yolk-sac larvae from the third trial. n = number of yolk-sac larvae measured.

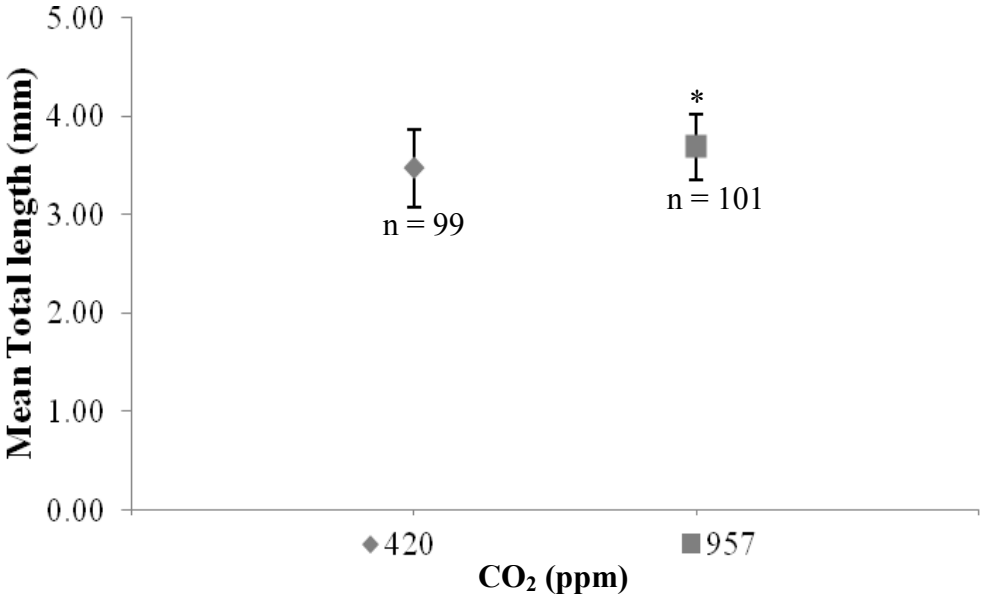

Figure 4.5 Mean total length (mm ± S.D.) of cobia yolk-sac larvae from the fourth trial. Asterisk indicates total length is significantly greater than control total length, n = number of yolk-sac larvae measured.

Chapter 5: Effects of Increased CO_2 on the Metabolism of Cobia Early Life History Stages

5.1 Conclusion

The overall goal of this dissertation was to determine if near future projections for hypercapnia would significantly impact the widely distributed marine fish, cobia during their early life-history stages under endogenous nutrition. Through the examination of the spermatozoa, embryo, and yolk-sac larvae metabolism via oxygen consumption it would appear that the spermatozoa and yolk-sac larvae were impacted by hypercapnia as indicated by decreased respiration rates but not significantly. Significant responses were found with other metrics. Spermatozoa velocity consistently decreased at 2920 ppm CO_2 relative to the control at 577 ppm CO_2. The total length of yolk-sac larvae increased at high CO_2 levels (800, 876, and 957 ppm) for three of the four trials but only the first and last were statistically significant. Total length was significantly less at 560 ppm CO_2, and there was not a significant difference in the rate of development of cobia embryos.

Variability in the data during the life stages examined was consistent with the literature (Stoss & Holtz 1983, Collins & Nelson 1993, Munday et al. 2009b, Hurst et al. 2013). In an effort to address the variability found in the data from this study, the number of replicates was increased and the experiments repeated as permitted by logistical constraints. An alternative methodology was applied for embryo respiration and the data were pooled when possible for all of the trials. It would be interesting to conduct the study by maintaining adults in a controlled hypercapnic environment, conduct fertilization experiments under the same hypercapnic conditions and then culture the larvae to later life-history stages to determine if impacts would occur. It has been shown that near future CO_2 levels can impact later life stages for cobia as indicated by

enlarged otoliths (Bignami et al. 2013), but would potential parental conditioning (Miller et al. 2012) mitigate such impacts?

The results of the experiments here demonstrate that hypercapnia does have the potential to impact cobia during the early life stages, but it also demonstrates that cobia have the potential to resist near-future perturbations. Due to the projected levels of near future CO_2 concentrations, studies should focus on the synergistic effect of sub-lethal impacts over multiple life stages. The effect of hypercapnia is species specific as the literature has shown (Stoss & Holtz 1983, Collins & Nelson 1993, Munday et al. 2009b, Franke & Clemmesen 2011, Baumann et al. 2012, Frommel et al. 2013, Hurst et al. 2013, Munday et al. 2013). The varied responses observed both in this study and the literature warrants continued investigation on the effects of hypercapnia.

5.2 Statistical power

The probability of a statistical test to correctly detect the treatment effect (i.e. reject the null hypothesis when the alternative hypothesis is correct) is known as the power of the test and typically has a desired level of 80% (Moore & McCabe 1999). The power of the statistical analyses conducted in the current study was quite variable, ranging from 4% in one of the 19-hr embryo development chi-square tests to 99% with a yolk-sac larvae total length ANOVA test (Table 5.3). The 100% power with a 9-hr embryo development chi-square test (Table 5.3) is uninteresting as this was from the trial which was confounded by temperature fluctuation and zero embryos were at the correct stage of development in the control. The Mann-Whitney U test was employed to analyze all respiration rates determined with the ENDECO respirometer. The small sample sizes

associated with the respirometer methodology resulted in low power for the Mann-Whitney U test, potentially close to zero (GraphPad 2013). The low power maybe the reason that a statistical significance was not shown for any data generated with this methodology. For a significant difference to be detected with the Mann-Whitney U test the sample size would likely need to have been greater than 12 (GraphPad 2013).

The power of a test can be improved by increasing α, increasing the sample size, increasing the impact of the treatment and/or decreasing the standard deviation, which is similar to increasing the sample size (Moore & McCabe 1999). In the current study, low power was addressed by increasing the replicates when logistically possible, repeating the experiments and pooling the raw data to increase the sample size when it was statistically permissible. In some cases such as with milt velocity and embryo respiration with the BOD method, the treatment level was unintentionally increased, which possibly contributed to increasing the impact of the treatment. The conclusions that were drawn from the results took into account the power of the statistical tests used.

Table 5.1 The power for the different statistical analyses used in this study. The subject matter tested, statistical test used, sample size, power of the statistical tests and chapter in which the analysis occurred. NA = not available

Parameter	Statistical Test	n (sample size)	Power (%)	Chapter
Spermatozoa respiration	Mann-Whitney U	2 replicates per $[CO_2]$	NA	2
Spermatozoa velocity	Mann-Whitney U	1860 per $[CO_2]$	NA	2
Spermatozoa velocity	Mann-Whitney U	1860 per $[CO_2]$	NA	2
Embryo respiration (respirometer)	Mann-Whitney U	10 replicates per $[CO_2]$	NA	3
Embryo respiration (BOD)	Linear regression	9 at ≈ 400 ppm 15 at ≈ 580 ppm 3 at ≈ 1000 ppm 15 at ≈ 2920 ppm	5.7	3
9-hr development	Chi-square test	174 at 440 ppm 107 at 950 ppm	100	3
19-hr development	Chi-square test	178 at 440 ppm 193 at 950 ppm	Not completed	3
9-hr development	Chi-square test	116 at 390 ppm 107 at 880 ppm	9	3
19-hr development	Chi-square test	212 at 390 ppm 140 at 880 ppm	4	3
9-hr development	Chi-square test	211 at 420 ppm 218 at 960 ppm	71	3
19-hr development	Chi-square test	182 at 420 ppm 243 at 960 ppm	22	3
Yolk-sac larvae respiration	t-test	4 per $[CO_2]$	5	4
Yolk-sac larvae total length	ANOVA	48 at 380 ppm 37 at 560 ppm 8 at 800 ppm	99	4
Yolk-sac larvae total length	Mann-Whitney U	69 at 443 ppm 50 at 949 ppm	NA	4
Yolk-sac larvae total length	Mann-Whitney U	95 at 393 ppm 100 at 876 ppm	NA	4
Yolk-sac larvae total length	t-test	99 at 420 ppm 101 at 957 ppm	98	4

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