Effects of CO_2-induced ocean acidification on the early development, growth, survival and skeletogenesis of the estuarine-dependant sciaenid *Argyrosomus japonicus*

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Dedicated to the fish

whose lives I took

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My favourite section, for I was boldly told that I could write however I wanted to. A mistake likely to be regretted! First and foremost, for the countless lives taken, I pray your sacrifice was not in vain and may ultimately lead to the conservation of your kind. Please do not haunt me or hurt my chances in the next life! Second, to the scientists I have reproached: forgive me, I mean you no offence. A true scientist and inspiration once said: "If we scientists cease to criticise each other we should all pack up shop and pursue something with a little more dignity, perhaps drama or philosophy". To those most influential of all, my incredible supervisors, Warren Potts, Nikki James, and Horst Kaiser, without whom none of this would have been possible. This thesis and the rest of my work is what it is today only because of you; whether that is a compliment or an insult I do not know; though I pray it is the former rather than the latter. Your time, patience, and most of all kindness, meant more to me than these pages could ever express. Carla Edworthy, my friend and research partner, thank you. Just as fish cannot swim without the water they are in, so too this thesis would have fallen short without your kind and caring support. Andre Bok, Lawrence Grant and Justin Kemp, I hope this thesis is a worthy compensation for your help, for it is the best thanks I could possibly offer. Finally, a nod to those brave souls who dared wade through mighty rivers, and brawled bravely against mosquito-infested swamps, solely to discover the drug which enlightened my senses and exhilarated my fatigue: here's to you, for introducing to us the most wonderful of addictions, coffee.

ABSTRACT

Although it is increasingly accepted that ocean acidification poses a considerable threat to marine organisms, little is known about the likely response of fishes to this phenomenon. While initial research concluded that adult fishes may be tolerant to changes predicted in the next 300 years, the response of early life stages to end-of-century CO_2 levels (~ 1100 µatm according to the IPCC RCP 8.5) remains unclear. To date, literature on the early growth and survival of fishes has yielded conflicting results, suggesting that vulnerability may be species dependant. The paucity of ocean acidification research on fishes is particularly evident when one considers larval skeletogenesis, with no robust studies on its impacts on bone and cartilage development. This study addresses the early life embryogenesis, hatching success, growth, skeletogenesis and survival of an estuarine-dependant species. Dusky kob (*Argyrosomus japonicus*) were reared in a control (pCO_2 = 327.50 ± 80.07 µatm at pH 8.15), intermediate (pCO_2 477.40 ± 59.46 µatm at pH 8.03) and high pCO_2 treatment (pCO_2 910.20 ± 136.45 µatm at pH 7.78) from egg to 29 days post-hatch (dph). Sixty individuals from each treatment were sacrificed at the egg stage and at 2, 6, 13, 18, 21 and 26 dph, measured and stained using an acid-free double-staining solution to prevent the deterioration of calcified matrices in fragile larval skeletons. The proportion of bone and cartilage was quantified at each stage using a novel pixel-counting method. Growth and skeletal development were identical between treatments until the onset of metamorphosis (21 dph). However, from the metamorphosis stage, the growth and skeletal development rate was significantly faster in the intermediate treatment and significantly slower in the high treatment when compared to the control treatment. By 26 dph, *A. japonicus* reared in high pCO_2 were, on average, 47.2% smaller than the control treatment, and the relative proportion of bone in the body was 45.3% lower in the high pCO_2 treatment when compared with the control. In addition, none of the fish in the high pCO_2 treatment survived after 26 dph. It appears that the combination of the increased energy requirements during metamorphosis and the increased energy cost associated with acid-base regulation may account for reduced growth, skeletogenesis and poor survival in high pCO_2. Regardless of the driver, the results of this study suggest that the pCO_2 levels predicted for the end of the century may have negative effects on the growth, skeletal development, and survival during metamorphosis.

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CHAPTER 1

1. GENERAL INTRODUCTION

Ocean acidification may be the most prominent threat to marine life this century (Caldeira and Wickett 2005, Orr et al. 2005, Fabry et al. 2008, Stocker et al. 2014). Anthropogenic decreases in surface ocean pH and concomitant reductions in carbonate states, commonly referred to as ocean acidification, are expected to adversely impact many marine organisms (Orr et al. 2005, Fabry et al. 2008, Doney et al. 2009). Ocean acidification is the inevitable consequence of the drastic increase in anthropogenic carbon dioxide (CO_2) and concomitant increased dissolution at the ocean surface, following the dawn of the industrial revolution (Caldeira and Wickett 2005). Estimates based on the most recent Intergovernmental Panel on Climate Change (IPCC) business-as-usual emission scenario (IPCC A2 scenario) predict that atmospheric CO_2 levels will further increase from current levels ~ 400 µatm to ~ 950 µatm by the end of the century (Stocker et al. 2014). The consequent increase in CO_2 absorption at the ocean surface is raising serious concerns for marine organisms, given that the current rate of ocean pH decrease is occurring at least ten times faster than at any time in the past 300 million years (Caldeira and Wickett 2003, Orr et al. 2005, Stocker et al. 2014).

Ocean acidification is not a new phenomenon and has been implicated in some of the most severe mass extinctions in the history of our planet (Clarkson et al. 2015), including the end-Permian and Paleocene-Eocene mass extinction events (Gibbs et al. 2010). Approximately 251 million years ago, the end-Permian mass extinction event resulted in the loss of as much as 95% of all species on earth, and the near disappearance of all marine life (Knoll et al. 1996, Hesselbo et al. 2000, Benton and Twitchett 2003). Recent findings suggest that these extinctions may have been caused by ocean acidification events driven by volcanic activity increasing atmospheric pCO_2. Given that the current carbon dioxide concentrations are increasing even faster than during the Permian mass extinction event (Caldeira and Wickett 2003, Orr et al. 2005, Hönisch et al. 2012, Stocker et al. 2014), there is a valid cause for concern (Figure 1.1). Vernon (2008) suggests that the correlation between elevated pCO_2 and past mass extinctions makes current ocean acidification by far the most alarming threat of all the predicted threats associated with anthropogenic climate change.

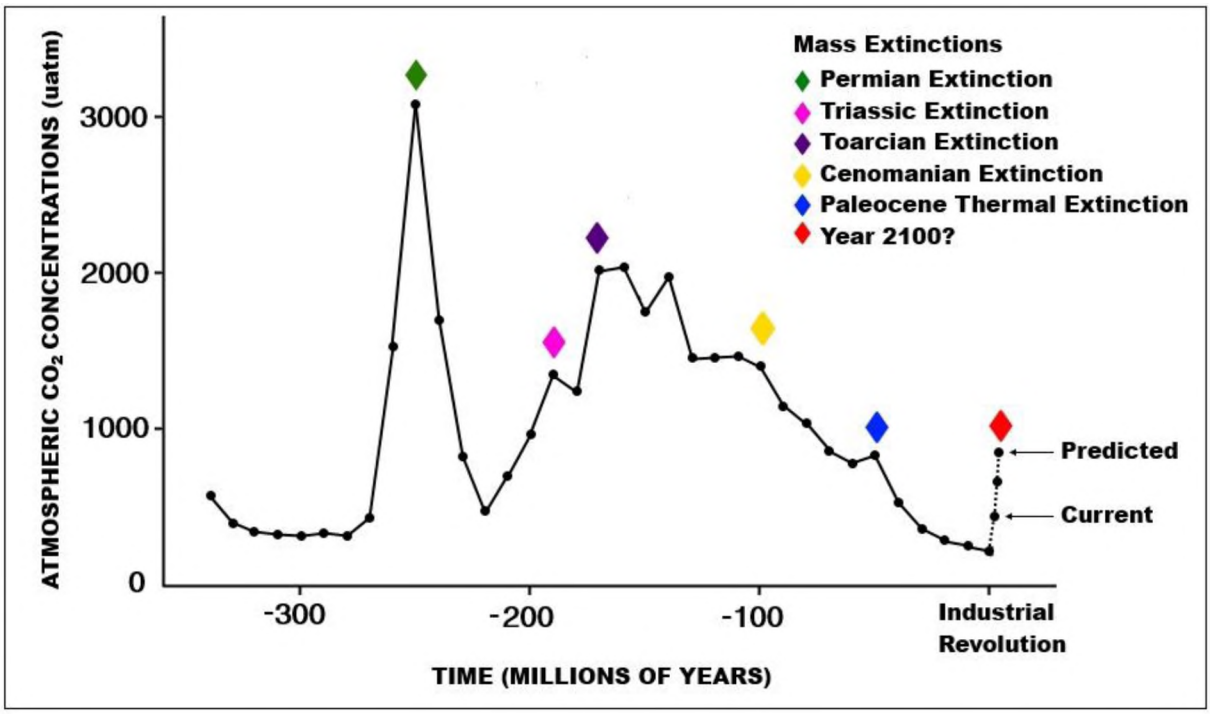


Figure 1.1. Comparing atmospheric carbon dioxide with past mass extinction events in the last 300 million years (Adapted from Ward 2006).

The chemical consequence of elevated atmospheric CO_2 is the formation of carbonic acid ($H_2CO_3^-$) and a free hydrogen ion (H^+) (Feely et al. 2009, Orr et al. 2005). Increased hydrogen ion concentration has already reduced ocean pH to 0.1 units lower than pre-industrial levels and is predicted to decrease ocean pH further by another 0.3 to 0.4 pH units by the end of the century (Orr et al. 2005). This change in ocean pH is equivalent to a 150% increase in ocean acidity relative to pre-industrial levels (Orr et al. 2005, Dupont et al. 2008). Alarmingly, the effects of ocean acidification are not limited to drastically reducing pH. Increasing hydrogen ions bind to carbonate to form bicarbonate (HCO_3^-), thereby lowering carbonate ion concentrations and lowering the saturation states of calcium carbonate and its polymorphs (i.e. different crystalline forms), calcite and aragonite (Fabry et al. 2008, Munday et al. 2008). Since calcite and aragonite are fundamental skeletal building blocks for most calcifying marine organisms (Fabry et al. 2008, Checkley et al. 2009, Zeebe 2012), the under-saturation of aragonite in high latitude surface waters (Orr et al. 2005) is expected to have adverse consequences for exo- and endoskeletal development (Fabry et al. 2008, Munday et al. 2008). Experimental evidence suggests that ocean acidification will impact marine calcifying organisms directly, through reduced calcification rates, and perhaps indirectly by reducing energy available for calcification due to elevated acid-base regulation costs (Fabry et al. 2008).

Despite the potential impacts of ocean acidification on many marine organisms, the response of perhaps our most important marine species, fishes, to the rapid changes predicted in ocean chemistry in the next few decades, remains relatively unclear (Baumann et al. 2012, Stiasny et al. 2016). This may be related to the novelty of ocean acidification research, with the first ocean acidification conference held only in 2004 (Brewer 2013). This knowledge gap is surprising, given the considerable amount of research investigating the response of fishes to global warming (Perry et al. 2005). While fishes may respond to warming temperatures by shifting their distribution patterns (Perry et al. 2005), the effects of ocean acidification are more uniform, inescapable and may potentially pose an even greater threat than global warming (Stiasny et al. 2016, Rosa et al. 2017). Consequently, understanding how fishes will respond to elevated levels of pCO_2 is essential for predicting how ocean acidification will impact on individual species and their adaptability.

To date ocean acidification research has focussed primarily on externally calcifying marine organisms, with adverse effects predicted for corals (e.g. Guinotte et al. 2006, Turley et al. 2007, Maier et al. 2012), shellfish (e.g. Gazeau et al. 2007, Miller et al. 2009, Talmage and Gobler 2010), marine plankton (e.g. Riebesell et al. 2007) and other invertebrates (e.g. Shirayama and Thornton 2005, Kurihara 2008). Fabry et al. (2008) suggest that of all marine organisms, fishes are the most unlikely to be affected by ocean acidification, with Ishimatsu et al. (2008) suggesting that adult fishes could tolerate the pCO_2 levels predicted for the next 300 years. The expected tolerance of adult fishes to the effects of ocean acidification is attributed to their highly effective acid-base regulation capabilities (Wood et al. 2003, Ishimatsu et al. 2008, Baker et al. 2015).

Although adult fishes are thought to be tolerant, the early life stages of some fish species may be more vulnerable than adults, with several studies suggesting adverse consequences for fishes reared in elevated pCO_2 (e.g. Baumann et al. 2012, Frommel et al. 2012, Chambers et al. 2014). The additional vulnerability of the early life stages of fishes may be attributed to an underdeveloped acid-base regulatory system, relatively larger surface-to-volume ratios, which are concomitant with increased homeostasis costs, and a high epithelial permeability required for early cutaneous respiration. This may impede internal physiochemical regulation (Baumann et al. 2012, Frommel et al. 2012, Heuer and Grosell 2016).

The primary goal of most ocean acidification studies is to predict the response of organisms to future pCO_2 levels (e.g. Baumann et al. 2012, Bignami et al. 2013, Perry et al. 2015). Several

indicators have been used to assess how fish will respond. Fish growth and survival are commonly used indicators, yet to date, the available literature on these indicators is conflicting. Some studies found reduced growth and survival in near-future pCO_2 (e.g. Baumann et al. 2012, Chambers et al. 2014, Pimentel et al. 2014a), while others suggest that ocean acidification has little to no effect (e.g. Munday et al. 2011, Bignami et al. 2013, Perry et al. 2015). While this may suggest that the impact of ocean acidification on growth and mortality varies between species, it is also possible that aspects of the experimental design, such as the timing (in terms of life stage) and duration of the study, may influence the results.

Compared to the vast literature on external calcifying organisms, few studies have investigated the skeletal development in internally calcifying organisms, such as fishes, and how these organisms respond to ocean acidification and the concomitant under-saturation of carbonate states. Furthermore, current literature examining the effect of elevated pCO_2 on fish early-stage skeletal development is limited both in number and type of indicators employed. To date, studies examining the development of fish skeletal structures in elevated pCO_2 have been limited to quantifying the presence of deformities (e.g. Ben-Asher et al. 2013, Frommel et al. 2014, Pimentel et al. 2014b, Perry et al. 2015), examining bone structure morphometrics (e.g. Munday et al. 2011), quantifying bone density (e.g. Kim et al. 2015) and measuring the calcium and phosphate content of bone (e.g. Martens et al. 2006). Unfortunately, comparisons between these studies are made difficult by variable levels of pCO_2 tested. For example, Martens et al. (2006) reared fishes at CO_2 concentrations that are not expected for the next 400 years (4700 to 16 600 uatm). The lack of comparability between studies makes it even more difficult to identify common patterns. While the long-term patterns are important, Heuer and Grosell (2014) highlighted the importance of testing pCO_2 levels predicted within the century because human concern rarely extends beyond their own lifetime.

Current literature suggests that the response of fishes to ocean acidification may vary, depending on life stage and perhaps life history of the test species (e.g. Munday et al. 2011, Baumann et al. 2012, Chambers et al. 2014). Consequently, this study selected dusky kob, *Argyrosomus japonicus,* as the study species of choice, due to the novelty of the species in ocean acidification research, as well as their estuarine dependant life history strategy. In South Africa, a number of marine species spawn at sea but depend on estuaries as nursery habitats (Whitfield 1998). These species generally have an estuarine juvenile phase and a predominantly marine egg, larval and adult phase (Wallace and Van der Elst 1975). Juveniles enter estuaries between 20 and 60 mm SL (standard length) and remain in estuaries for between

one and three years, after which they return to the sea (Whitfield 1994). When entering estuaries, these species must adapt to the highly dynamic estuarine environment (Breitburg et al. 2009) which is very different from the stable marine environment (Lüthi et al. 2008). Hence species which are adapted to estuarine environments during the vulnerable egg and larval stage should be less vulnerable to the effects of ocean acidification than estuarine-dependant species with a marine egg and larval phase (Lonthair et al. 2017). Whether this vulnerability extends to fishes that have evolved with a pre-estuarine pelagic egg and larval stage remains to be tested. The present study examined whether elevated levels of pCO_2 affected *Argyrosomus japonicus* during the earliest life stages, i.e. the marine life stage.

Argyrosomus japonicus is an estuarine-dependant species, with a temperate, near circumglobal distribution (Griffiths 1996, Griffiths and Heemstra 1995) (Figure 1.2).

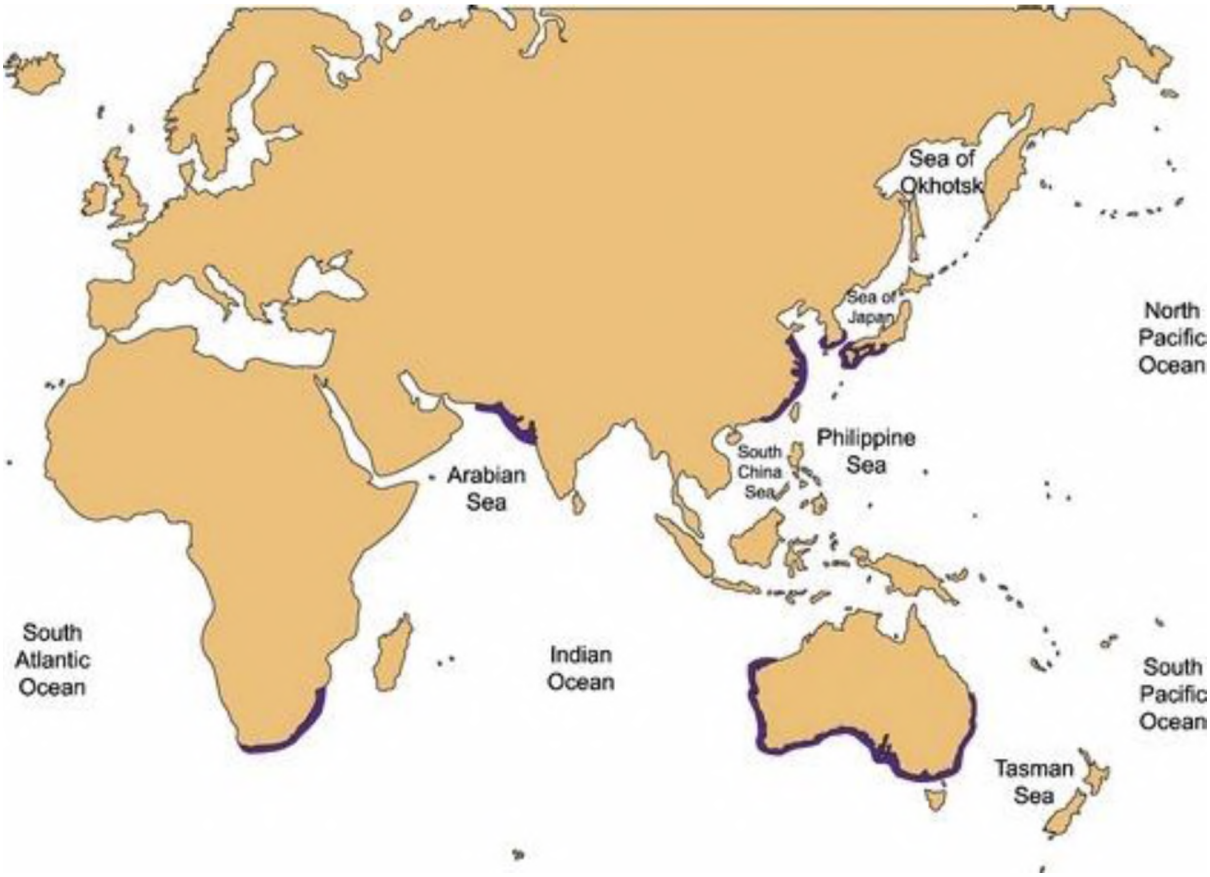


Figure 1.2. World map showing *Argyrosomus japonicus* distribution (Silberschneider and Gray 2008).

Furthermore, *A. japonicus* is a slow-growing and long-lived (up to 42 years) sciaenid (Griffiths 1996, Griffiths and Heemstra 1995). This carnivorous predator can grow up to 2 m TL (total

length) and reach weights of up to 80 kg (Griffiths 1996). Adult *A. japonicus* predominantly inhabit the near-shore marine environment and spawn on inshore reefs shallower than a 100 m (Griffiths 1996, Childs and Fennessy 2013). After spawning, larvae are thought to drift along the warm Agulhas Current and recruit into estuaries at approximately 20 to 30 mm (~ 4 weeks) (Griffiths 1996). These euryhaline fish show a strong dependence on estuarine habitats up until early adulthood (6 years/ ~ 100 cm TL), with an increasing affinity for the ocean environment thereafter (Childs and Fennessy 2013), although adults have been recorded feeding in the lower reaches of estuaries (Griffiths 1996, Cowley et al. 2008). Owing to its large size and palatability, *A. japonicus* is an important commercial and recreational fishery species in South Africa, Australia, China and Japan (Griffiths 1996). Heavy exploitation is common throughout its distribution and the *A. japonicus* population in South African waters was considered collapsed as early as the mid-1990s (Griffiths 1996). *Argyrosomus japonicus* is also a prominent aquaculture species, in both Australia and South Africa (Griffiths 1996, Drapeau et al. 2004). Its high commercial value, potential for aquaculture, and vulnerable status make any research regarding early growth and development invaluable for its management and conservation.

Aims and objectives

The aim of this study was to determine the potential effects of ocean acidification on the early life stages of *A. japonicus* by quantifying and comparing embryogenesis, hatching success, growth, development, survival, and skeletogenesis of *A. japonicus* individuals reared in present pCO_2 levels, and those predicted for 2050 and 2100. Near future levels of carbon dioxide treatments were tested as suggested by previous fish ocean acidification literature (Ishimatsu et al. 2008) and as humans are generally more concerned with changes that may affect them within their lifetime.

This thesis is divided into two main research chapters: Chapter Two examines the impact of elevated pCO_2 on the embryogenesis, hatching success, development, growth, and survival of early life stage *A. japonicus*. Chapter Three uses a multi-method approach, including a novel method for contrasting cartilage and bone development under the three pCO_2 scenarios. Chapter Four summarises the findings of the experimental chapters, examines the drivers of the impacts of elevated pCO_2 on early life stage dusky kob, contextualises the findings in terms of global knowledge, and recommends future work to improve our understanding of the impacts of ocean acidification on fishes.

CHAPTER 2

The effects of CO_2-induced acidification on Argyrosomus japonicus embryogenesis, hatching success, growth, morphometric development, and survival.

2.1. INTRODUCTION

The impact of ocean acidification on fishes remains far from understood. Initial fish ocean acidification research suggested that adult fishes are tolerant of carbon dioxide levels predicted for the next 300 years (Caldeira and Wickett 2003, Fabry et al. 2008, Ishimatsu et al. 2008) and led to the assumption that fishes are not vulnerable to the near-future effects of ocean acidification (Stiasny et al. 2016). However, more recent research with a focus on fish early development in near-future CO_2 conditions have identified a range of potential impacts (Baumann et al. 2012, Frommel et al. 2012, Chambers et al. 2014). These impacts include reduced growth rates (e.g. Baumann et al. 2012, Miller et al. 2012, Chambers et al. 2014), changes in fish morphometry (e.g. Chambers et al. 2014), decreased survival (e.g. Baumann et al. 2012, Miller et al. 2012, Chambers et al. 2014, Stiasny et al. 2016) and compromised embryogenesis (e.g. Baumann et al. 2012, Chambers et al. 2014, Rosa et al. 2014). Early-stage vulnerability is thought to arise from an underdeveloped acid-base regulatory mechanism, relatively larger surface-to-volume ratio, a concomitant increase in the costs of homeostasis and a higher physiological vulnerability arising from high epithelial permeability required for early cutaneous respiration (Pörtner et al. 2004, Baumann et al. 2012, Heuer and Grosell 2016). However, the exact drivers of reduced early-life growth and survival in future CO_2 conditions remain unclear (Baumann et al. 2012).

Elevated carbon dioxide is expected to increase acid-base regulation costs, thereby reducing the energy available for growth and development. Heuer and Grosell (2016) proposed that Gulf toadfish (*Opsanus beta*) are capable of upregulating metabolism to deal with unfavourable environmental conditions. However, this upregulation in metabolism to compensate for elevated acid-base regulation costs has been shown to come at a significant cost or energy trade-off (Heuer and Grosell 2016). Miller et al. (2012) observed an increase in the metabolic rate of cinnamon clownfish (*Amphiprion melanopus*) reared in elevated pCO_2 (~ 1000 µatm), which corresponded with a decrease in their growth and survival. These findings are not isolated to fishes. Wood et al. (2008) found that heightened pCO_2 resulted in an increase in the metabolic rate of the brittle star (*Amphiura filiformis*) and concomitantly reduced muscle

development and impaired muscle function. Based on these findings, it is likely that exposure to elevated CO_2 will result in an increase in the metabolic rate of fish, however, how this elevated metabolic rate may affect the growth and survival of *Argyrosomus japonicus* remains unclear.

Current literature on the effects of near future levels ($\leq$ 1000 µatm) of elevated pCO_2 on larval marine fish early development are conflicting (Pörtner et al. 2004, Ishimatsu et al. 2008), with some studies reporting negative impacts of ocean acidification on early life stages, while others have found no response (Munday et al. 2009, Bignami et al. 2013, Frommel et al. 2013). For example, Munday et al. (2009) found elevated pCO_2 (1030 µatm) had no effect on the embryonic duration, egg survival and size at hatching, growth and survival of the tropical reef fish, orange clownfish (*Amphiprion percula),* whereas Baumann et al. (2012) found significant decreases in both growth and survival of inland silverside, *Menidia beryllina,* in a hypercapnic environment (~ 1000 µatm). However, Rossi et al. (2015) found that the growth rates of barramundi, *Lates calcarifer*, increased at elevated (1675.1 µatm) levels of pCO_2. The variation in these findings suggests that the impacts of ocean acidification during the early life stages may differ between fish species and may be influenced by its life history strategy. Furthermore, based on the available research, it is possible that biogeography and habitat association may also influence the response (Munday et al. 2011, Lonthair et al. 2017).

Hatching success is generally investigated in conjunction with growth and survival, since embryogenesis is thought to strongly influence the success of early development (Sayer et al. 1993, Ballagh 2011). Embryogenesis can be divided into the degree of embryo development, egg surface area, and oil globule area. Few studies have examined the effect of elevated pCO_2 on embryogenesis, and hatching success in conjunction with growth, development and survival (Ishimatsu et al. 2008, Munday et al. 2009, Frommel et al. 2012). Similar to the literature on growth and survival, current knowledge on the effects of elevated pCO_2 on embryogenesis, and hatching success of marine fishes is conflicting. Decreased hatching success and early survival have been found in some studies (e.g. Miller et al. 2012, Pimentel et al. 2014a, Rosa et al. 2014); however, several studies found that elevated pCO_2 exposure had no influence on the hatching success or hatching time of larvae (e.g. Munday et al. 2009, Franke and Clemmesen 2011, Frommel et al. 2012, Hurst et al. 2013). This may be surprising as several studies on freshwater species have suggested that even small changes in acidity can significantly increase hatching time and compromise hatching success (e.g. Ingersoll et al. 1990, Sayer et al. 1993).

In this experiment, the response of the model species *Argyrosomus japonicus* to near-future pCO_2 was tested by rearing larvae for 29 days from eggs in three pCO_2 treatments, including current pCO_2 and levels predicted by the year 2050 and 2100, and examining embryogenesis, hatching success, morphometry and survival. Where morphometric deductions in elevated pCO_2 studies to date have been limited to length and sometimes weight measurements (Chambers et al. 2014), this chapter examines the potential effects of elevated pCO_2 on *A. japonicus* morphometry by examining length, height, width, yolk area, stomach area, oil globule area, and eye area.

2.2. METHODS AND MATERIALS

2.2.1. EXPERIMENTAL SYSTEM

All experiments were conducted at a shore-based fish farm (Pure Ocean Fish Farm (Pty) Ltd., situated in East London, South Africa, between April and May 2016. Spawned *A. japonicus* eggs were obtained from Pure Ocean broodstock (F1 generation) induced through intramuscular injection of 50 $\mu g.kg^{-1}$ LHRHa analogue.

Directly post-spawning *A. japonicus* eggs were collected from egg collection baskets situated within the broodstock tank. Fertilised eggs (positively buoyant) were stocked in nine 1000 L cylindro-conical plastic tanks at a stocking density of 15 larvae.L^{-1}. Low stocking density helped to minimise stress concomitant with overcrowding (Montero et al. 1999). Tanks were disinfected by adding 12.5% sodium hypochlorite 24 hours before the experiment commenced. Sea water was left to circulate with aeration for 10 minutes and 3.7 g of sodium thiosulfate was added to neutralise the reaction. Chlorine test reagent tested negative for residual chlorine (Moretti et al. 1999).

The experimental system consisted of three treatments with three replicate tanks each (Figure 2.1). Each tank was fitted with an automated CO_2 gas proportionator valve system (DAQ-S; Loligo Systems Inc.), and calibrated pH feedback controller (WTW SenTix 81 pH electrode). This system was based on the standard method of regulating pCO_2 (CO_2 and air mixture: Afrox MSDS no. 994-324) input based on desired treatment pH levels (e.g. Michaelidis et al. 2005, Anthony et al. 2008, Munday et al. 2009). Carbonate system chemistry was calculated with the programme CO2CYS, based on the total dissolved inorganic carbon, salinity, alkalinity,

temperature and pH to estimate pCO_2 (µatm), bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), total alkalinity (A_T), and calcium concentration (saturation states of both aragonite and calcite) (Lewis and Wallace 1998, Pierrot et al. 2006) (Table 2.2). Dissociation constants were in accordance with Roy et al. (1993). Water quality, including pH, oxygen, salinity, temperature, carbonate chemistry, alkalinity, total dissolved solids was measured daily. Temperature, dissolved oxygen (DO), and total dissolved solids (TDS) were measured with a Hanna Instruments multi-meter (Aqua Lytic AL15). Alkalinity for each tank was estimated using titration with Automatic Titration System (Hanna Instruments HI902C). Carbonate system chemistry was calculated with the CO2CYS programme using salinity, alkalinity, temperature, and pH to estimate pCO_2 (µatm), total dissolved inorganic carbon (C_T), bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), total alkalinity (A_T), calcium concentration (saturation states of both aragonite and calcite) (Lewis and Wallace 1998, Pierrot et al. 2006). Dissociation constants were in accordance with Roy et al. (1993). Calcium concentration was estimated from salinity, and carbonate (CO_3^{2-}), which was calculated from dissolved inorganic carbon (DIC) and total alkalinity (Table 2.2). Instrument calibration was performed prior to the water quality testing.

Carbon dioxide treatments were derived from the IPCC (International Panel on Climate Change) "Most likely" or "Business as usual" (IS92) scenario for current pCO_2, year 2050 pCO_2, and year 2100 pCO_2 (Stocker et al. 2014). The three treatments were:
i) Current pCO_2 (control) = ~ 327.50 ± 80.07 µatm, pH = 8.15;
ii) Year 2050 pCO_2 - pCO_2 = ~ 477.40 ± 59.46 µatm; pH = 8.03;
iii) Year 2100 pCO_2 - pCO_2 = ~ 910.20 ± 136.45 µatm; pH = 7.8.

Treatment replicates were assigned per a randomised block design (Figure 2.1). Tanks were constantly aerated by means of a blower (Aerotech 1.1 KW) and temperature was maintained at 25 °C by temperature control boxes attached to aquarium heaters (Aquaheater 210 Ac 220 ~ 240 V, 50 – 60 Hz). Photoperiod was artificially maintained at 16:8 hours light-dark cycle, with a maximum light intensity of 9 watts per square metre at the water surface as suggested ideal for *A. japonicus* larval rearing (Ballagh et al. 2011). A 10% water exchange regime was employed on the 7th and 14th dph, followed with a daily 20% exchange thereafter.

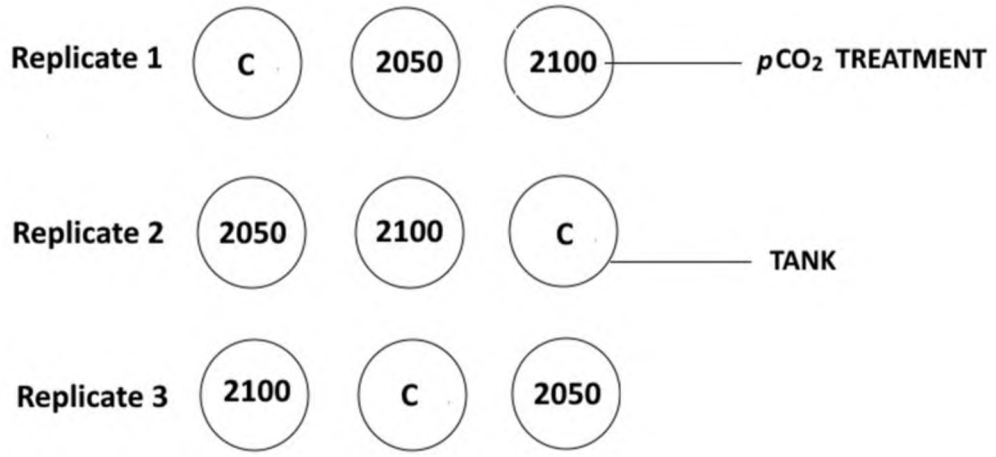


Figure 2.1. Randomised block system design consisting of nine 1000 litre tanks with three allocated pCO_2 treatments to each tank at Pure Ocean Fish Farm. Carbon dioxide treatments are indicated as C: Current pCO_2; 2050: year 2050 pCO_2 and 2100 indicating year 2100 pCO_2.

Dead larvae were carefully siphoned from the bottom of the tank at midday when most larvae are near the surface. *Argyrosomus japonicus* were fed live rotifers (*Brachionus plicatilis*) twice daily (7 AM and 4 PM) for the first three weeks post-hatch. Rotifer densities were increased from 2 to 20 rotifers per mL in accordance with the Pure Ocean Fish Farm *A. japonicus* rearing protocol. Artemia (*Artemia franciscana*) were stocked (2 to 5/mL) from 7 dph (pre-flexion) to 25 dph (settlement). Live feed was enriched with dead *Nannochloropsis* spp. from 2 dph, at a concentration of 1 ml/million rotifers in the tank (Lubzens et al. 1995). The green water rearing method was selected since it has been shown to enhance larval feeding success (Chesney 2005, Shields and Lupatsch 2012). The use of dead *Nannochloropsis* reduced the risk of respiration-related pH changes in the tanks. Skretting Larval Feed was introduced from 21 dph.

2.2.2. EXPERIMENTAL PROCEDURE

Hatching success was estimated from fertilised *A. japonicus* eggs (n = 100) collected 1 hour post-spawning and randomly placed in fine mesh net mesocosms (25 × 15 cm) floating in each experimental tank. The mesocosms were removed at 26 hours post-spawning (hps). Hatchlings and unhatched eggs were collected in labelled petri dishes and euthanized using an overdose of 2-phenoxyethanol. Proportional hatching success of eggs was then compared between control and acidified treatments.

Egg development was described from the 100 eggs collected from each tank at 14 hps. Each egg was examined using a Leica EZ4 HD dissecting microscope under 35× magnification and

its developmental phase (no development, partial development, and complete larval development) was recorded. Eggs were then photographed and the photographs were calibrated for morphological measurements using the open-source software FIJI and LAS EZ. The egg area (EA) and oil globule area (OA) were measured with the freehand tracing tool in FIJI.

Larval development was described from specimens collected (n = 30) from each experimental tank at 10:00 AM on 0, 2, 8, 13, 18, 21, 26 and 29 dph (Table 2.1). These sampling days corresponded to key life stages, i.e. egg, hatchlings, pre-flexion, flexion, post-flexion, the onset of metamorphosis on 21 dph and settlement (defined as a shift from pelagic to a demersal life style in this thesis) at 29 dph, for the development of this species (Table 2.1). After collection, larvae were immediately euthanized using an overdose of 2-phenoxyethanol (400 mg L^{-1}) (King et al. 2005) and stored in 90% EtOH for future analysis. Digital images were collected (as described above) at a magnification ranging from 8 - 35 X (depending on the developmental stage). The stage of development was recorded and a total of 10 geometric morphometric measurements were made using the straight line or freehand tracing tool in the FIJI package. These measurements included total length (TL), standard length (SL), body depth at vent (BD), head width (HW), head depth (HD), eye area (EA), stomach area (SA), yolk area (YA), oil globule area and caudal fin length (CL) (Figure 2.2). Development was expressed as the percentage of larvae belonging to each stage in each age (dph) class.

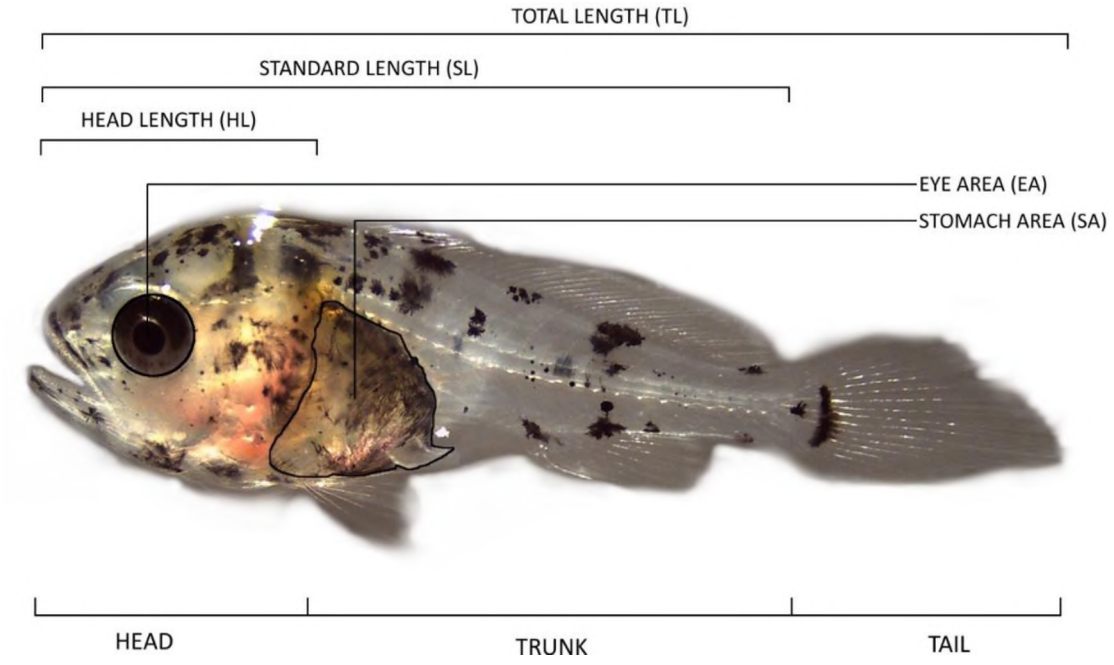


Figure 2.2. Image of a post-flexion *Argyrosomus japonicus* larvae showing the variables measured to describe their morphological development.

Table 2.1. Images of the various early ontogenetic stages of *Argyrosomus japonicus* highlighting their age, stage and distinguishing features.

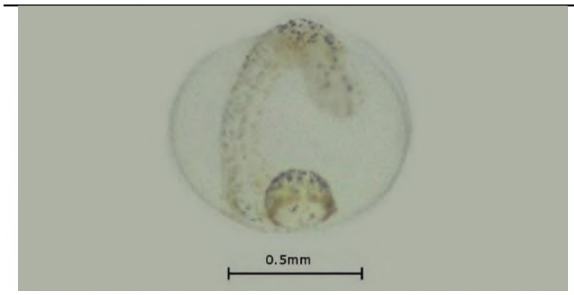
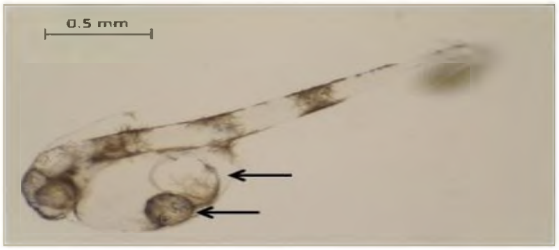
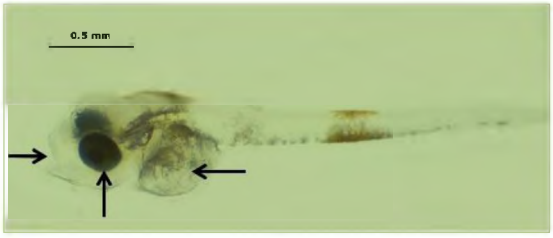
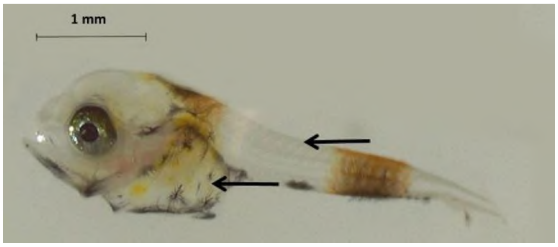
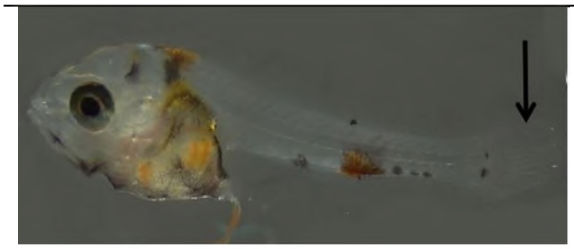
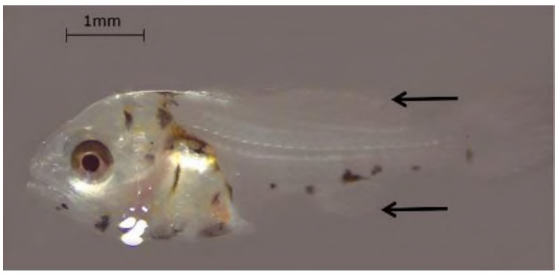
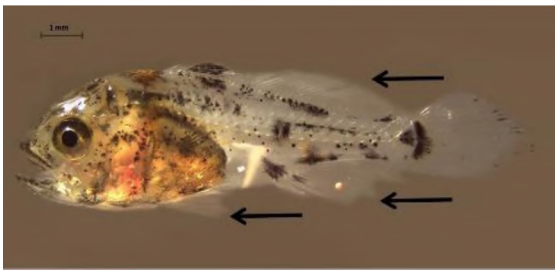
IMAGE	LIFE STAGE
	**Egg stage (12 hours old)** The period from spawning to hatching. At 20 hps (hours post-spawning) the embryo was in the final stage of development and had visible eyes but no differentiated mouth. TL: 2.1 ± 0.02 mm.
	**Early yolk-sac stage (0 dph)** Yolk sack present. Oil globule clearly visible. TL: 2.71 ± 0.07 mm.
	**Yolk-sac stage (2 dph)** Yolk sack present. Oil globule reduced. Differentiated mouth and developed eyes. TL: 2.92 ± 0.03 mm.
	**Pre-flexion (8 dph)** Yolk sack replaced by stomach. No tail flexion. Myomeres start to form. TL: 3.98 ± 0.04 mm.
	**Flexion (13 dph)** Tail flexed. Caudal fin rays start to form. TL: 4.77 ± 0.26 mm.

Table 2.1. Continued. Images of the various early ontogenetic stages of *Argyrosomus japonicus* highlighting their age, stage and distinguishing features.

IMAGE	LIFE STAGE
	**Post-flexion (18 dph)** Caudal complex fully developed. TL: 4.77 ± 0.26 mm.
	**Onset of metamorphosis (21 dph)** Dorsal and anal fins start to form. 5.54 ± 0.16 mm.
	**Settlement (29 dph)** Dorsal, anal and pelvic fins fully developed. 6.96 ± 0.62 mm.

The experimental tanks were drained into a net (600 µm mesh) at the end of the 29-day experiment. All surviving larvae were enumerated and survival was calculated as mean percentage of survivors per treatment.

2.2.3. DATA ANALYSIS

Data were tested to conform to the assumption of homogeneity of variance using a Levene's test, and normality using a Sharpiro-Wilk test. Data were transformed when necessary to meet the assumptions of normality and equal variance. Where data did not meet assumptions, the appropriate non-parametric test was used. Statistical analyses were performed using a significance level of 0.05 and a confidence interval of 95%, using Statistica (10.0) software (StatSoft Inc., Tulsa, OK, USA). Water quality parameters were analysed separately to test for differences between the main effects of "pCO_2 treatment" over "dph" by means of factorial analysis of variance (FAOV's). Egg development was compared between treatments (n = 30) as the percentage of fully developed (ready to hatch) larvae in the egg at 14 hours post-spawning by means of one-way analysis of variance (ANOVA).

Egg area was compared between treatments (n = 30) by one-way ANOVA. The variance distributions for oil globule area were not normally distributed (Sharpiro Wilk test: $P < 0.05$) and were not improved by transformations. Therefore, non-parametric Kruskal-Wallis median tests were used to determine if these morphometric traits differed among pCO_2 treatments.

Hatching success was estimated as the mean percentage of successful hatches in each pCO_2 treatment mesocosm (n = 100). Hatching success was compared between treatments using one-way ANOVA.

Growth was expressed as the change in mean total length over days post-hatch. Factorial ANOVA was used to test the effects of pCO_2 level on growth, development and geometric morphometrics. *Post hoc* multiple comparison tests (Tukey-Kramer test) were used to detect significant differences between treatment replicates.

To avoid the common problem associated with treatment allometric growth variation, all morphometric measurements were size-adjusted to total length using the following equation (adapted from: Reimchen et al. 1985, Senar et al. 1994, Simon et al. 2010):

$$Y'ij = \log Yij - \mathrm{m}j\,(\log(\frac{\mathrm{TL}i}{\overline{\mathrm{TL}}})$$

where $Y'ij$ is the size-adjusted value of morphometric measurement j, for individual i. Yij is the original morphometric measure, mj is the pooler regression coefficient of log Y as a function of log TL (total length). TLi is the total length of individual i, and $\overline{\mathrm{TL}}$ is the mean total length. The efficacy of the size transformation was determined from the coefficient of determination (R^2), i.e. the log Y' as a function of log TL regression, where R^2 values below 0.05 were considered to have limited size bias.

Size-adjusted morphometric variables were then subjected to principal component analysis (PCA) to investigate the relationship between elevated CO_2 and body shape using the statistical package PAST Version 2.16 (Hammer et al. 2001).

Survival was described based on the number of *A. japonicus* alive on the final day of the 29-day experiment. Proportional survivorship was then compared between control and acidified treatments with a one-way ANOVA. The percentage survival was estimated as:

Survival (%) = $(\frac{x}{(yi - z)} \times 100)$, where x is the number of survivors, y_i is the initial number of larvae and z is the number of fish sampled.

2.3. RESULTS

2.3.1. WATER CHEMISTRY

Mean temperature, salinity, and dissolved oxygen did not differ significantly between pCO_2 treatments throughout the course of the experiment (Factorial ANOVA: $F_{90, 54}$, $P < 0.05$) (Table 2.2). However, there were significant differences in the pH, calcite concentration, and aragonite saturation (Table 2.2).

Table 2.2. Water quality parameters for the three pCO_2 treatments for rearing *Argyrosomus japonicus* during the 29-day experiment. * indicates a significant treatment effect (Factorial ANOVA: $F_{19, 54}$, $P < 0.05$).

PARAMETER	CONTROL pCO_2	2050 pCO_2	2100 pCO_2	*P* VALUE
pH	8.16 ± 0.08	8.01 ± 0.04	7.78 ± 0.06	*
pCO_2 *(ppm)*	329.48 ± 79.71	478.75 ± 59.27	909.43 ± 136.04	*
$\Omega_{calcite}$	6.73 ± 1.17	4.99 ± 0.52	3.18 ± 0.53	*
$\Omega_{aragonite}$	4.42 ± 0.77	3.28 ± 0.35	2.09 ± 0.35	*
Alkalinity (TA)	2561.33 ± 190.36	2484.72 ± 133.68	2494.14 ± 124.33	*
Salinity	35.0 ± 0	35.0 ± 0	35.0 ± 0	
Temperature (°C)	23.57 ± 0.79	23.65 ± 0.84	23.49 ± 0.66	
Dissolved oxygen	7.4 ± 0.32	7.27 ± 0.31	7.21 ± 0.24	

2.3.2. EGG DEVELOPMENT

Argyrosomus japonicus embryos had developed into the form of a larval fish and had begun to deplete the yolk sac at 14 hours post-spawning (hps). Although there were no statistically significant differences between the percentage of fully developed larvae in the eggs for each treatment (One-way ANOVA: $F_{2, 7} = 1.62$, $P > 0.05$), the mean number of eggs with a developed embryo was highest in the 2100 pCO_2 treatment (76.01% ± 14.11), followed by the 2050 pCO_2 treatment (64.67% ± 49.94) and the current pCO_2 treatment (26.67% ± 43.03) (Figure 2.3).

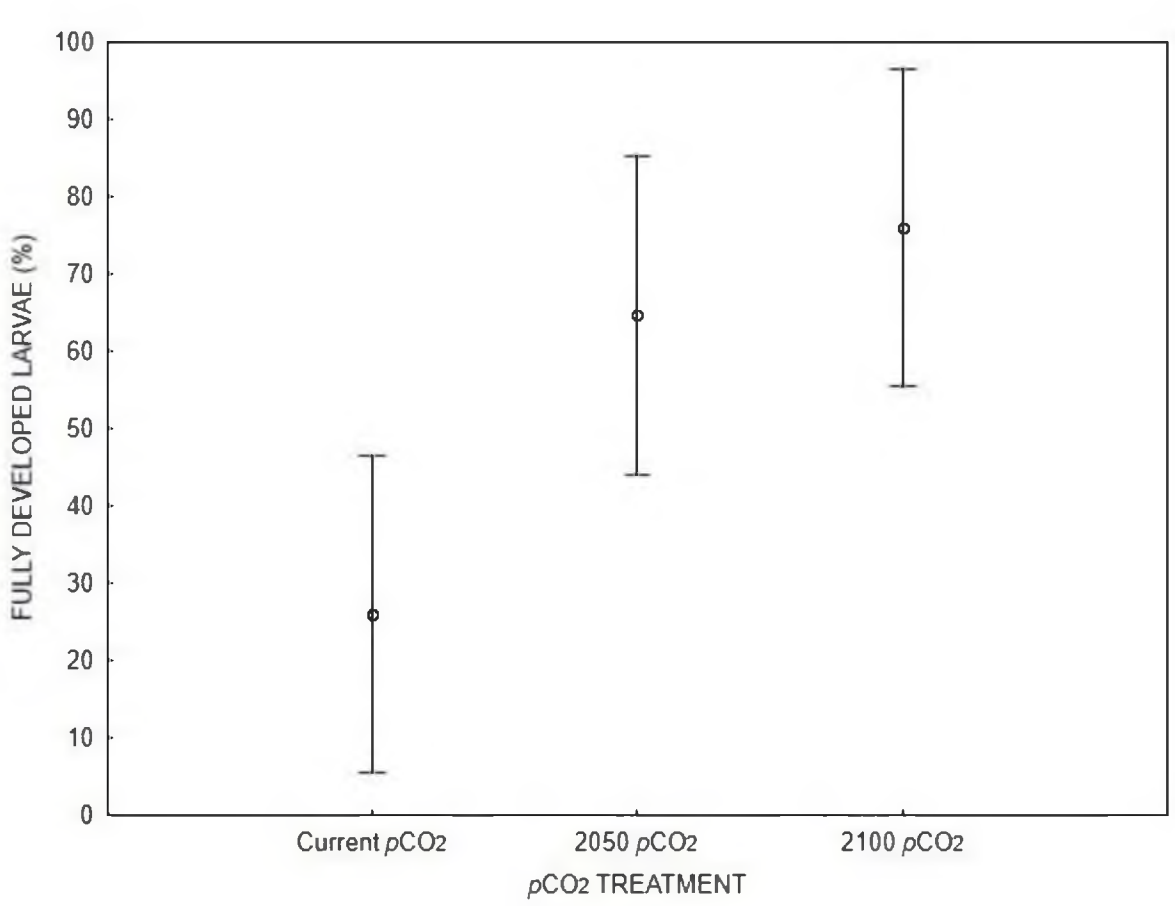


Figure 2.3. Comparing the percentage of fully developed *Argyrosomus japonicus* embryos at 14 hours post-spawning between current and predicted levels of pCO_2 for the years 2050 and 2100.

2.3.3. EGG AND OIL GLOBULE AREA

Mean egg area was lowest (0.61 ± 0.05 mm^2) in the current pCO_2 treatment and highest (0.66 ± 0.06 mm^2) in the intermediate pCO_2 treatment and was found to be significantly different between these treatments (One-way ANOVA: $F_{2, 267} = 25.57$, $P < 0.001$) (Figure 2.4). *Post hoc* Tukey tests indicated that both the 2050 and 2100 pCO_2 treatments differed significantly from the current pCO_2 treatment, but not from each other ($P < 0.05$) (Figure 2.4).

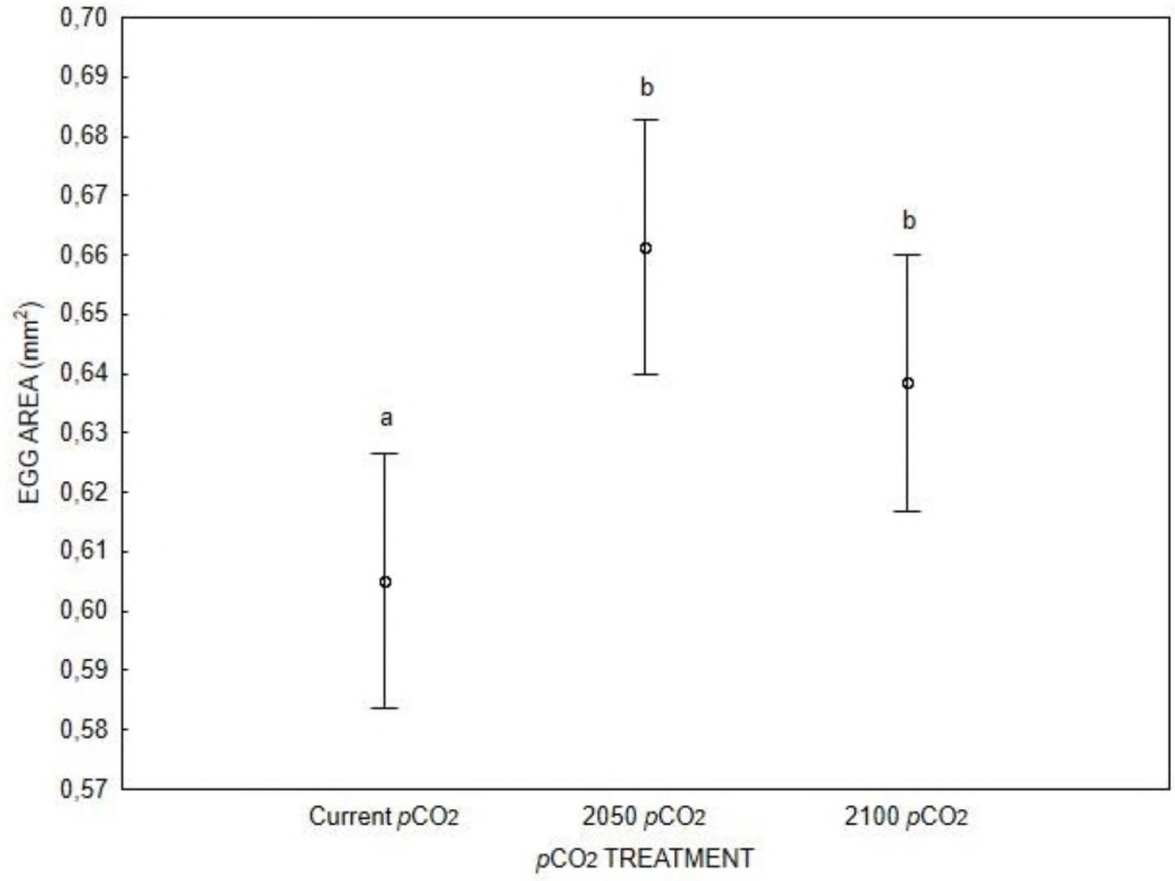


Figure 2.4. Comparing the surface area of *Argyrosomus japonicus* eggs at 14 hours post-spawning between current and predicted levels of pCO_2 for the years 2050 and 2100. Different letters indicate significant differences between treatment means as informed from *post hoc* Tukey tests ($P < 0.05$).

The mean oil globule area was ~ 0.6 mm^2 for all three treatments and did not differ significantly between pCO_2 treatment at 14 hps (Kruskal-Wallis: $H_{2, 270} = 0.44$, $P > 0.05$) (Figure 2.5).

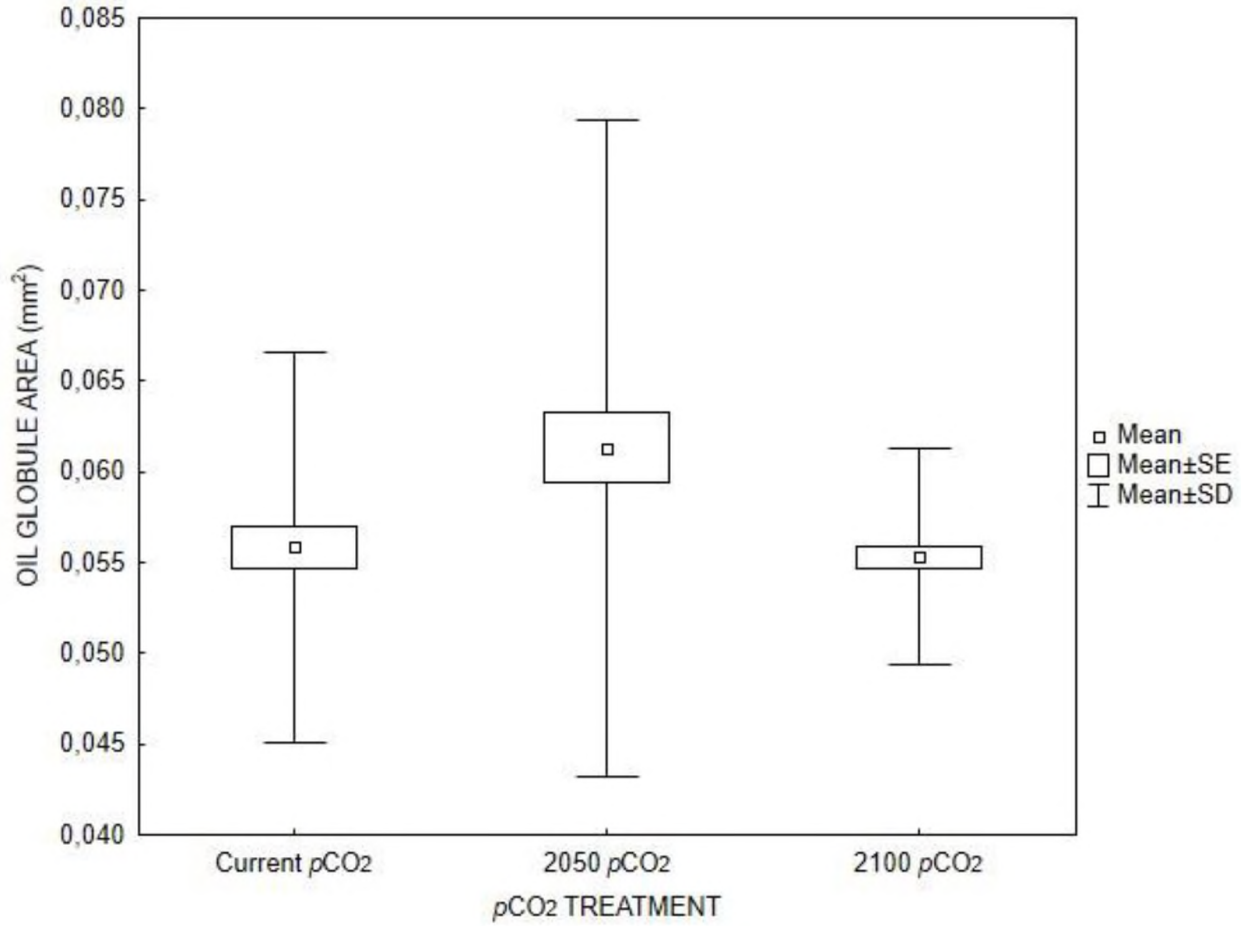


Figure 2.5. Oil globule area (mm^2) of *Argyrosomus japonicus* embryo reared in current, intermediate and high pCO_2 levels at 14 hps.

2.3.4. HATCHING SUCCESS

Hatching success ranged from 55.3 ± 2.7% in the current pCO_2 treatment to 58.2 ± 4.0% and 58.1 ± 1.0% at the 2050 pCO_2 and 2100 pCO_2 treatments, respectively (Figure 2.6). Although egg survivorship was variable, pCO_2 treatment did not significantly affect the percentage of successful hatches (One-way ANOVA: $F_{2, 267} = 1.25$, $P > 0.05$).

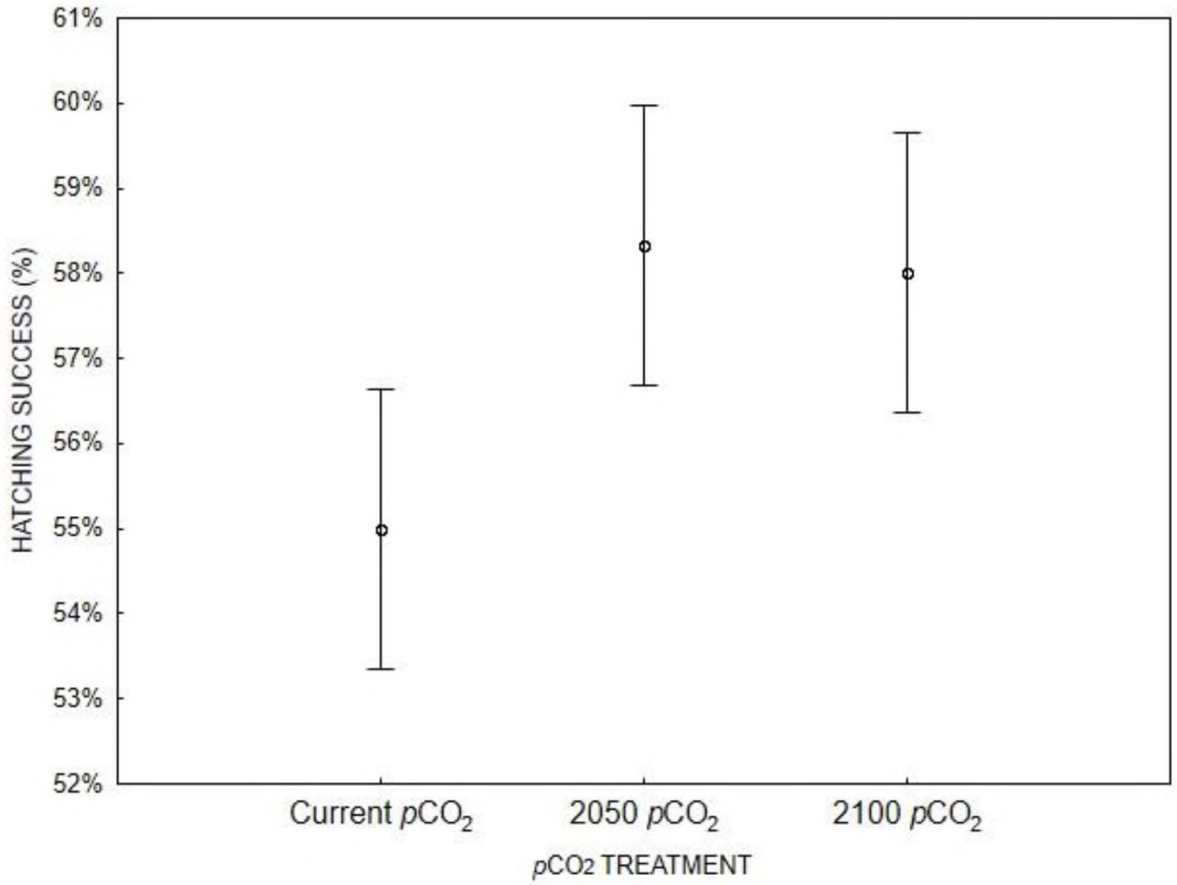


Figure 2.6. Proportion of successful *Argyrosomus japonicus* hatchlings 26 hours post-spawning at current and predicted levels of pCO_2 for the years 2050 and 2100.

2.3.5. GROWTH

Hatching size (TL) ranged from 2.55 ± 0.31 mm (2100 pCO_2) to 2.71 ± 0.06 mm (current pCO_2). There was no significant difference in the mean size (TL) at hatching or any time after that until 21 dph (Factorial ANOVA: $F_{19, 54} = 24.59$, $P > 0.05$) (Figure 2.7). There was, however, a significant difference in mean size (TL) from 21 dph (Factorial ANOVA: $F_{19, 54} = 24.59$, $P < 0.001$), with the mean TL of fish reared in the intermediate 2050 pCO_2 treatment being significantly larger than the current and high 2100 pCO_2 treatments (Tukey, $P < 0.001$) (Figure 2.7). By the 26th dph, the mean size of individuals in the 2100 pCO_2 treatment was significantly smaller (6.10 ± 0.01 mm) than the current pCO_2 (7.74 ± 0.33 mm) treatment, while individuals in the 2050 pCO_2 treatment were significantly larger (12.43 ± 0.67 mm) than the other two treatments (Figure 2.7). No further comparisons could be made between all three treatments as there were no survivors in the 2100 pCO_2 treatment on 29 dph (Figure 2.7).

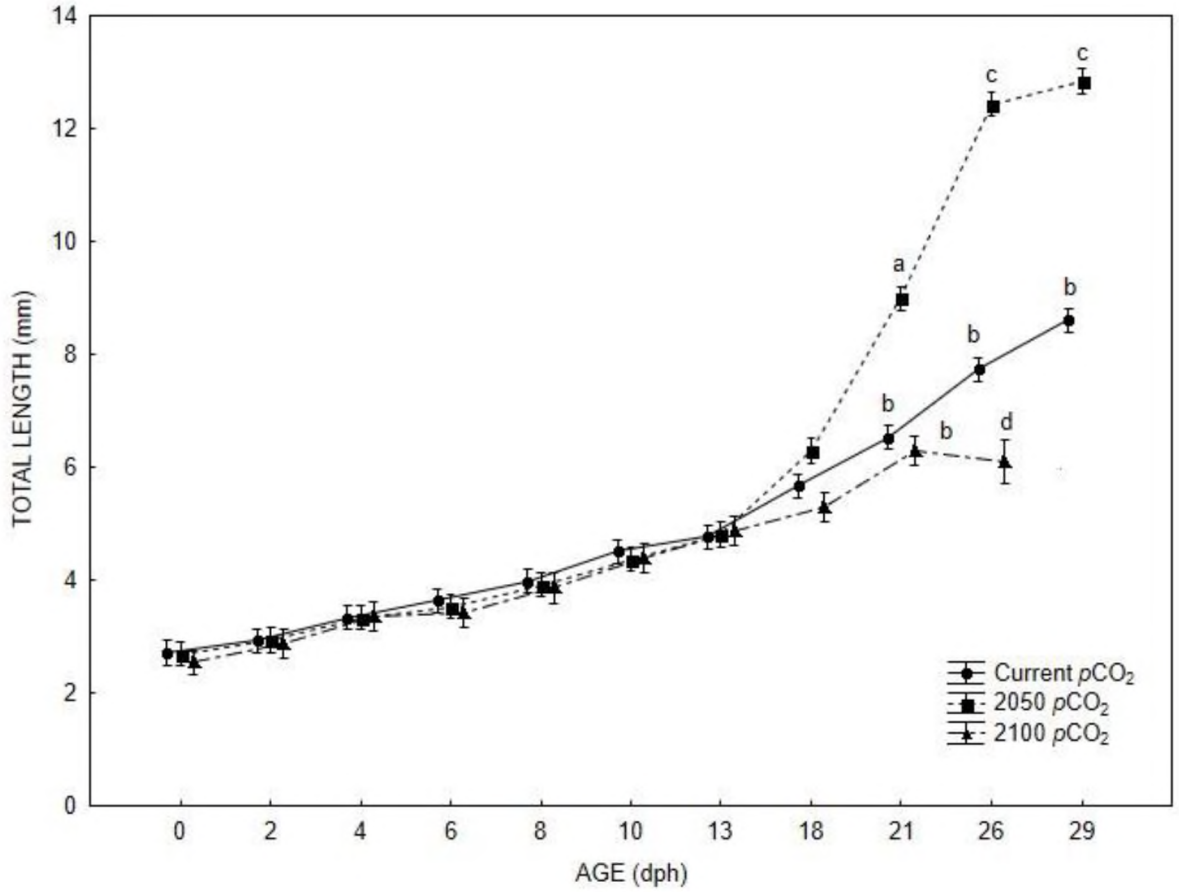


Figure 2.7. Early-life growth of *Argyrosomus japonicus* reared at current and predicted levels of pCO_2 for the years 2050 and 2100. There were no survivors in the 2100 pCO_2 treatment on day 29. Data points were collected on the same day; presented scattered for easier interpretation. Different letters indicate significant differences between treatment means (*Post hoc* Tukey tests, $P < 0.05$).

2.3.6. DEVELOPMENT

Development followed a similar pattern in all treatments until 21 dph (Figure 2.8). By 26 dph, all fish in the 2050 pCO_2 treatment had reached metamorphosis, while post-flexion larvae dominated the current (control) pCO_2 (53.3%) and 2100 pCO_2 (96.2%) treatments. By the end of the 29-day experiment, no fish from the current or high 2100 pCO_2 treatments had completed full metamorphosis (Figure 2.8) and all the fish in the 2100 pCO_2 treatment had died. However, a small percentage (11%) of individuals in the 2050 pCO_2 treatment had completed metamorphosis and were in the early juvenile stage (Figure 2.8). Despite these differences, there was a significant treatment effect on the proportion of larvae at key ontogenetic stages (Factorial ANOVA: $F_{11, 34} = 4.51$, $P < 0.001$) after 21 dph.

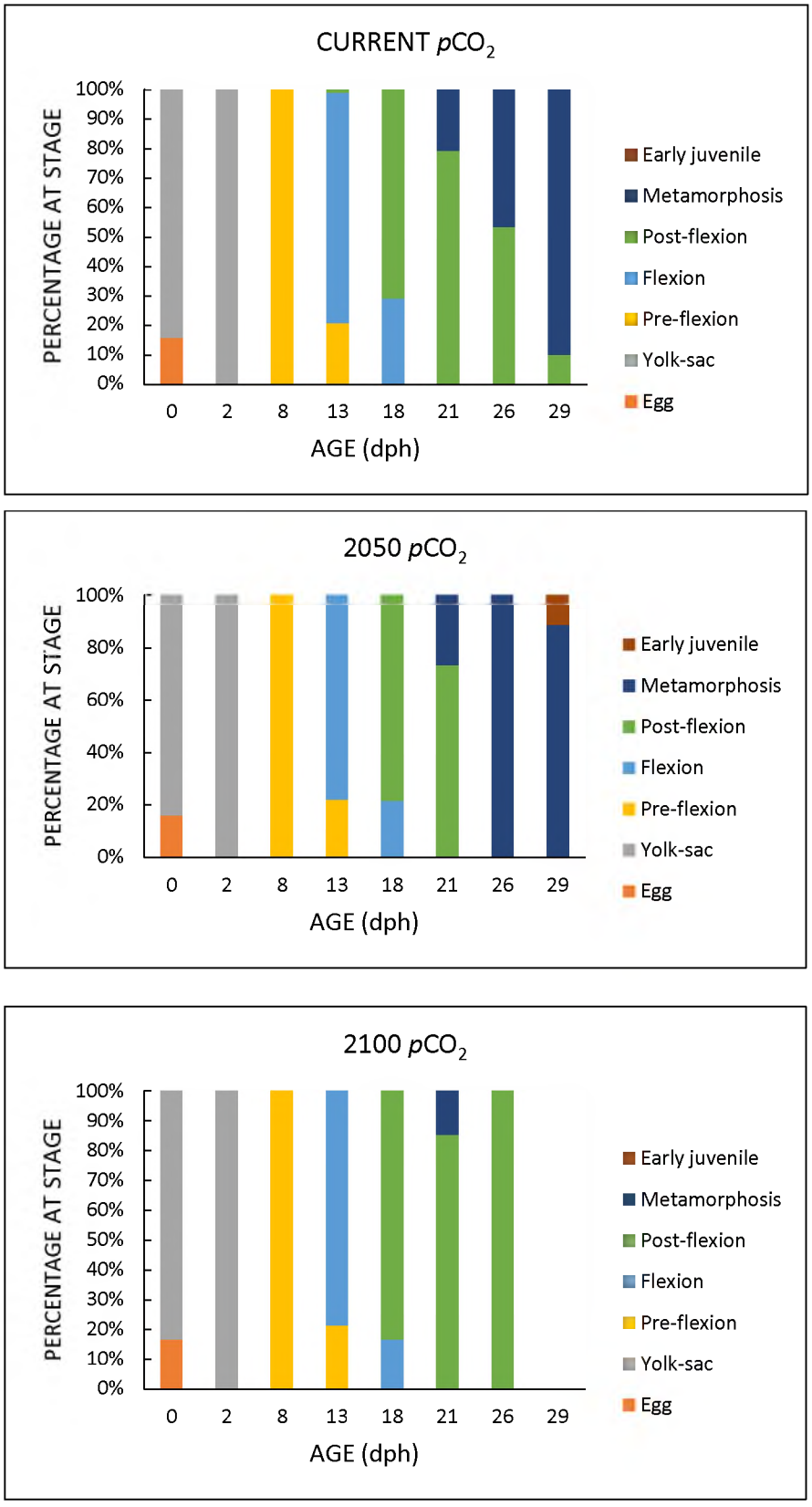


Figure 2.8. Comparing the relative proportion of *Argyrosomus japonicus* at each key ontogenetic development stage between current and predicted levels of pCO_2 for the years 2050 and 2100.

2.3.7. MORPHOLOGICAL VARIABLES

Larvae reared in the high pCO_2 treatment displayed broad morphological variation at hatching (0 dph), when compared to the other treatments (Figure 2.9). The component loadings of PC1 indicated that this could be attributed primarily to variation in the stomach area (0.58) and head length (0.48). Morphological variation was less pronounced between treatments between 2 and 18 dph (Figure 2.9). However, by 21 dph, morphological variation was greater in the high pCO_2 treatment than in the others. However, this variability could not be attributed to either of the principal components. Individuals from the 2050 and 2100 pCO_2 treatment groupings were significantly different from one another on 26 dph (Loadings PC1: HL = 0.65 and EA = 0.64; PC2: SA = 0.71 and HL = 0.45) (Figure 2.9). The groupings were not significantly different by 29 dph and this could most likely be attributed to the large morphological variation observed in individuals reared in the 2050 pCO_2 treatment (Figure 2.9).

2.3.8. SURVIVAL

There were no *A. japonicus* survivors by the end of the 29-day study period in the 2100 pCO_2 treatment. Mean survival was significantly higher in the 2050 pCO_2 treatment (150.67 ± 123.6 individuals per tank) than in the current (44 ± 40.15 individuals per tank) pCO_2 treatment (T-test: $P < 0.01$).

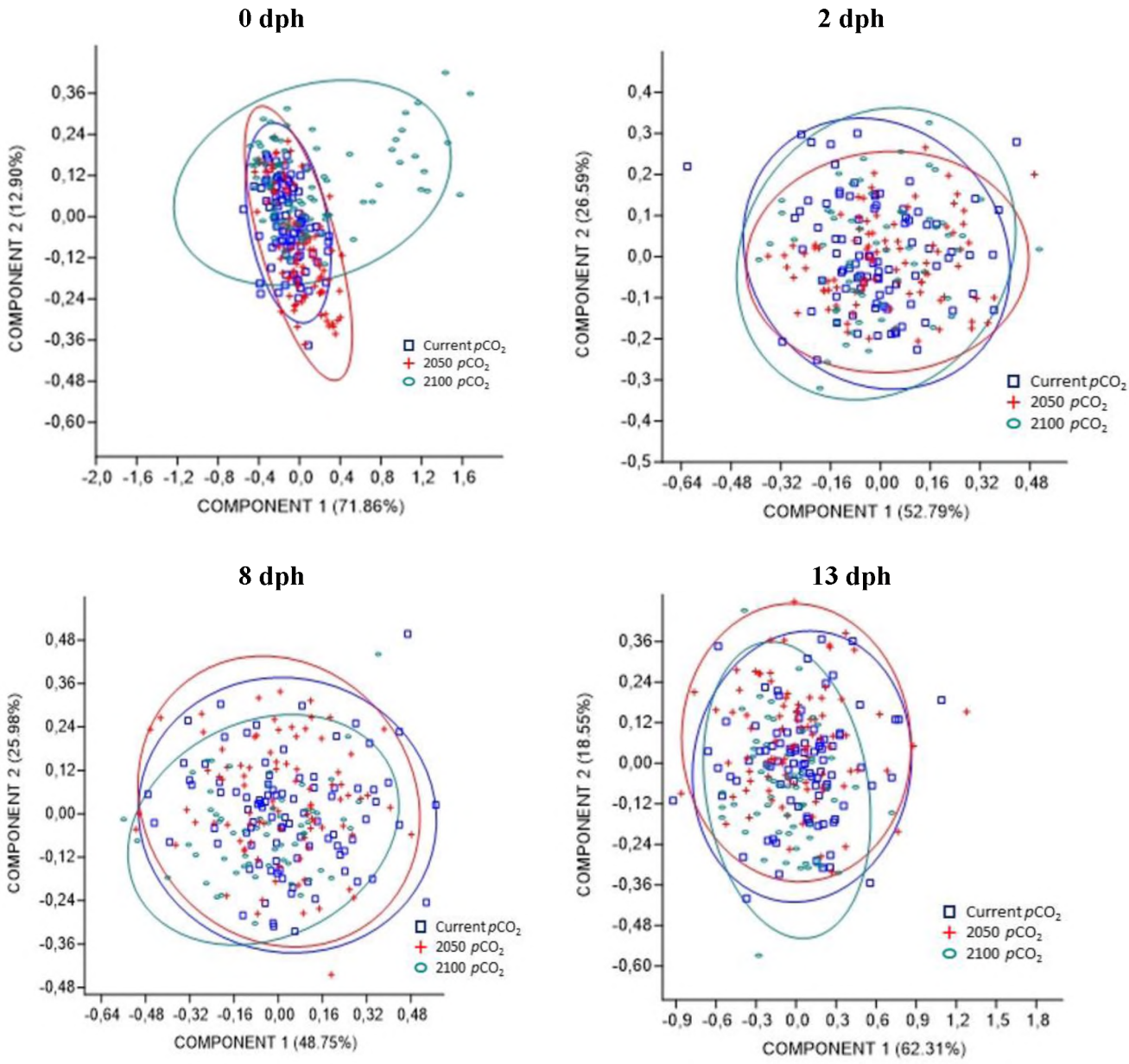


Figure 2.9. Principal component analysis comparing the relationship between size-adjusted morphometric measurements at 0, 2, 8 and 13 days post-hatch (dph) for *Argyrosomus japonicus* reared at current and predicted levels of pCO_2 for the years 2050 and 2100.

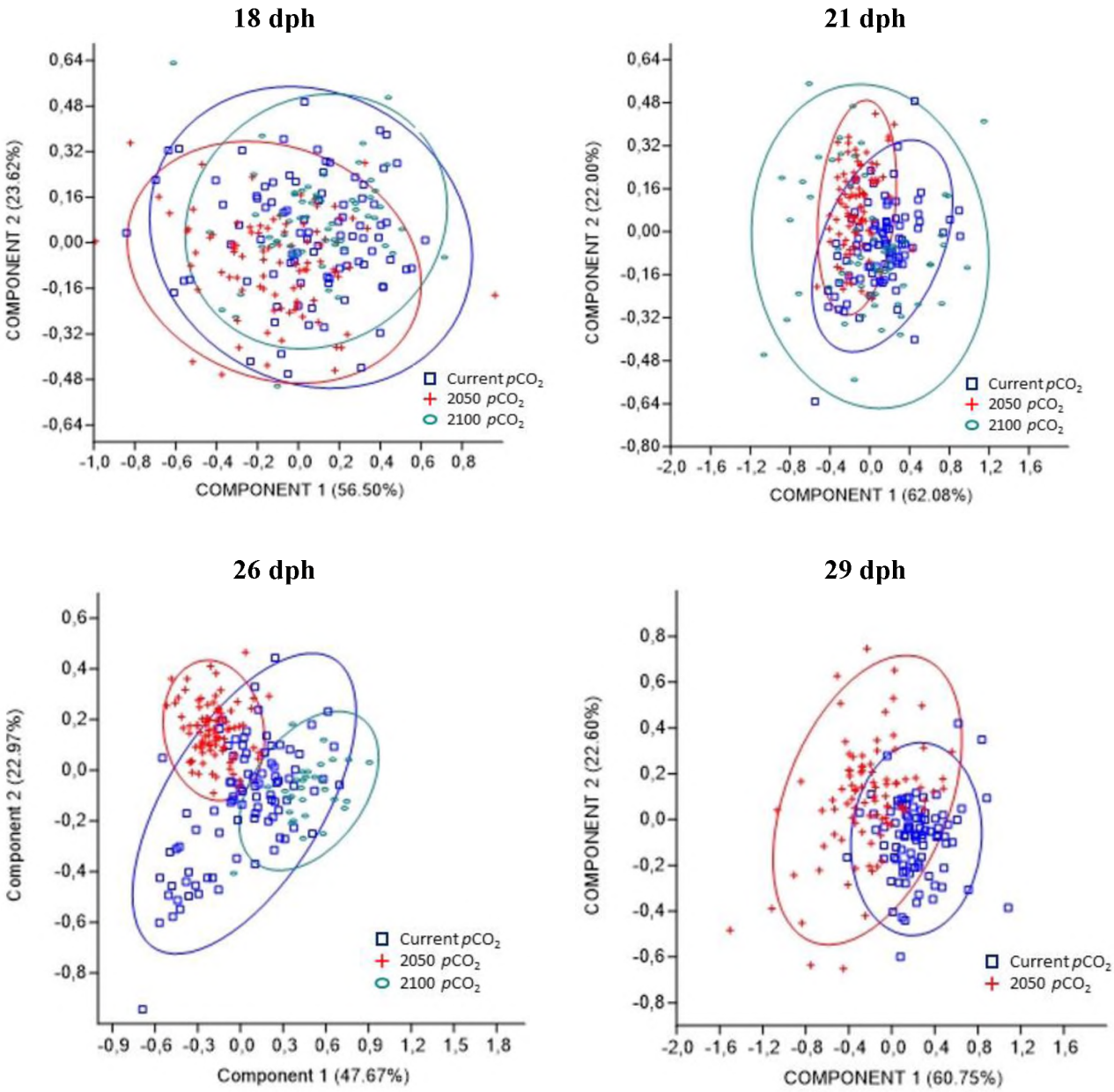


Figure 2.9. Continued. Principal component analysis comparing the relationship between size-adjusted morphometric measurements for *Argyrosomus japonicus* at 18, 21, 26 and 29 days post-hatch (dph) reared at current and predicted levels of pCO_2 for the years 2050 and 2100.

2.4. DISCUSSION

This is the first study to investigate the developmental response of a large estuarine-dependant species to elevated pCO_2 during the marine phase, and the results suggest that the early-life stage may be sensitive to the pCO_2 predicted for the end of this century. By 26 dph, individuals reared in the high 2100 pCO_2 treatment were, on average, 47.2% smaller (total length), with slower development compared to those in the current pCO_2 treatment. Furthermore, none of the fish in the high pCO_2 treatment survived beyond metamorphosis. Interestingly, fish reared at intermediate pCO_2 levels had the fastest growth, most rapid development and best survival rate.

These findings concur with several other studies that have suggested that fishes may be vulnerable to the pCO_2 levels expected for the end of the century during early ontogeny (e.g. Bauman et al. 2012, Chambers et al. 2014, Rosa et al. 2014). For example, Baumann et al. (2012) found that that elevated pCO_2 (~ 1000 µatm) compromised the early life stage survival and growth of estuarine *Menidia beryllina*. Rosa et al. (2014) found that elevated carbon dioxide (1020 µatm) reduced the early survival and hatching success of juvenile bamboo sharks, *Chiloscyllium punctatum*. Chambers et al. (2014) concluded that elevated pCO_2 (~ 1860 µatm) significantly reduced the length and survival of the early life stages of summer flounder, *Paralichthys dentatus*.

Besides impacting growth, development and survival there was also evidence of other responses through the developmental phases. The egg surface area was significantly larger in the high 2100 pCO_2 treatment. In a review of freshwater fish species Sayer et al. (1993) suggest that differences in egg size in acidified conditions are generally related to the rate of chorion hardening and concomitant water uptake. Consequently, larger egg volume in high pCO_2 may be attributed to slower hardening of the chorion and a consequent increased absorption of water (Jung et al. 2012). Lønning et al. (1984) demonstrated the importance of calcium for the hardening of the chorion. Limited calcium states at high CO_2 (Fabry et al. 2008) may have resulted in slower hardening of the chorion and consequent increases in egg volume in the 2100 pCO_2 treatment. Regardless of the driver, the larger eggs in the high pCO_2 treatment did not result in any significant difference in the size of hatchlings. This is not unusual, as egg size and larval body mass and/or length are not always related (Sayer et al. 1993). Interestingly, although not significant, the larvae in the high pCO_2 treatment were the smallest at hatching when compared to the other treatments, with the highest shape variability of all the treatments.

Few studies have compared the morphometric shape of early fishes in different pCO_2 conditions (e.g. Chambers et al. 2014). Interestingly, there appeared to be variability in the size-standardised morphology of individuals during the hatching phase (0 dph), with greater morphological variability, particularly along the PC1 axis, which mostly represented the stomach area and head length measurements. However, the observed variation in the high 2100 pCO_2 treatment was limited to hatchlings, as there was no further treatment effect evident on fish morphology until 21 dph (Figure 2.9). At the onset of metamorphosis (21 dph) morphological variability increased in the high pCO_2 treatment along both axes. This variation in morphology may, however, not reflect a difference in the larval shape, but instead may indicate the diversity in the developmental stages present in each treatment. For example, the morphological variation on day 26 was narrow in the 2100 pCO_2 treatment (Figure 2.9), which comprised individuals belonging only the post-flexion stage (Figure 2.8). In contrast, morphological variation was broader in the current treatment (Figure 2.9) as it comprised post-flexion larvae and individuals that had completed metamorphosis (Figure 2.8). Therefore, to compare changes in morphology between treatments, the results of the PCA should only be compared when all individuals in all treatments belong to the same life stage. In this study, the life stages were uniform between treatments from 2 dph until 18 dph. The PCA during this period showed clear overlap between treatments (Figure 2.8), suggesting that there was limited morphological variability between treatments and that different rates of larval development may account for the perceived variability in morphology. These findings are in accordance with those of Chambers et al. (2014) who found no effect of elevated pCO_2 (1860 µatm) on the shape of *Paralichthys dentatus*. The developmental delay evident in high pCO_2 is, however, cause for concern and could explain the reduced survival as rate of development and survival are often interlinked (Munday et al. 2009, Rossi et al. 2015).

Differences in development between treatments may reflect differential growth rates. From 21 dph, there were significant differences in the mean size and development of individuals between treatments, with significantly smaller and less-developed individuals present in the high 2100 pCO_2 treatment. These findings differ from the reduced growth and survival post-hatching observed by Baumann et al. (2012) and Rosa et al. (2014) and challenge the notion that elevated pCO_2 impacts are only restricted to the earliest stages of fish development. Consequently, these findings highlight the importance of testing the effects of elevated pCO_2 on all stages of development in order to fully comprehend the vulnerability of fishes to ocean acidification. For example, Bignami et al. (2013) found no effect on cobia exposed to 800 and

2100 µatm pCO_2; however, this study was unable to expose the early larval stages (larvae were only exposed to elevated pCO_2 from 2 dph) or the late metamorphosis stages to elevated pCO_2 conditions. The exclusion of the stages considered most sensitive by this and other studies (e.g. Baumann et al. 2012) complicates comparisons between results with these studies. Consequently, it is recommended that future ocean acidification studies on fish early development expose fish eggs to elevated pCO_2 immediately after hatch and extend the study duration well past metamorphosis, to draw robust conclusions.

Reduced growth has also been observed in the high pCO_2 treatments of several other studies on marine fishes (e.g. Baumann et al. 2012, Miller et al. 2012, Chambers et al. 2014). Interestingly, increased growth at pCO_2 levels similar to the highest treatment in this study (~ 1000 µatm) have been observed in some fish species (e.g. Munday et al. 2009, Chambers et al. 2014, Rossi et al. 2015), while others show no growth response (e.g. Franke and Clemmesen 2011, Munday et al. 2011, Hurst et al. 2013). These conflicting results suggest that changes in larval growth patterns in response to hypercapnia are species-specific and complicate predicting the response of fishes to ocean acidification.

In this study, the differentiation in growth and development began at the onset of metamorphosis, which involves radical restructuring of larval morphology. Metamorphosis is considered an energetically expensive stage as the morpho-physiological changes from larvae to juvenile fishes takes place (Rossi et al.2015). In *A. japonicus*, this phase also corresponds with a shift from a pelagic to a demersal lifestyle (Ballagh et al. 2011). In a parallel study Edworthy (2017) found a significant increase in the standard metabolic rate across all three treatments during this stage. The high physiological demand during metamorphosis suggests that fishes in this stage may be less tolerant to excessive acid-base regulation costs, and explains potential vulnerability to physiological stressors such as elevated pCO_2 (Doherty et al. 2004). Rossi et al. (2015) similarly suggest that the effects of elevated pCO_2 (1675.1 µatm) are most pronounced in barramundi (*Lates calcarifer*) during metamorphosis, with reduced swimming speeds and heightened anxiety at this stage.

The driver for the observed differences between pCO_2 treatments during metamorphosis remains unclear. Various studies have suggested larval fishes increase their metabolic rate during exposure to elevated pCO_2 to compensate for increased acid-base regulation energy costs (Pimentel et al. 2014a, Heuer and Grosell 2016). Fivelstad et al. (1999) found that Atlantic salmon (*Salmo salar*) smolt, exposed to very high levels of pCO_2 (~ 12 000 µatm), increased

their gill ventilation rate by 125%, possibly indicating elevated acid-base regulation costs. Wood et al. (2002) found that the energetic cost of acid-base regulation amongst tilapia (*Alcolapia grahami*) in the acidified Lake Magadi cost up to 50% of their baseline metabolism. It is therefore possible that the heightened energetic demand during metamorphosis and the additional costs of acid-base regulation in the high pCO_2 conditions exceeded the metabolic capabilities of *A. japonicus*. This is supported by a parallel metabolic study by Edworthy (2017), where periods of high metabolic demand coincided with the reduced development at metamorphosis observed in the high pCO_2 treatment in this study (see Figure 4.2. in Chapter 4). Heuer and Grosell (2016) suggest that fishes shift energy away from important life processes to maintain a constant body pH; this is a likely explanation for the observed reduction in the growth and development of *A. japonicus* in the high 2100 pCO_2 treatment.

In contrast to the reduced growth and development observed in the high pCO_2 treatment, growth and development was fastest in the intermediate (year 2050) pCO_2 treatment. By 29 dph, fish from the intermediate treatment were, on average, 49% longer (total length) compared to fish from the current pCO_2 treatment, and 12.5% of *A. japonicus* from the intermediate treatment were already in the early juvenile stage of development by 29 dph. This is not the first study to observe increased growth in elevated pCO_2 treatment during early fish development (e.g. Wood et al. 2008, Munday et al. 2009, Bignami et al. 2013, Chambers et al. 2014, Kim et al. 2015, Rossi et al. 2015). However, the mechanism driving faster growth in conditions of increased pCO_2 remains unclear and requires further investigation (Ishimatsu et al. 2008).

Besides faster development amongst larval barramundi (*Lates calcarifer*), individuals reared in elevated CO_2 (1675 µatm) had higher levels of anxiety and exhibited slower swimming speeds (Rossi et al. 2015). This change in behaviour may be indicative of an energy conservation strategy induced by heightened acid-base regulation costs in elevated pCO_2. Munday et al. (2009) found orange clownfish, *Amphiprion percula,* were larger when reared in elevated pCO_2 (1030 ppm) and suggested that the increased growth may be related to increased dietary intake, in response to the heightened metabolic demand for acid-base regulation in acidified conditions. The findings of this study suggest that *A. japonicus* exposed to intermediate levels of pCO_2 (~ 550 µatm) may have higher metabolic rates and therefore increased growth rates, but this increased growth may depend on successful feeding. Although this study maintained the optimal conditions for growth and survival, these conditions will most likely not be found in the natural environment. The natural environment is characterised

by fluctuations in food availability and temperature (which influences activity), consequently it is possible that the growth and survival results may differ in the natural environment. Survival may be further influenced by predation in the natural environment. Predator avoidance has been shown to be affected by elevated carbon dioxide resulting in higher mortalities (Cripps et al. 2011, Ferrari et al. 2011). Dixson et al. (2010) found that *Amphiprion percula* larvae were more attracted to predators in elevated pCO_2 conditions. As the outcomes in the natural environment could be significantly different from a laboratory-based study, this study suggests the need for further research assessing the impact of both food availability and predators on survival in elevated pCO_2 studies.

In conclusion, these findings suggest the growth, development, and survival of *A. japonicus* may be negatively affected by carbon dioxide levels expected by the end of the century. It appears that the greatest impact of the elevated pCO_2 will be felt during times of high energy requirement, such as metamorphosis, and ultimately the ability of fishes to survive past this stage will determine the impacts on recruitment and population sustainability in future ocean conditions.

CHAPTER 3

The effects of CO_2-induced acidification on Argyrosomus japonicus cartilage and bone development.

3.1. INTRODUCTION

Ocean acidification is thought to challenge the calcification rates of many marine organisms (e.g. Orr et al. 2005, Fabry et al. 2008, Miller et al. 2009), with several recent studies reporting reduced calcification rates in externally calcifying marine organisms in response to elevated pCO_2 (e.g. Fabry et al. 2008, Moy et al. 2009, Talmage and Gobler 2010). Despite the growing concern for calcifying organisms, few studies have assessed the effect of ocean acidification on internal calcifying organisms, such as fishes (Frommel et al. 2012, Kim et al. 2015). Recent studies that investigated the skeletal development of fishes in future-predicted pCO_2 suggested that ocean acidification may have adverse consequences for some fish species (e.g. Chambers et al. 2014, Pimentel et al. 2014b, Kim et al. 2015). These effects include reduced cranio-facial elements (e.g. Chambers et al. 2014), increased number of deformities (e.g. Pimentel et al. 2014b) and decreased bone density (e.g. Kim et al. 2015), which are thought to be attributed to reduced calcium carbonate saturation states (Munday et al. 2011).

Several studies have found a link between ocean acidification and increased craniofacial and skeletal malformations (e.g. Chambers et al. 2014, Pimentel et al. 2014b, Perry et al. 2015). The most commonly impacted skeletal elements appear to be the abdominal and caudal vertebrae, with abnormalities including fusions, compression, deformed haemal and neural spines, deformed arches and vertebra malformations such as scoliosis, lordosis and kyphosis (Perry et al. 2015, Pimentel et al. 2014b, Pimentel et al. 2016). An increase in the occurrence of deformities, or impaired skeletogenesis, will adversely impact on fish growth and survival and ultimately, on recruitment success (Pörtner et al. 2004, Fabry et al. 2008). Consequently, ocean acidification poses major ecophysiological challenges to larval fishes and may potentially result in substantial declines in adult fish populations (Pimentel et al. 2014b).

The effect of ocean acidification on skeletal development is expected to be most pronounced in the early life stages of fishes (Baumann et al. 2012). Newly deposited minerals for skeletal development are also considered labile compared to old mineral deposits, thus increasing early stage vulnerability to the effects of decreasing pH and concomitant acidosis (Taylor and van Dyke 1985). Acidosis has been shown to decalcify, corrode and weaken small calcium

carbonate structures (Rombough 1997, Pörtner and Farrell 2008); this may lead to corroded or weakened calcium structures in acidic conditions and potentially induce a higher occurrence of deformities amongst larval fishes (Perry et al. 2015).

Not only is fish skeletogenesis considered vulnerable to acidosis, but also to the costs associated with increased acid-base regulation (Munday et al. 2008, Frommel et al. 2012). Early skeletogenesis is energetically demanding and associated with a high metabolic cost (Pimentel et al. 2014b). This may become problematic in elevated pCO_2 conditions, which has been shown to induce metabolic depression, limiting the energy available for skeletogenesis (Pimentel et al. 2014a, Heuer and Grosell 2016).

Up to now, most studies examining the impact of ocean acidification on skeletal development have been limited to quantifying skeletal deformities (e.g. Ben-Asher et al. 2013, Frommel et al. 2014, Pimentel et al. 2014b), comparing size of bone structures (e.g. Munday et al. 2009) and density (e.g. Kim et al. 2015). Current literature fails to addressed potential alterations in skeletal development patterns and this limits our understanding of the impacts of ocean acidification on skeletogenesis. Furthermore, none of these studies have closely examined and compared patterns of chondrification and ossification. The lack of chondrification and ossification data can most probably be attributed to the difficulties associated with viewing and quantifying cartilage and bone tissue development in larval fishes. Consequently, the current indicators used to assess skeletogenesis limit our understanding of the impacts of ocean acidification on larval fishes.

Currently, the most common method for viewing skeletal structures of fishes in ocean acidification studies is staining and clearing (e.g. Munday et al. 2011, Chambers et al. 2014, Pimentel et al. 2014b), and digital X-ray inspection (e.g. Ben-Asher et al. 2013, Perry et al. 2015). X-ray radiology is a practical method for detecting perceptible skeletal abnormalities in adult fishes (Fisher et al. 2003), but it is limited to detecting morphometric skeletal malformations and cannot quantify differences in bone or cartilage developmental patterns. Furthermore, this method is less effective in detecting subtle differences in larval skeletons.

Staining and clearing is a far more effective screening tool for detecting subtle skeletal differences among larval fishes and presents the opportunity to analyse bone and cartilage separately. However, to date, the most common method for staining and clearing incorporates the use of an acidic alcian blue solution (pH ranges between 1.7 and 2.4) to dye cartilage (Bancroft and Gamble 2008). This may be problematic since it has been suggested that the

acidic alcian blue solution causes deterioration of newly formed calcified matrices in the fragile, early larval fish skeletons and can lead to ambiguous conclusions (Gavaia et al. 2000, Walker and Kimmel 2007). Consequently, this study developed a non-acidic alcian blue stain, based on the addition of a salt ($MgCl_2$) as proposed by Scott and Dorling (1965), instead of acid. This staining method, although not commonly used, is considered suitable for staining larval cartilage (Bancroft and Gamble 2008). Consequently, a non-acidic alcian blue-alizarin red double stain was developed, based on the staining methods proposed by Scott and Dorling (1965), Gavaia et al. (2000), Walker and Kimmel (2007) and Darias et al. (2010). This double-staining technique has been found to be effective for examining early skeletal development (Walker and Kimmel 2007, Darias et al. 2010) but, despite its obvious suitability, has never been used in the context of ocean acidification research.

Meristic counts of the skeletal elements have been proposed as a powerful tool for evaluating the skeletal system in larval fishes (Boglione et al. 2001). Gisbert et al. (2014) suggest that early meristic counts of fishes, such as vertebrae or fin rays, are susceptible to environmental conditions up until metamorphosis. Following metamorphosis, meristic characters become fixed and remain unchanged, regardless of environmental conditions (Lindsey 1988). Torres-Núñez et al. (2014) propose that anal and dorsal fin ray counts are the most diagnostic meristic elements in the Turbot, *Scopthalmus maximus,* when the species is exposed to changes in temperature. However, up to now, no studies have used meristic counts of the anal and dorsal fin rays and spines of larval fishes to evaluate the impact of elevated pCO_2.

The aim of this chapter is to compare the skeletal response of early life stages of a marine spawning, estuarine-dependent species that is exposed to levels of hypercapnia expected in the near future. To do this, *A. japonicus* were reared from egg in current, and predicted (for 2050 and 2100) levels of pCO_2. Skeletal development was compared during five key life stages (yolk-feeding, pre-flexion, flexion, post-flexion and early juvenile). Specimens were either prepared for examination using clearing and staining with a non-acidic stain or X-ray analysis with digital X-ray inspection, and the patterns of skeletogenesis were compared between pCO_2 treatments by enumerating the number of deformities, comparing ray and fin meristic measurements, and quantifying the degree of chondrification and ossification using a pixel analysis tool.

3.2. MATERIALS AND METHODS

Details of the study site, study species, experimental protocol, water quality and treatment for this experiment are outlined in the Material and Methods section of Chapter Two.

Sixty *A. japonicus* larvae were collected from each replicate tank (180 per treatment) at key ontogenetic windows on 2, 6, 13, 18, 21 and 26 dph (Table 3.1). Larvae were euthanized using an overdose of 2-phenoxyethanol (400 mg L^{-1}) (King et al. 2005) and equally divided between two preparation techniques. Skeletal elements were inspected using either staining and clearing with an acid-free stain, or X-ray analysis.

3.2.1. PREPARATION

3.2.1.1. STAINING AND CLEARING

This study adopted the acid-free staining protocol originally proposed by Scott and Dorling (1965), with the early larval staining suggested by Gavaia et al. (2000) and combined it with the double-stain methods proposed by Walker and Kimmel et al. (2007). *Argyrosomus japonicus* staining proved far more effective without pre-fixation (e.g. Gavaiva et al. 2000, Walker and Kimmel et al. 2007). Furthermore, this study extended both staining and clearing duration and intensified alcian blue and alizarin red concentrations from the staining protocol proposed by Walker and Kimmel et al. (2007). Clearer stained skeletons were visible with the inclusion of a pre-ethanol incubation step, which supports the notion that hydration dilutes the stain (Potthoff 1984). Larvae were immersed in 70% ethanol for 20 minutes before being transferred to a stain solution (Table 3.2) that consisted of 5 ml of 95 mM $MgCl_2$ with 4.3 g of alcian blue 8 GX dissolved in 70% EtOH for cartilage staining, and 0.05 ml of 0.6% alizarin red S to stain bone. Staining solutions were consistently prepared pre-staining to ensure pixel colour comparability between treatments (Darias et al. 2010). Solutions were mixed by means of a rotary evaporator and magnetic stirrer.

Fish were immersed and staining occurred overnight on a controlled platform shaker (Labcon 3100 u). This was done to avoid a decrease in pH around specimens, improve solution penetration, and ensure equal mixing and distribution. Optimal incubation times for each stage of *A. japonicus* ontogeny were pre-determined during a pilot study (stages of ontogeny are shown in Table 2.1 and incubation times in Table 3.2). Bleaching and clearing followed the methods proposed by Walker and Kimmel (2007), with reduced bleach concentrations and incubation durations for smaller specimens (Table 3.2). Stained specimens were photographed

with a Leica EZ4 HD dissecting microscope (magnification was adjusted to specimen size) for later analysis.

Table 3.1. Skeletogenesis of *A. japonicus* at key stages of ontogeny and days post-hatch (dph), with total length (TL) and standard deviation (SD) shown.

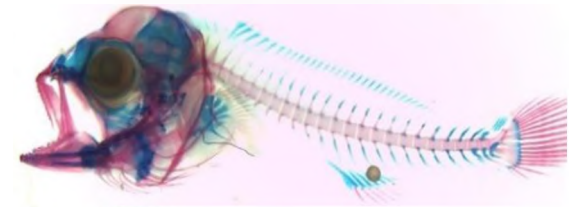
DPH	LIFE STAGE	STAINED SPECIMEN	TL ± SD
0	Hatchling		2.71 ± 0.07
2	Yolk feeding		2.92 ± 0.03
5	Pre-flexion		4.35 ± 0.19
6	Pre-flexion		4.77 ± 0.26
13	Flexion		4.77 ± 0.26
18	Post-flexion		5.23 ± 0.19
21	Early juvenile (onset of metamorphosis)		5.54 ± 0.16
26	Settlement		6.96 ± 0.62

Table 3.2. Incubation times for each solution during non-acidic double-staining of *A. japonicus* larvae at key stages of ontogeny.

Ontogenetic stage	**Hatching**	**Yolk stage**	**Pre-flexion**	**Notochord flexion**	**Post-flexion**	**Settlement**
DPH	0 DPH	2 DPH	5 DPH	13 DPH	18 DPH	26 DPH
Total length	2.6 – 2.8 mm TL	2.8 – 3.00 mm TL	3.2 – 4.5 mm TL	5 – 6 mm TL	6 – 7 mm TL	7 – 13 mm TL
Incubation times at stage						
Dehydration	5 minutes	10 minutes	15 minutes	20 minutes	20 minutes	20 minutes
Double stain	6 hours	10 hours	14 hours	20 hours	24 hours	26 hours
Bleaching	8 minutes	10 minutes	15 minutes	20 minutes	25 minutes	30 minutes
Clearing	6 hours	12 hours	16 hours	24 hours	48 hours	60 hours

3.2.1.2. X - RAY

Thirty of the sixty specimens collected from each replicate were stored in 70% ethanol and X-rayed at the South African Institute of Aquatic Biodiversity's digital X-Ray inspection system (Inspex 20i, 40 – 90 kV) one month after the completion of the experiment. *Argyrosomus japonicus* larvae were placed on a tray, in a lateral position facing left and digital X-ray images taken of the entire fish. Digital x-ray images were then examined for signs of deformities.

3.2.2. INDICATORS

3.2.2.1. DEFORMITIES

Skeletal deformities were quantified through the inspection of stained and x-rayed images of *A. japonicus* larvae in accordance with previously established malformation detection methods (Gavaia et al. 2002, Javidan and Schilling 2004, Pimentel et al. 2014b). Deformities were classified as either mild (1 point) or extreme (2 points) (Table 3.3). Deformities such as scoliosis, lordosis, kyphosis and vertebral fusions were considered mild, whereas any malformation that would severely inhibit fish survival or success was deemed extreme. Special attention was paid to the development of the pharyngeal elements and dentary, because of the prominence of skeletal abnormalities in this area (Neuhauss et al. 1996, Kimmel et al. 1998,

Javidan and Schilling 2004). Elements investigated included cranial abnormalities, such as opercular and cranium deformities, fusion and compression, deformed spines, arches and vertebral malformations.

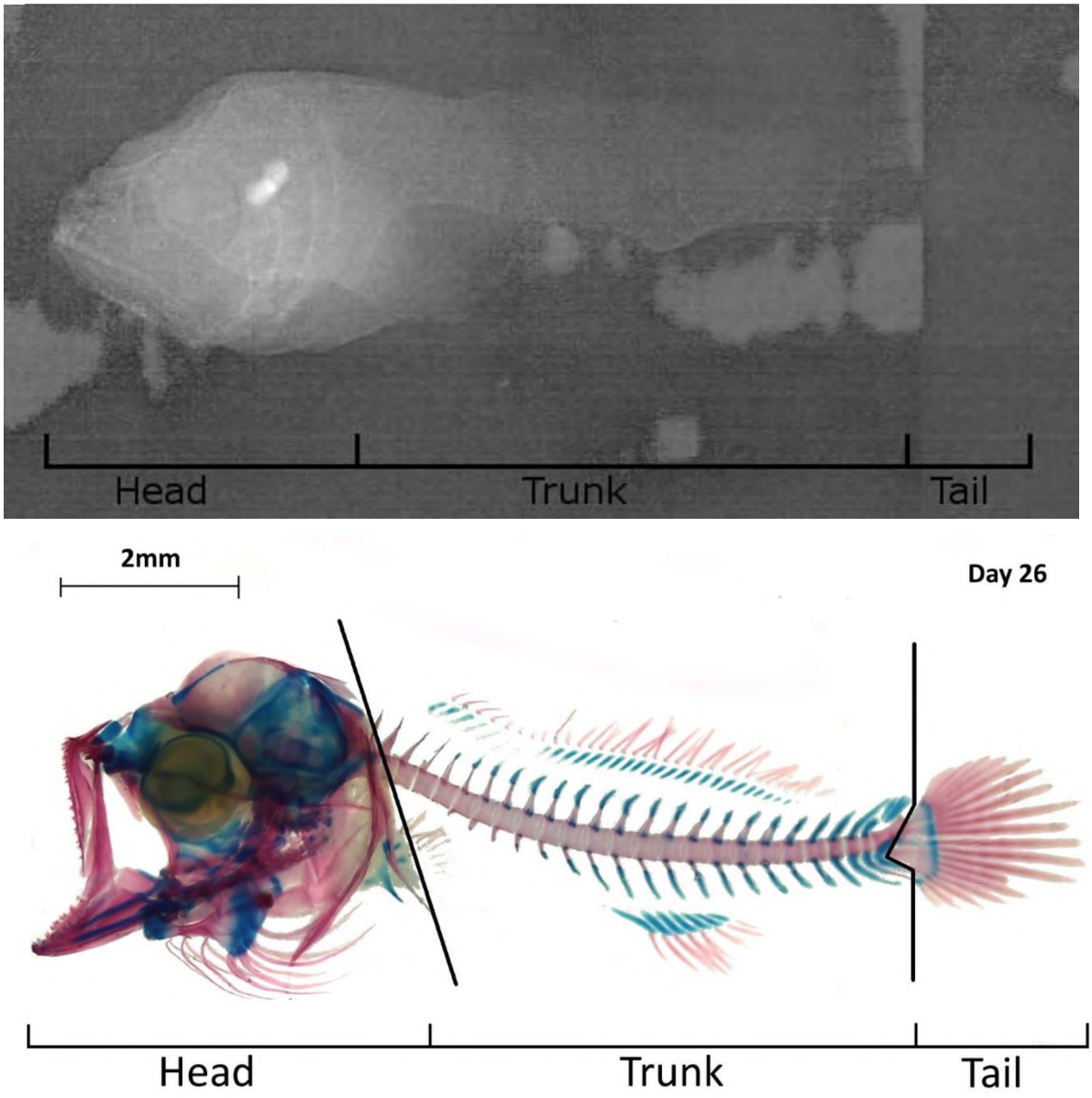


Figure 3.1. X-ray and stained skeleton of *A. japonicus* larvae at metamorphosis (26 dph, 9.59 mm TL) showing the head, trunk and tail sections for deformity and pixel analysis.

Table 3.3. Most common impact on skeletal elements in elevated pCO_2 research (Javidan and Schilling 2004, Pimentel et al. 2014b).

DEFORMITIES	DESCRIPTION
Skull (Craniofacial deformities)	
Jaw deformity	Malformed and/or reduced maxillary, pre-maxillary, angular and/or dentary bones
Deformed operculum	Deformed opercular, ceratobranchial and ceratohyal bones
Fusion	Abnormally fused cranial elements
Compression	Abnormally narrow or shortened
Reduced or elongated structures	Most common to pharyngeal skeleton (Javidan and Schilling 2004).
Unknown	
Trunk (Vertebral column)	
Vertebral body malformation	Torsion and/or malformation of one or more vertebrae (Pimentel et al. 2014b)
Lordosis	Excessive inward vertebral curvature (Pimentel et al. 2014b)
Scoliosis	Side-to-side vertebral curvature
Kyphosis	Excessive outward vertebral curvature
Vertebral fusion	Partial or total fusion of two or more vertebrae (Pimentel et al. 2014b)
Unknown	
Fins (pectoral, pelvic and dorsal)	
Malformed hypural	Deformed, absent, asymmetric, fused or supernumerary
Malformed epural	Deformed, absent, asymmetric, fused or supernumerary
Malformed parahypural	Deformed, absent, asymmetric, fused or supernumerary
Malformed fin rays	Deformed, absent, asymmetric, fused or supernumerary
Malformed pterygiophores	Deformed, absent, fused or supernumerary

3.2.2.2. MERISTICS

Meristic counts, including anal, dorsal, and caudal fin rays and spines were collected from *A. japonicus* on the final day of staining (26 dph). These counts were then compared between pCO_2 treatments to test the plastic response of *A. japonicus* to elevated pCO_2. As a result of the difficulty in differentiating between spines and rays at this stage of development, spines and rays were counted collectively.

3.2.2.3. PIXEL ANALYSIS

Skeletal ossification and chondrification was quantified using a pixel analysis as described by Darias et al. (2010) and Gisbert et al. (2014). The electrostatic reaction between alcian and cartilage stains cartilaginous structures blue, whereas alizarin S stains bone red (Taylor and Van Dyke 1985, Walker and Kimmel 2007). Red and blue pixels, reflecting skeletal ossification and chondrification, were quantified at five key stages of ontogeny (2, 6, 13, 18, 21, 26 DPH) each reflecting a key stage of skeletogenesis. The number of red and blue pixels were enumerated in the head, trunk, and tail sections of stained larvae at the five stages, using the pixel analysis method proposed by Darias et al. (2010). Image analysis was undertaken in the image analysis software GIMP. Here the area of interest (head, trunk, or tail) was selected and isolated. Then the number of red and blue pixels were enumerated using the colour select tool, which was set at a constant threshold of 20%. This ensured quantifying only near blue or red colour ranges. To eliminate size bias, the number of red and blue pixels in each region were then divided by the total number of pixels in the entire fish measured using the same method but at maximum threshold to give a proportional value using the equation:

$$\text{Mean head/trunk/tail ossification} = \left(\frac{x}{y} \times 100\right)$$

where x is the number of red pixels counted in the head, y the total number pixels counted in the entire stained specimen.

3.2.3. STATISTICAL ANALYSES

3.2.3.1. DEFORMITIES

Deformities were expressed as treatment deformity percentage (incidence and degree of skeletal deformities divided by total deformities). The mean deformity scores were compared between pCO_2 treatment by means of a One-Way ANOVA (homogeneity of data, Levene's test). If significant treatment interactions were detected, the Tukey multiple-comparison test

was used to detect differences between replicates (Zar 1974). Statistical analyses were performed in the Statistica package, version 10.0 (StatSoft Inc., Tulsa, OK, USA).

3.2.3.2. RAY AND SPINE DEVELOPMENT

Owing to the non-parametric distribution of meristic data, treatment mean counts were compared using a Kruskal-Wallis (KW) test.

3.2.3.3. PIXEL ANALYSIS

Chondrification and ossification were all analysed separately in the head, trunk, and tail to test for differences between the main effects of "pCO_2 treatment" and "dph" and the interaction between these two effects by means of factorial analysis of variance. *Post hoc* multiple comparison tests (Tukey-Kramer test) were used to detect significant differences between treatment replicates. A Shapiro-Wilk (Shapiro and Wilk 1965) and Levene's test (Levene 1960) were used to test the normality of residuals and homogeneity of variance, respectively. Sphericity tests were conducted to test for equality of variance of all levels of independent variables and, when necessary, corrected degrees of freedom (df) values were used (Greenhouse and Geisser 1959; Huynh and Feldt 1970). Where necessary, data were transformed (log, reciprocal, sqrt. arcsine) to improve distribution of residuals. When transformation did not improve homogeneity of the variance, this study accepted the probability of committing a Type 1 error and being slightly more than the nominal value α. As pointed out by Ott and Longnecker (1993), where the sample sizes are nearly equal, as in this case (n = 30), the variances may be markedly different and the P-values for an analysis of variance will still be only mildly distorted.

3.3. RESULTS

3.3.1. DEFORMITIES

The X-ray imagery showed poor detail in comparison to staining and clearing during early skeletogenesis (Figure 3.1). Consequently, deformities and all other analyses were conducted from digital images of stained and cleared specimens. In total, only four deformities from 1620 sampled larvae were recorded during this study. All deformities were detected in the stained and cleared specimens. These included an absent operculum (Figure 3.2, A), branchiostegal ray abnormalities (Figure 3.2, B), malformed hemal and dorsal arch formations (Figure 3.2, C),

and compressed skull development (Figure 3.2, D). Deformity A and B belonged to year 2050 and year 2100 pCO_2 treatments, respectively, while C and D were observed in the current pCO_2 treatment. Deformities A, B and C were only visible on fish at 26 dph, while deformity D was observed on 10 dph. No significant treatment effect was found on number or degree of deformities.

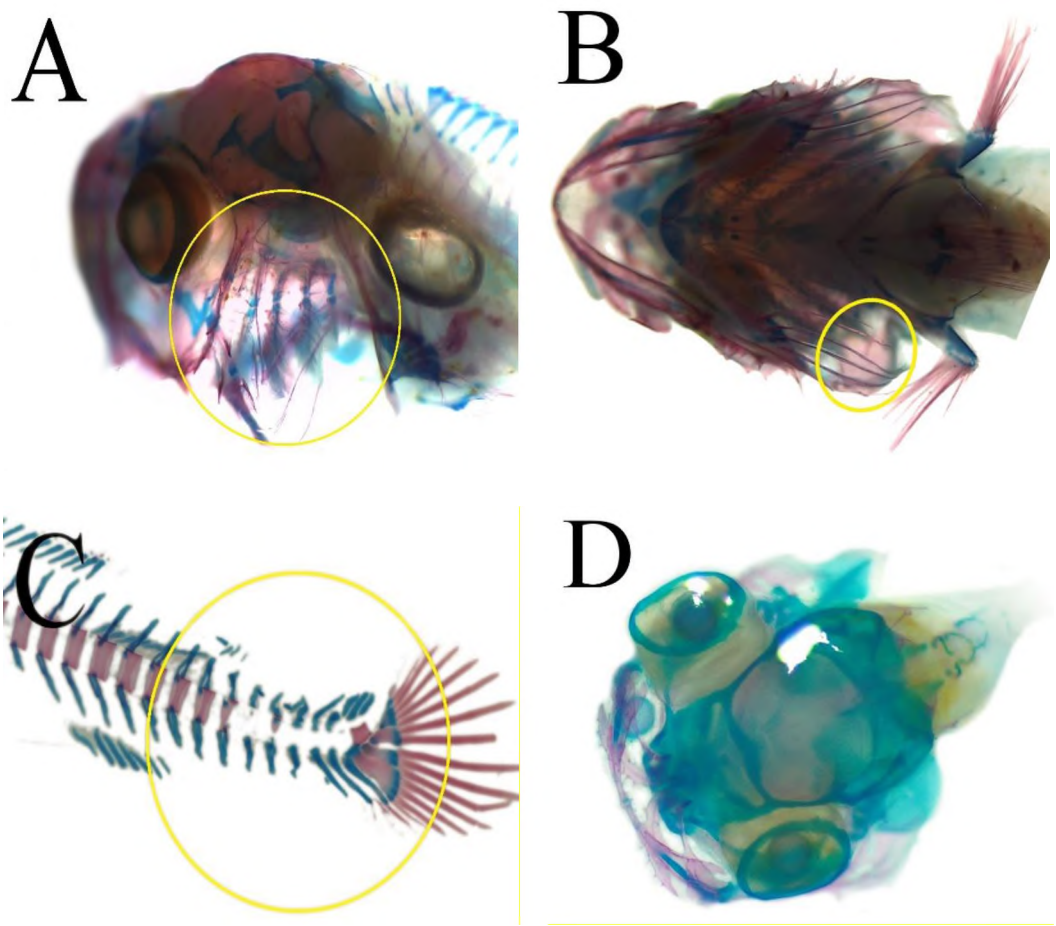


Figure 3.2. Skeletal deformities in non-acidic double-stained *Argyrosomus japonicus* reared at current and predicted levels of pCO_2 for the years 2050 and 2100. A = absent operculum, B = branchiostegal ray abnormalities, C = malformed hemal and dorsal arch formations, D = compressed skull development.

3.3.2. RAY AND SPINE DEVELOPMENT

Fin development proceeds with the formation of the pectoral (2 dph), to the caudal (13 dph), dorsal and anal (21 dph) and pelvic fin support (26 dph). By 26 dph, the average number of rays and spines was 36 for dorsal fins, 9 for anal fins and 24 for caudal fin rays. Complete dorsal fin development was common in both current and 2050 pCO_2 treatments by 26 dph (Table 3.4). Although the fin meristic data did not differ significantly between pCO_2 treatment on 26 dph (Table 3.4), no 2100 pCO_2 treatment fish had fully completed spine and ray

development in the dorsal or caudal fins (Table 3.4). Furthermore 25% of the 2100 pCO_2 treatment fish had no visible dorsal fin development and 10% were without any anal fin development by 26 dph (Table 3.4).

Table 3.4. Dorsal, anal, and caudal fin meristic counts at 26 dph of *A. japonicus* larvae reared in three pCO_2 treatments, showing treatment mean, range, and number of fish with no respective fin development.

Treatment	Dorsal			Anal			Caudal		
	$\bar{x}$	Range	No dev.	$\bar{x}$	Range	No dev.	$\bar{x}$	Range	No dev.
Current pCO_2	36	27 – 40	0	9	8 – 9	0	23	16 – 29	0
2050 pCO_2	37	32 – 40	0	9	8 – 9	0	27	22 – 29	0
2100 pCO_2	29	19 – 37	5	9	8 – 9	2	20	18 – 27	0

3.3.3. PIXEL ANALYSIS

3.3.3.1. CHONDRIFICATION

Chondrification was first visible in the neurocranium including the palatoquadrate, ceratohyal and Meckel's cartilage on the day of hatching (Figure 3.3 A). This occurred concurrently with the development of the eye lens (Figure 3.3 B). Chondrification progressed to the visceral skeleton and branchial elements in yolk stage larvae from 2 dph (1–1.5 mm TL). Chondrification rapidly expanded throughout the skull from 4 dph (3.3–3.4 mm TL). This expansion occurred primarily from the branchial cartilages, along with the dentary and articulating structures (Figure 3.3).

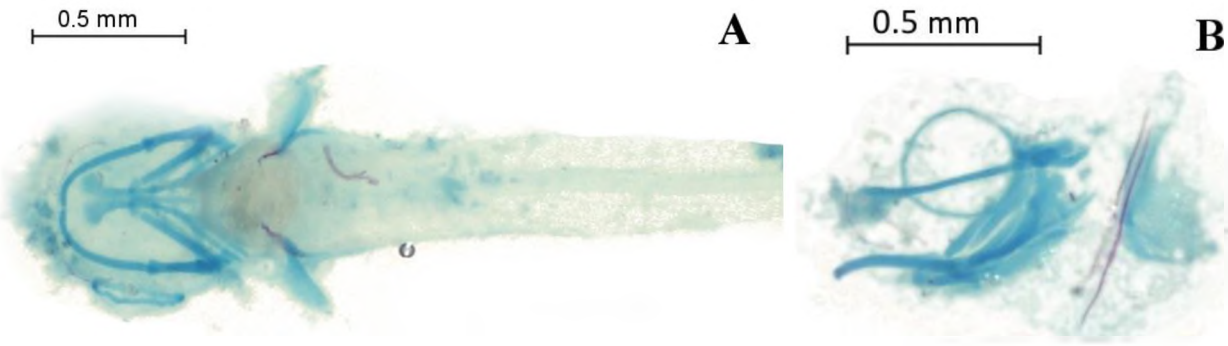


Figure 3.3. Non-acidic double-stained *A. japonicus* larvae 18 hours post-hatch, showing cartilage development focused on the dentary and articulating elements. Bone was stained red with alizarin red, while cartilage was stained blue with alcian blue.

Chondrification remained limited to the skull until 9 dph, when the caudal hypurals stained blue and signalled the start of skeletal development in the tail (Figure 3.4).

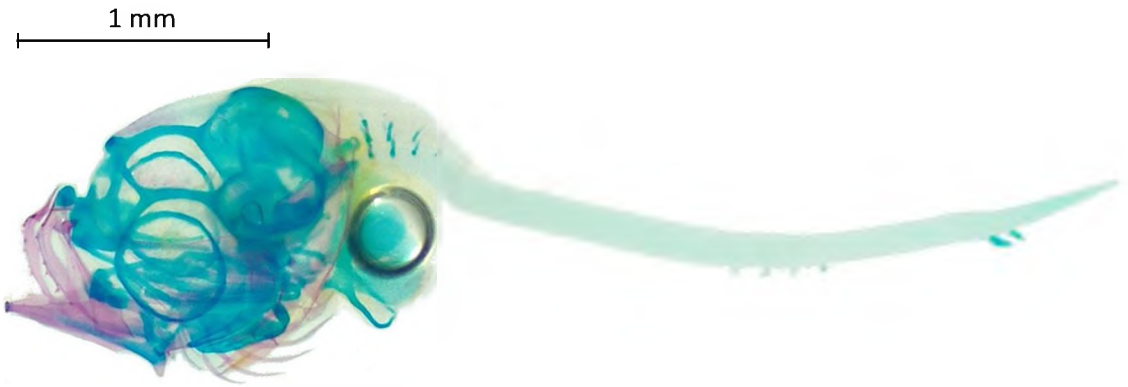


Figure 3.4. Non-acidic double-stained *A. japonicus* at 9 dph, showing the first signs of tail skeletal development.

Stained structures were evident in each section of the skeleton (skull, vertebral column, and caudal complex) from 13 dph and the time at which flexion occurred was not different between treatments. The neural and haemal arches stained blue at 13 dph in the trunk section and were followed by the development of the ribs and radial cartilage on 18 dph (Figure 3.5).

Figure 3.5. Non-acidic double-stained *A. japonicus* at 18 dph, showing the first signs of ribs and radial cartilage development.

Head

Relative chondrification of the head followed a similar pattern in all treatments until day 21 post-hatch (Figure 3.6.). Here, relative chondrification increased substantially in fish subjected to the current and 2050 pCO_2 treatments, but remained similar for fish in the 2100 pCO_2 treatments. At 21 dph, the relative head chondrification was 15.8% and 15.9% higher in the current and 2050 pCO_2 treatments, respectively, when compared with the 2100 pCO_2 treatment (Figure 3.6). However, this was less pronounced by 26 dph, with the relative head chondrification 4.7% and 5.2% higher in the current and 2050 pCO_2 treatment, respectively, when compared with the 2100 pCO_2 treatment. A factorial ANOVA ($F_{10, 28} = 1.71$, $P > 0.05$) indicated that the mean relative head chondrification of the larvae in the 2100 pCO_2 treatment was significantly lower than larvae in both the current and 2050 pCO_2 treatments on the 21 dph (Tukey's, $P < 0.05$) (Figure 3.6).

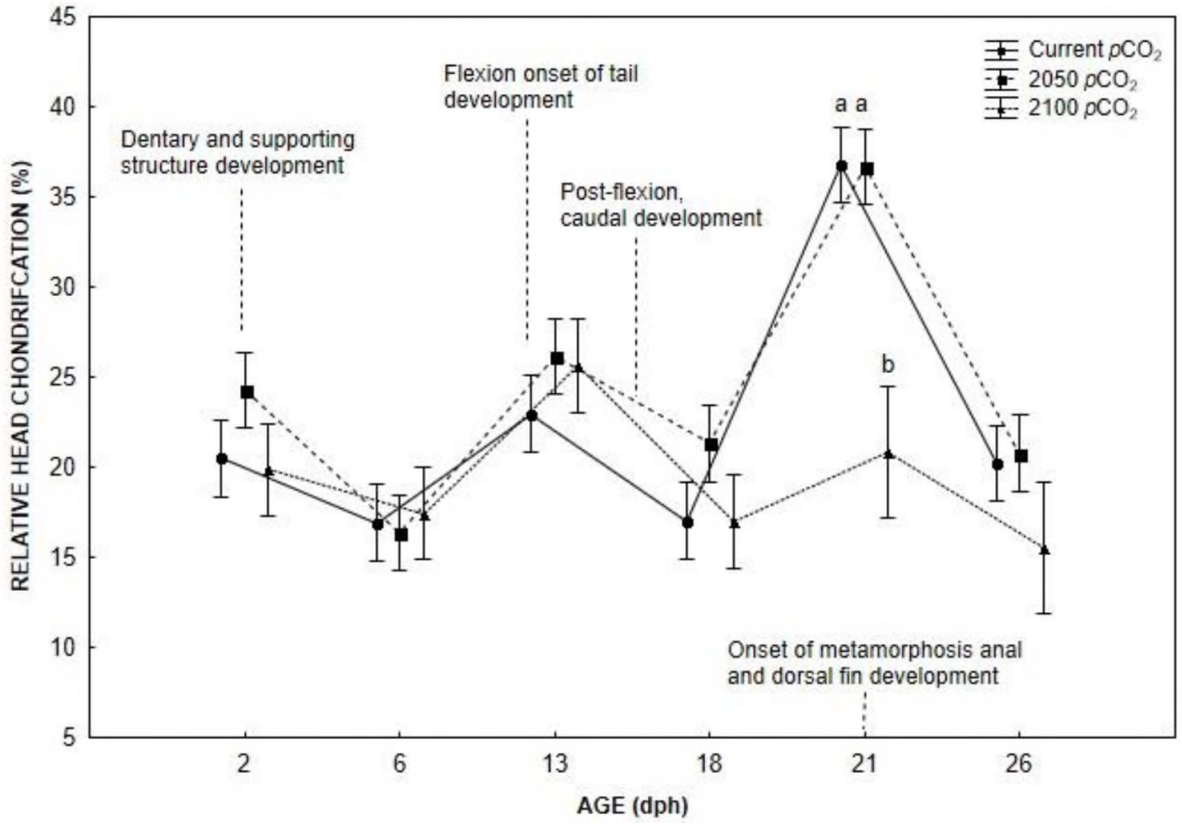


Figure 3.6. Changes in the relative proportion of cartilage (as a function of pixels) in the head of *Argyrosomus japonicus* reared at current and predicted levels of pCO_2 for the years 2050 and 2100. Vertical dotted lines indicate main developmental events of skeletogenesis. Weighted means (± S.E.) of relative head chondrification averaged across pCO_2 treatment replicates (n = 90). Data points were collected on the same day; presented scattered for easier interpretation. Different letters signify differences between treatment means (Tukey's, $P <$ 0.05).

Trunk

As with the head, relative chondrification in the trunk was homogeneous in all treatments until 21 dph (Figure 3.7.). Although the relative chondrification in the trunk varied greatest at the onset of metamorphosis (21dph), at the completion of metamorphosis (26 dph) the 2050 and current pCO_2 treatments were 3% and 1.7% higher, respectively, compared to the 2100 pCO_2 treatment (Figure 3.7). There was a significant interaction between mean trunk chondrification and time (dph) (Factorial ANOVA: $F_{10, 28} = 2.45$, $P < 0.05$) and a significant effect of both treatment and time (dph) on average chondrification (Figure 3.7). Tukey's *post hoc* test indicated that relative chondrification was significantly greater for fish subjected to the 2050 pCO_2 treatment (Tukeys, $P < 0.05$) at 21 dph (Figure 3.7).

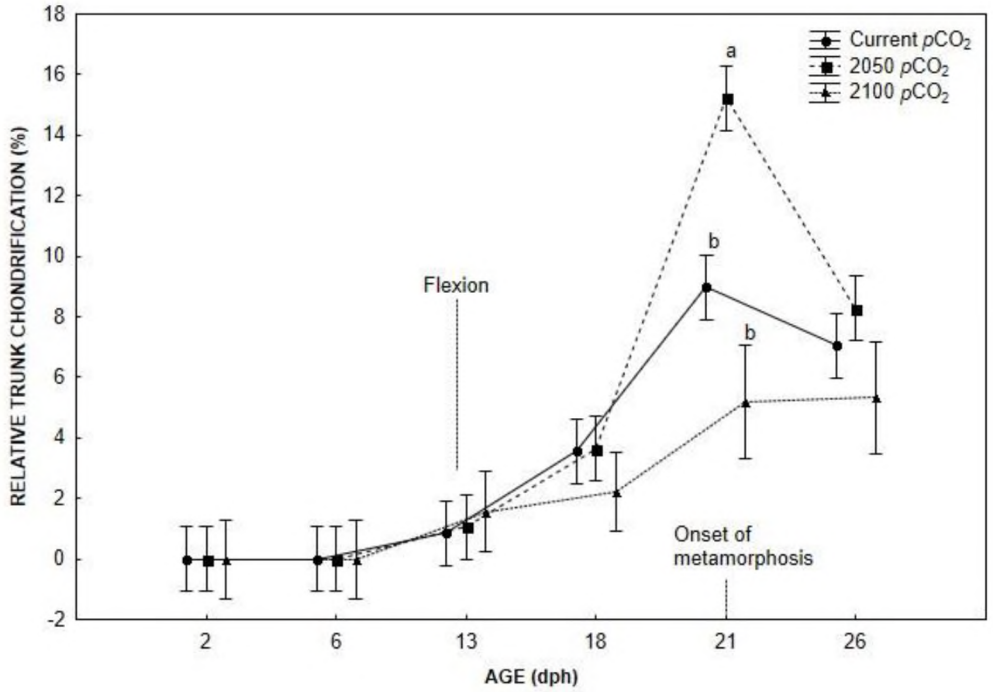


Figure 3.7. Changes in the relative proportion of cartilage (as a function of fish size) in the trunk of *Argyrosomus japonicus* reared at current and predicted levels of pCO_2 for the years 2050 and 2100. Weighted means (± S.E.) of relative trunk chondrification averaged across pCO_2 treatment replicates (n = 90). Data points were collected on the same day; presented scattered for easier interpretation. Different letters signify differences between treatment means (Tukey's, $P < 0.05$).

Tail

The pattern of relative chondrification in the tail was similar for the current and year 2050 pCO_2 treatment and, although comparatively retarded in the 2100 pCO_2 treatment, there was no significant difference in relative chondrification between treatment averages (Tukey's, $P < 0.05$) (Figure 3.8). No significant interactions were observed in tail chondrification between pCO_2 treatment and time (dph) (Factorial ANOVA: $F_{10, 28} = 0.45$, $P > 0.05$) (Figure 3.8).

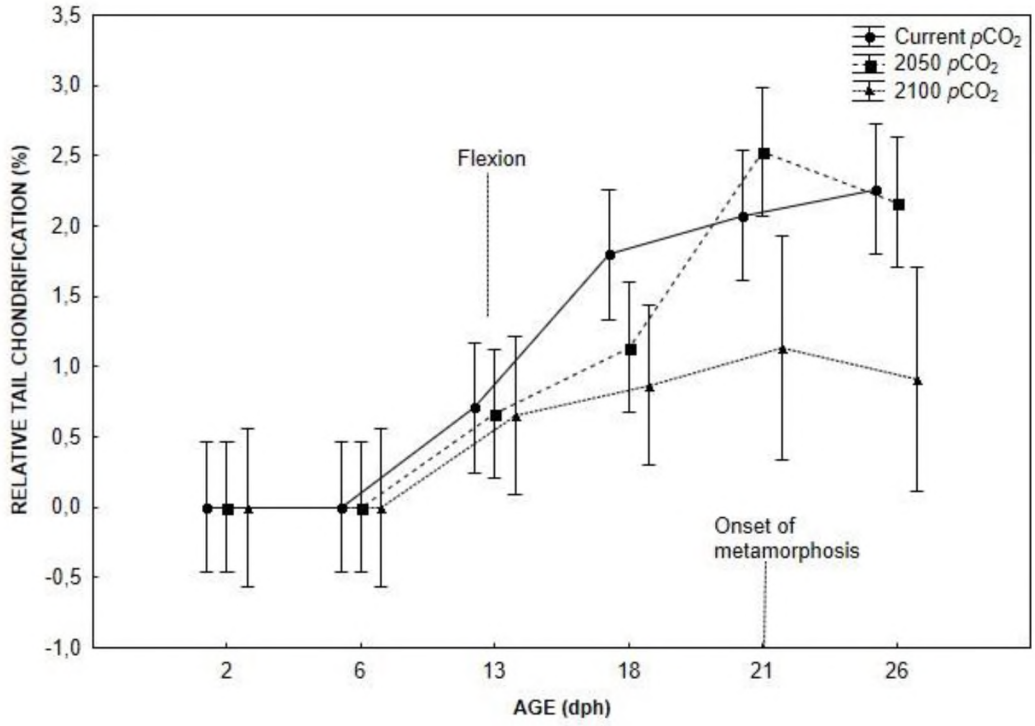


Figure 3.8. Changes in the relative proportion of cartilage (as a function of fish size) in the tail of *Argyrosomus japonicus* reared at current and predicted levels of pCO_2 for the years 2050 and 2100. Data points were collected on the same day; presented scattered for easier interpretation. Weighted means (± S.E.) of relative tail chondrification averaged across pCO_2 treatment replicates (n = 90).

3.3.3.2. OSSIFICATION

The first sign of ossification (red stain) occurred at eighteen hours post-hatch, in fish with a mean length of ~ 1.3–1.5 mm TL, with the formation of the cleithrum appearing as a fine hair-like structure behind the pectoral fin (Figure 3.3). Ossification progressed to the maxilla and premaxilla at 4 dph (~ 3.1–3.8 mm TL). Both the maxilla and pectoral base ossified without a cartilaginous precursor. The quantity of ossified structures increased rapidly from 4 dph. At 6 dph, dentary and branchials begin to ossify, and by 9 dph, ossification had spread to the hyosymplectic, palatoquadrate, mandibular and parasphenoid, which are used for jaw articulation. Ossification remained limited to the skull until 12 dph (~ 4 to 4.2 mm TL), with the first sign of non-cranial ossification occurring with the formation of the caudal fin rays at 10 dph. The onset of flexion occurred at 10 dph; however, the transition from notochord to a vertebral column was only visible from 13 dph (Figure 3.9). Flexion was generally completed by 15 dph in all treatments.

Figure 3.9. Non-acidic double-stained *A. japonicus* at 13 dph, showing flexion and the onset of caudal fin ray ossification.

Post-flexion (completed flexion of the tail) occurred at 16 dph and was characterised by greater trunk and tail ossification. Fin development was preceded by proximal formation of a cartilaginous fin base, followed by ossified (stained red) rays. Fin ossification occurred at the anal, dorsal, and pectoral fins during metamorphosis (21 dph), while the dermal plates (e.g. parietal and frontal) ossified from 26 dph in the head (Figure 3.10).

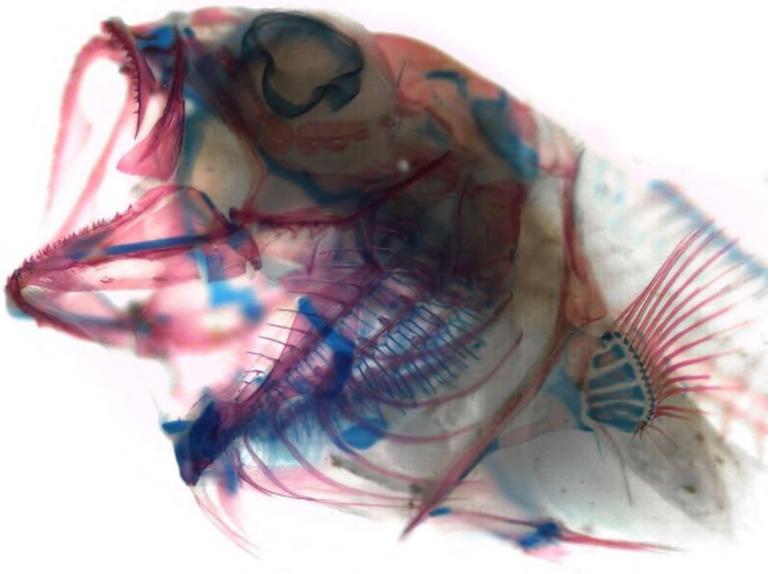


Figure 3.10. Non-acidic double-stained *A. japonicus* at 29 dph, showing the ossification of the dermal plates and the almost complete ossification of the head post-metamorphosis.

Head

Relative ossification of the head followed a similar pattern in all treatments (Tukey's, $P > 0.05$) until day 21 post-hatch (Figure 3.11). Relative ossification within the head was 7.6% and 20.2% higher in the current and 2050 pCO_2 treatments, respectively, compared with the 2100 pCO_2 treatment by 26 dph (Figure 3.11). There was a significant interaction between pCO_2 treatment and head ossification (number of red pixels in head / total pixels in skeleton × 100) (Factorial ANOVA: $F_{10, 28} = 13{,}66$, $P < 0.05$) (Figure 3.11). A Tukey's *post hoc* test indicated that relative ossification was significantly greater in fish subjected to the 2050 pCO_2 treatments and significantly retarded in the year 2100 pCO_2 treatments, compared to the current treatment (Figure 3.11).

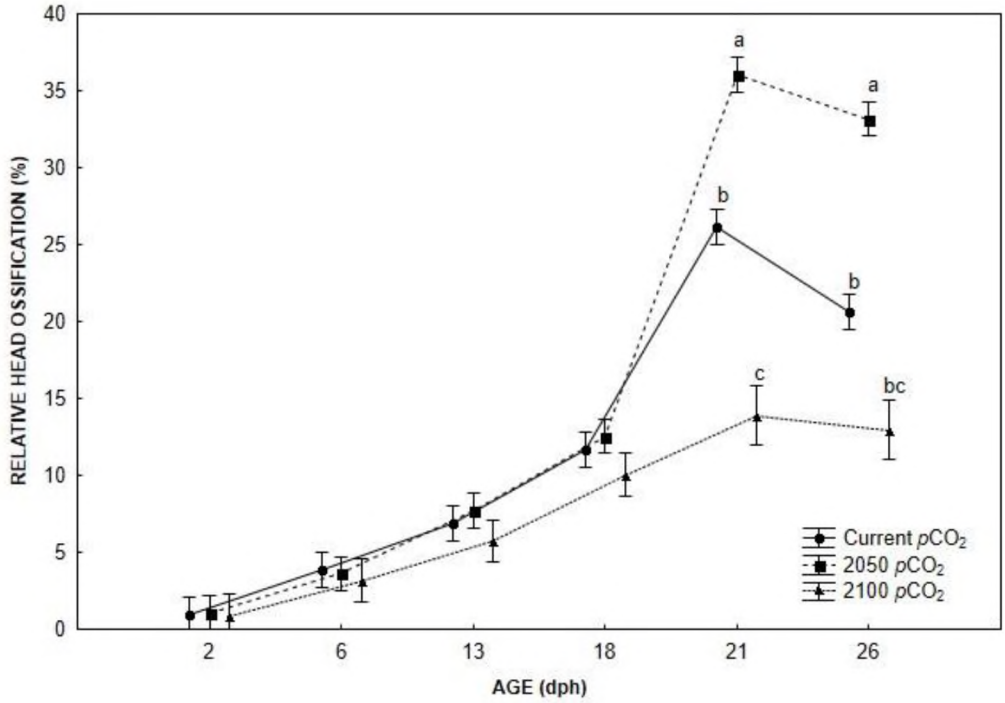


Figure 3.11. Changes in the relative ossification (as a function of fish size) in the head of *Argyrosomus japonicus* reared at current and predicted levels of pCO_2 for the years 2050 and 2100. Data points were collected on the same day; presented scattered for easier interpretation. Different letters signify differences between treatment means (Tukey's, $P < 0.05$).

Trunk

Relative ossification of the trunk followed a similar pattern in all treatments until 18 dph (Figure 3.12.) and was 6.2% and 12.8% higher in the current and 2050 pCO_2 treatment, respectively, compared with the 2100 pCO_2 treatment by day 21 (Figure 3.12). There was a significant interaction between the main effects pCO_2 treatment and time (dph) for mean

relative trunk ossification (Factorial ANOVA: $F_{10, 28} = 17.83$, $P < 0.05$) (Figure 3.12). A Tukey's *post hoc* test indicated that relative ossification was significantly greater in the 2050 pCO_2 treatment and significantly retarded in the 2100 pCO_2 treatment, compared with the current treatment from 21 dph (Tukey's, $P < 0.05$) (Figure 3.12.).

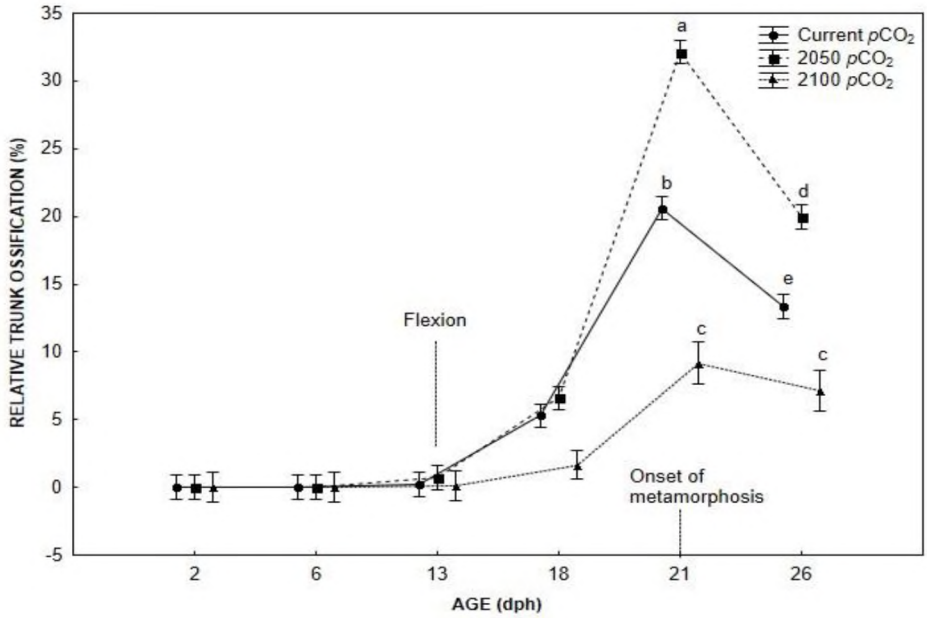


Figure 3.12. Changes in the relative proportion of bone (as a function of fish size) in the trunk of *Argyrosomus japonicus* reared at current and predicted levels of pCO_2 for the years 2050 and 2100. Data points were collected on the same day; presented scattered for easier interpretation. Different letters signify differences between treatment means (Tukey's, $P < 0.05$).

Tail

Relative ossification of the tail was similar for all treatments up until 13 dph (Figure 3.13), after which it slowed in the 2100 pCO_2 treatment. From 21 dph relative ossification of the tail was significantly retarded in the 2100 pCO_2 treatment compared to the 2050 pCO_2 treatment (Tukey's, $P < 0.05$), though neither differed significantly from the current treatment (Tukey's, $P > 0.05$) (Figure 3.13). By 26dph, the relative ossification in the tail was 4.5% and 11.6% lower in the current and 2100 pCO_2 treatments, respectively, compared to the 2050 pCO_2 treatment. A significant interaction was observed between the main effects of pCO_2 treatment and dph for mean relative tail ossification (Factorial ANOVA: $F_{10, 28} = 3.88$, $P < 0.05$) (Figure 3.13), with a Tukey's *post hoc* test indicating that relative tail ossification was significantly

different between the 2100 pCO_2 treatment and the 2050 pCO_2 treatment, but neither were different from the current treatment.

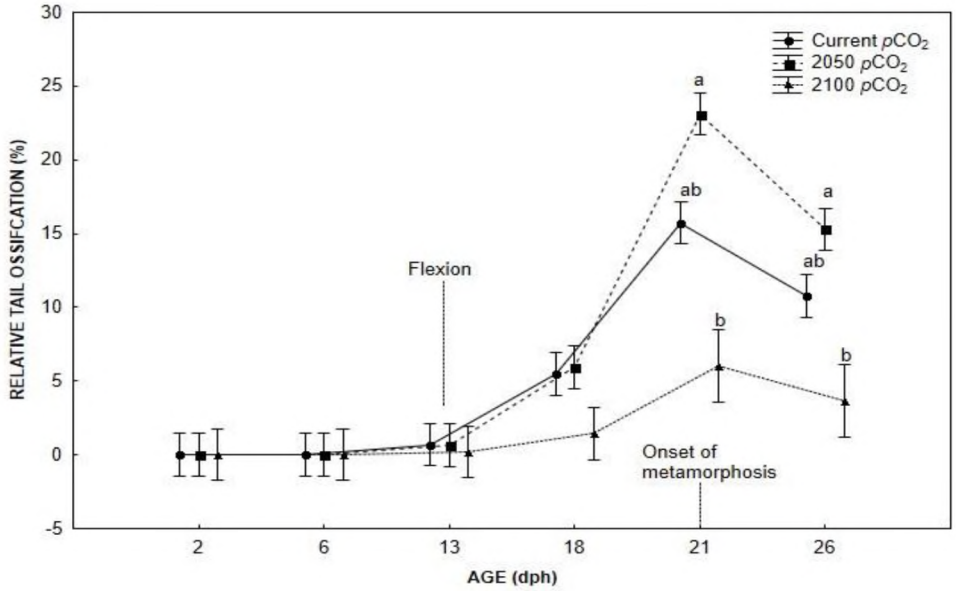


Figure 3.13. Changes in the relative proportion of bone (as a function of fish size) in the tail of *Argyrosomus japonicus* reared at current and predicted levels of pCO_2 for the years 2050 and 2100. Data points were collected on the same day; presented scattered for easier interpretation. Different letters signify differences between treatment means (Tukey's, $P < 0.05$).

3.4. DISCUSSION

High pCO_2 predicted by the end of the century adversely impacted on the skeletal development of *A. japonicus*, with larvae reared in this treatment having significantly lower relative average chondrification in the head and trunk compared to fish reared in the current and mid-century treatments. This vulnerability is further evident in terms of ossification, where the proportion of bone in the head, trunk and tail was 45.3% lower in the high pCO_2 treatment when compared to the current treatment at 26 dph (metamorphosis). These results suggest that levels of pCO_2 predicted by the end of this century may negatively impact *A. japonicus* skeletal development, which could have adverse consequences for growth, survival and recruitment success.

Pixel analysis found that the relative bone and cartilage development pattern remained homogenous up until 21 dph, after which there was a rapid increase in both the relative cartilage and bone. This period was also characterised by significant treatment differentiation, with reduced skeletal development evident in the high pCO_2 treatment. This is surprising, given that

the ion regulatory mechanisms of the gills and intestine were expected to be more developed at this stage, thereby ameliorating the impacts of acidification (Claiborne et al. 2002, Evans et al. 2005, Michaelidis et al. 2007). This finding challenges the notion that the skeletal effects of ocean acidification will be most evident during the earliest developmental stages and suggests that future experiments on skeletal development extend the study duration past the completion of metamorphosis. In the case of *A. japonicus*, this would be past 26 dph.

The exact mechanisms responsible for the observed differences in the skeletal development of fishes exposed to altered seawater chemistry remains unclear (Baumann et al. 2012, Fabry et al. 2008). The predominant argument in the literature suggests that the under-saturation of essential carbonate ions (such as Ca^{2+}) in a future ocean, coupled with underdeveloped ion regulation by larval fishes is likely to impair skeletogenesis (Feely et al. 2004, Orr et al. 2005, Fabry et al. 2008, Ishimatsu et al. 2008, Pimentel et al. 2014b). This is attributed to the high Ca^{2+} demand for the development and maintenance of the skeletal system, especially during early ontogenesis when ion regulation is thought to be underdeveloped (Munday et al. 2009, Munday et al. 2011, Pimentel et al. 2014b, Perry et al. 2015).

The onset of metamorphosis (21 dph) coincided with a significant increase in ossification throughout the skeleton, in addition to fin and supporting structure development. Here rapid increases in both cartilage and bone development were observed in larvae in the current and intermediate treatment, but not in the high pCO_2 treatment. There was also a decrease in growth during this phase in the high pCO_2 treatment (Chapter 2). As discussed in relation to impaired growth and development, it is likely that the poor skeletal development of *A. japonicus* after 21 dph resulted from a shift in energy allocation away from skeletal development to acid-base regulation in elevated pCO_2 conditions.

In contrast to the decreased ossification observed in the 2100 pCO_2 treatment, the relative ossification over all body parts was 47.8% higher in the 2050 pCO_2 treatment than in the current pCO_2 treatment. This has not been found in other studies, where skeletal development is either compromised (e.g. Perry et al. 2015, Pimentel et al. 2014b, Pimentel et al. 2016) or unaffected (e.g. Munday et al. 2011, Ben-Asher et al. 2013, Frommel et al. 2014) by elevated pCO_2. Chambers et al. (2014) found larger cranio-facial features of summer flounder, *Paralichthys dentatus* reared in elevated CO_2 (1860 ppm pCO_2) at metamorphosis. However, Chambers et al. (2014) did not standardise their skeletal elements to length. As such, larger cranio-facial

elements may be attributed to the larger flounder size observed in elevated pCO_2 at metamorphosis.

Increased metabolic rates may favour faster skeletal development in an ideal environment, such as in a laboratory-based study, where food is not limited and predation energy costs are reduced. However, elevated metabolic rate is a short-term adaptive strategy, which evolved to deal with short-term eco-physiological changes and thus is not advantageous in the long term (e.g. Wood et al. 2008, Rosa et al. 2014). Consequently, it is not surprising that high pCO_2 metabolic adaptations usually come at a cost in growth, development, skeletogenesis, and may ultimately reduce survival. These costs commonly include a reduction in protein synthesis (Hand 1991). For example, Wood et al. (2008) linked higher calcification rates in the ophiuroid brittle star, *Amphiura filiformis,* to an elevated metabolism when exposed to year 2100 pCO_2. However, this upregulation in calcification came at a cost of reduced muscle mass and impaired muscle function (Wood et al. 2008). Consequently, increases in relative ossification could result in indirect costs during development, thereby negatively affecting fishes reared in the high pCO_2 treatment in the long run. To test this hypothesis, it is recommended that the current study be repeated with the incorporation of food limited treatments and concomitant protein analyses to fully comprehend the impacts of elevated pCO_2.

Ossification appeared to be more affected by high pCO_2 than chondrification. The proportion of bone in the head, trunk and tail was 45.3% lower between the high pCO_2 treatment and the current pCO_2 treatment, while cartilage was only 2.6% lower by the end of the study. This may indicate that bone tissue development is more vulnerable to the effects of ocean acidification. There is currently no literature to support this finding as this is the first ocean acidification study to separately investigate cartilage and bone development in a fish. Cartilage generally consists of unmineralised cartilage and crystals of calcium-deficient hydroxyapatite ($Ca_9HPO_4(PO_4)5OH$), along with considerable amounts of amino acids, phosphoserine, and other biological compounds (Dorozhkin and Epple 2002, Witten et al. 2010). Furthermore, cartilage requires less Ca^{2+} for mineralisation than bone (Witten et al. 2010) and this may explain the observed differences. Interestingly, this could explain why the Chondrichtyes, possessing a cartilaginous endoskeleton, remained relatively unaffected by the Permian mass extinction event (Kriwet et al. 2008, Rosa et al. 2017), which was thought to have been driven by ocean acidification (Koot 2013). Unfortunately, our current knowledge on the cartilage development in Osteoichthyes remains limited. However, skeletal consistency (and composition) appears to range from cartilage-like connective tissue during early development,

to bone-like cartilage during metamorphosis. This is common in teleosts where osteogenesis occurs via endochondral ossification (where cartilage is replaced by bone) (Javidan and Schilling 2004). This would support the hypothesis that the ossification stage during metamorphosis is the stage most vulnerable to the impacts of ocean acidification.

Phosphate is generally the limiting mineral during fish skeletal development (Bodansky et al. 1931). Fish bones consist primarily of calcium-phosphate in the form of hydroxyapatite ($Ca_5(PO_4)3OH$), not calcium carbonate, like the shells of most marine invertebrates (Dorozhkin and Epple 2002, Munday et al. 2011). Munday et al. (2011) suggest that skeletal development in fishes is less likely to be impacted by ocean acidification as the bioavailability of calcium phosphate is less vulnerable than calcium carbonate. Although phosphate might not be directly impacted by ocean acidification, phosphate plays a major role in fish metabolism and may have indirect impacts on energy allocation (Halver and Hardy 2002). Phosphate availability may be reduced in high pCO_2 due to heightened metabolic phosphate demand for acid-base regulation, thereby hindering development of the calcium phosphate skeleton. The bioavailability of minerals may also be indirectly affected by ocean acidification through the influence of pH on biochemical assimilation. Phosphate, essential for growth, skeletal development, and metabolism, is predominantly absorbed in the intestinal tract from the diet by means of enzymatic digestion (Halver and Hardy 2002). However, the activity of this enzyme (alkaline phosphatase), responsible for phosphate absorption, has been shown to decrease rapidly with decreasing pH (Yamada and Suzumura 2010, Pimentel et al. 2015). Consequently, fish phosphate assimilation may be negatively affected by ocean acidification, which may explain the adverse consequences observed in the growth and the skeletal development in the high pCO_2 treatment in this study. Interestingly, even though phosphate is present in bone and cartilage, there is a relatively higher phosphate demand during bone formation than there is in cartilage formation (Bodansky et al. 1931).

To date, the few studies that have addressed the effects of ocean acidification on fish skeletogenesis, have focused either on deformities (Ben-Asher et al. 2013, Pimentel et al. 2014b, Frommel et al. 2014, Perry et al. 2015, Chambers et al. 2014), or morphometrics (Munday et al. 2011) as indicators of skeletogenesis. Deformities have been detected using either digital X-ray inspection (e.g. Perry et al. 2015), or by staining and clearing (e.g. Pimentel et al. 2014b). This study found that X-ray imagery (Inspex 20i, 40 – 90 kV) was unclear during fish early skeletogenesis and provided far less detail than stained specimens. Although it is possible that more advanced X-ray technology may provide better resolution, the results from

this study indicate that non-acidic staining may be the optimal method for detecting and quantifying deformities.

Although ocean acidification had a negative effect on the skeletogenesis of *A. japonicus,* there was no significant treatment effect on deformities. Consequently, quantifying the response of larval fish to elevated pCO_2 may not be best assessed by the occurrence of deformities method. Current literature on skeletal deformities resulting from elevated pCO_2 also give conflicting results, with half the studies concluding significant increases in deformities (Chambers et al. 2014, Pimentel et al. 2014b, Perry et al. 2015), and the other half concluding no effect (Munday et al. 2011, Ben-Asher et al. 2013, Frommel et al. 2014). For example, Pimentel et al. (2014a) found significantly more deformities in sole larvae (*Solea senegalensis*) exposed to elevated pCO_2, whereas Frommel et al. (2014) found no evidence for skeletal deformities in their study on Atlantic herring (*Clupea harengus*). Therefore, deformities may be an effective indicator in some species, such as *S. senegalensis* that are known for their relatively high incidence of skeletal deformities during ontogenesis (Boglino et al. 2012). *Argyrosomus japonicus,* however, have a relatively low occurrence of deformities (L. Grant, Pure Ocean Mariculture, pers. comm. 2016) and, with only four detected in this study, it is likely that deformities will be a poor indicator of the impacts of ocean acidification on this species. Deformities are generally associated with genetics and occasionally malnutrition, with the link between deformities and ecophysiology rarely made (Lall and Lewis-McCrea 2007). Consequently, future studies examining the potential effects of ocean acidification should take a multi-method approach rather than solely examining skeletal deformities as an indicator of skeletal development.

This study found no evidence to suggest that pCO_2 treatment may influence the meristic counts (Table 3.4). The number of anal, dorsal or caudal fin rays have been suggested as being the most susceptible to environmental change (Gisbert et al. 2014, Torres-Núñez et al. 2014). Meristic variation is a common indicator used to investigate osteological variation in different environments (Lindsey 1988, Boglione et al. 2001). Meristic osteology may be influenced by developmental plasticity in response to environmental influences; however, genes are known to play a dominant role (Forsman and Berggren 2016). Furthermore, given the findings, meristic count analysis may not have been the best indicator for testing the impacts of ocean acidification on the skeletal development of *A. japonicus.* These findings suggest that ocean acidification related effects are not always detectable at a phenotypic level and suggest that future studies should incorporate additional indicators.

The results from this study suggest that staining and clearing represents a powerful indicator for identifying deformities during fish early development. While conventional staining methods used in previous studies (Chambers et al. 2014, Frommel et al. 2014, Pimentel et al. 2014b) were suitable for detecting deformities and for morphometric analyses, they lacked the clarity required for pixel analysis (Figure 3.14). Furthermore, conventional staining techniques make use of a highly acidic alcian blue stain (pH < 3) and this may result in the deterioration of newly formed calcified matrices of the fragile larval fish skeletons tested and could lead to ambiguous deductions (Gavaia et al. 2000, Walker and Kimmel 2007). It is suggested that future studies examining the impacts of ocean acidification on the skeletal development of fishes should consider the use of the non-acidic stain originally proposed by Scott and Dorling (1965) with adaptations to improve clarity (Figure 3.14). When used in combination with appropriate imaging, this method can provide the same, if not better, clarity than CT scanning, at reduced costs.

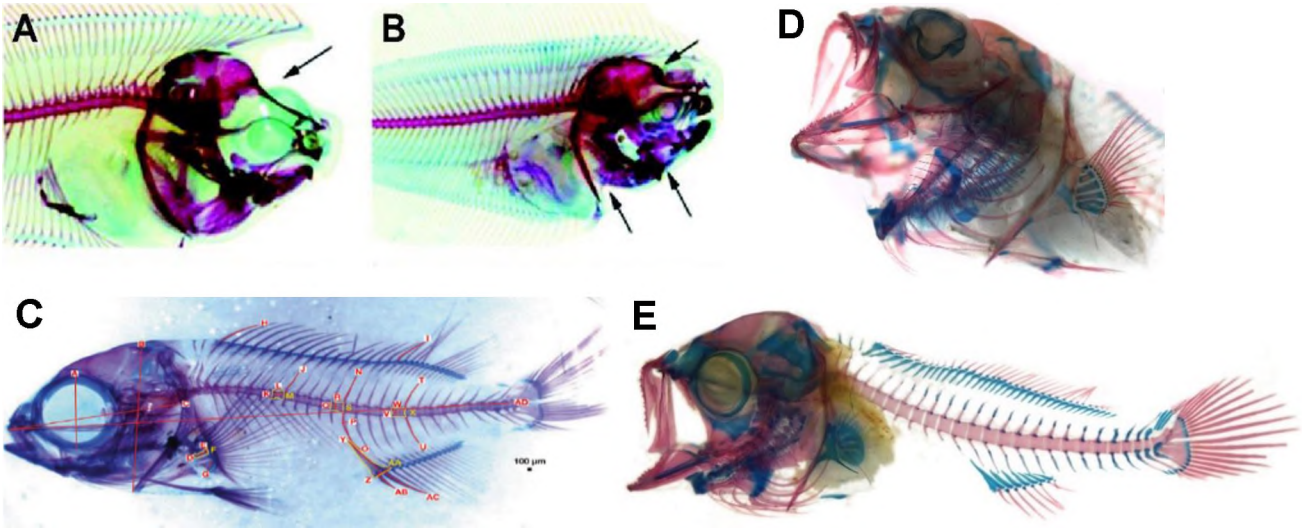


Figure 3.14. Comparing conventional acid-stained fish A, B (Pimentel et al. 2014) and C (Munday et al. 2011) with the non-acidic double stain adapted for this study (D and E).

The efficacy of the non-acidic stain in distinguishing between bone and cartilage (Figure 3.14 DE) presented an opportunity to develop a higher resolution indicator for skeletal development in the larval skeleton. Contrary to literature quantifying fish skeletal development in elevated pCO_2 based on either visual inspection and classification of skeletal abnormalities (Pimentel et al. 2014b), or morphometric measurements of skeletal structures (Munday et al. 2011), this study used a pixel-counting method proposed by Darias et al. (2010) to assess relative proportion of bone and cartilage during early development. Although this method was originally developed to quantify ossification in small European sea bass (*Dicentrarchus*

labrax) larvae reared in different environmental or nutritional conditions, it provides a robust method for use in ocean acidification research.

In conclusion, the findings of this study support the growing body of evidence that elevated pCO_2 will negatively affect fish skeletal development (e.g. Chambers et al. 2014, Pimentel et al. 2014b, Perry et al. 2015). In the case of this marine spawning estuarine-dependent species, reduced bone and cartilage development will have adverse consequences on development, fitness, and performance and will potentially influence behaviour, feeding, success, predator avoidance, and ultimately, recruitment and population sustainability.

CHAPTER 4

4. GENERAL DISCUSSION

Ever increasing anthropogenic CO_2 absorption at the ocean surface poses a critical threat to marine organisms globally (Kleypas et al. 2005, Fabry et al. 2008, Bignami et al. 2013). Although literature suggesting adverse consequences for externally calcifying marine organisms is expanding rapidly, the response of fishes to near-future predicted CO_2 remains unclear (Baumann et al. 2012, Frommel et al. 2012). However, a growing body of research recognises the vulnerability of fishes during their early life stages to the CO_2 levels predicted by the end of this century (e.g. Baumann et al. 2012, Chambers et al. 2014, Pimentel et al. 2014b, Pimentel et al. 2016).

To date, research on the survival, development, and growth response of fishes to increased pCO_2 has been limited to a few species and has yielded conflicting results. Furthermore, few of these studies have considered the interrelationships between patterns of survival, growth, skeletal development, and their underlying metabolic drivers. The paucity in fish ocean acidification research is particularly evident when one examines research on the effects of elevated pCO_2 on early skeletal development, which limits our understanding of how fish early stages are likely to respond to ocean acidification. This study addressed a critical gap in the skeletal development research (Munday et al. 2011, Pimentel et al. 2014b, Perry et al. 2015) and proposes a more detailed method of analysing the vulnerability of fish skeletogenesis to elevated pCO_2. This study adopted a novel, multi-method approach, and suggests that embryogenesis, hatching success, development, growth and survival, as well as skeletogenesis of *A. japonicus* may be sensitive to pCO_2 levels predicted by the end of this century.

Approaches to assessing skeletal health in ocean acidification studies

Deformities have been used as the predominant indicator of skeletal "health" in ocean acidification research, with mixed results (e.g. Ben-Asher et al. 2013, Pimentel et al. 2014b, Perry et al. 2015). Although this may be indicative of inter-species variability in responses to elevated pCO_2, results from this study suggest that it may reflect the susceptibility of the early life stages of different species to deformities. For example, cobia (*Rachycentron canadum*) are highly susceptible to deformities in captive environments (Mclean et al 2008), compared with *A. japonicus*, which seldom display deformities in aquaculture environments (Ballagh et al.

2011) as is evident from the few deformities observed in elevated pCO_2 during this study (Chapter 3). This suggests that deformities may not be a robust indicator of the effect of elevated pCO_2 on fish skeletal development. Furthermore, this indicator was not effective in detecting more subtle or gradual changes in larval skeletal development. Similarly, the morphometric approach to analysing skeletal development in elevated pCO_2 studies (e.g. Munday et al. 2011) is limited to quantifying structures that are easy to measure and, as such, excludes important skeletal elements in the head. Consequently, this method may not be the most effective at detecting subtle changes in skeletal development. Finally, these measurements are largely correlated to size, and a lack of size standardization may bias the results.

The novel pixel-counting method introduced by this study allowed for quantification of subtle changes in larval bone and cartilage development and may be particularly useful for future ocean acidification skeletal research on fishes. Interestingly, the pixel method would not have been possible without the development of an acid-free double-staining and clearing solution. This stain provided superior clarity without the potential bias of bone degeneration, which may occur with the use of traditional acid staining, a technique used in most fish ocean acidification studies (e.g. Munday et al. 2011, Chambers et al. 2014, Frommel et al. 2014, Pimentel et al. 2014b). The combination of these methods proved a powerful indicator of subtle changes in early skeletal development where deformities and morphometric measurements did not provide sufficient resolution to quantify potential differences. Furthermore, this method had the unique advantage of quantifying both relative bone and cartilage development at key stages of ontogenesis providing a more accurate assessment of the vulnerability of individual species to ocean acidification.

Vulnerability of A. japonicus to ocean acidification

One of the most noticeable findings of this study was the lack of differences for all indicators between treatments during the early part of the experiment, up until 21 dph. These findings contradict the early vulnerability to elevated pCO_2 suggested by Baumann et al. (2012) who found reduced early growth and survival in estuarine silverside (*Menidia beryllina*), as well as adverse consequences of short-term elevated pCO_2 on the metabolism and swimming of dolphinfish (*Coryphaena hippurus*) (Pimentel et al. 2014b). Results from this study suggest that pre-metamorphosis *A. japonicus* are more tolerant of pCO_2 induced acidification (~ 910 µatm) predicted by the end of the century than those at metamorphosis, where they responded to the effects of both the mid-century (2050) and end-of-century (2100) pCO_2 levels.

Growth, development and skeletogenesis were significantly lower in the high pCO_2 treatment and significantly faster in mid-century treatment than in the control.

By 26 dph, *A. japonicus* individuals reared in high pCO_2 (2100) were, on average, 47.2% smaller than the control treatment, and the relative proportion of bone in the body was 45.3% lower in the high pCO_2 treatment than in the control. Of most concern, however, was the lack of *A. japonicus* survivors post-metamorphosis (29 dph) in the high pCO_2 treatment. The lack of survivors post-metamorphosis may reflect the inverse relationship between early growth and survival (e.g. Rossi et al. 2015). Rossi et al. (2015) found a similar correlation between growth and survival in barramundi (*Lates calcarifer*) reared in elevated pCO_2 (1675 µatm), where slower growth reduced the chances of successful settlement and ultimately increased mortalities. This is further supported by Franke and Clemmensen (2011), who suggested that slower growth resulted in decreased feeding success and increased predation mortality of Atlantic herring (*Clupea harengus L*). These findings are in accordance with several other studies that suggest reduced growth and survival of fishes in near future pCO_2 levels (e.g. Baumann et al. 2012, Chambers et al. 2014, Miller et al. 2012). Although the exact driver for reduced survival in high pCO_2 remains unclear, the results of this study suggest that retarded development may have ultimately led to decreased survival.

The skeletal development trends observed in elevated pCO_2 were particularly interesting, with reduced skeletal development already evident by 18 dph (Chapter 3, Figures 3.11, 3.12, 3.13) followed by reductions in growth evident by 26 dph (Chapter 2, Figure 2.7). This suggests that the observed reductions in skeletal development preceded reductions in growth. It is axiomatic that somatic growth spurts can only occur after the development of the supporting skeleton. Consequently, fishes from the high pCO_2 treatment would have to develop the supporting skeleton before growth and muscle development could take place. Retarded skeletogenesis would ultimately prolong the larval development and particularly, in this case, the period of metamorphosis for fishes in high pCO_2. Similarly, in a study on barramundi, *Lates calcarifer,* Rossi et al. (2015) found that prolonged larval duration resulted in decreased survival. Because the effects of elevated pCO_2 are evident in skeletal development before they are evident in larval fish growth, it is possible that retarded skeletal development could ultimately be a driver of reduced growth and survival.

The vulnerability observed at metamorphosis is of interest. Metamorphosis is a transitional stage where fish larvae undergo abrupt head and fin development (Figure 4.1), including the

ossification of the anal, dorsal, and pectoral fins, and the dermal plates (parietal, frontal, etc.) in the head (Chapter 3, Ossification). The development of the fins would most likely support manoeuvrability, while the dermal plate development would result in a larger buccal cavity volume, which is necessary for improved suction feeding (Chambers et al. 2014). These developments should increase feeding success, thereby providing the dietary energy required for this developmentally expensive stage (Rossi et al. 2015). At metamorphosis, this study observed reduced relative ossification in the head, trunk, and fins in the high pCO_2 treatment compared to the current treatment (Figure 4.1). Impaired head skeletal development may result in an underdeveloped jaw and a comparatively smaller buccal cavity volume. This may ultimately decrease the effectiveness of the suction feeding mechanism employed by *A. japonicus*. Chambers et al. (2014) similarly observed reduced cranio-facial features and delayed mandible development at metamorphosis among summer flounder, *Paralichthys dentatus* reared in elevated CO_2 (1860 ppm pCO_2) when compared to ambient-CO_2 reared fish. Furthermore, the reduced fin development observed in the high pCO_2 at metamorphosis may have adverse consequences for larvae locomotion/swimming and concomitant feeding success (e.g. Pimentel et al. 2014a). This is supported by Rossi et al. (2015), who found that barramundi (*Lates calcarifer*) reared in elevated pCO_2 (1675 µatm) exhibited reduced swimming speeds at metamorphosis. Similarly, Chambers et al. (2014) observed reduced growth and survival of summer flounder, *Paralichthys dentatus,* post-metamorphosis in high pCO_2. Consequently, reduced feeding success, due to either slower swimming speeds or smaller buccal cavity volumes, would ultimately reduce treatment competitiveness and decrease the dietary energy for growth and development, thereby adversely impacting on the individual's hierarchy in the food web and chances of survival (Pimentel et al. 2014a).

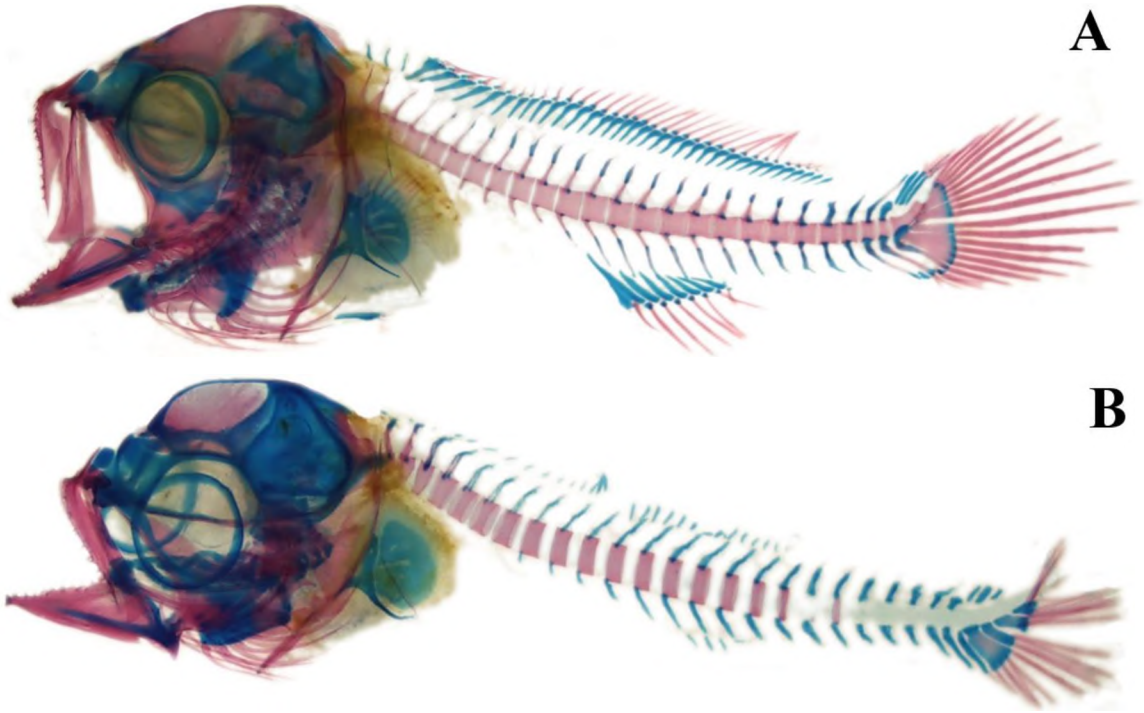


Figure 4.1. Comparing the difference in skeletal development of *Argyrosomus japonicus* reared in current CO_2 (A) and CO_2 expected by the end of the 21st century (B) at 26 days post-hatch.

While various studies have addressed the effect of elevated pCO_2 on fish early development, the mechanism driving the observed changes in fish growth, development and skeletogenesis remains unclear (Baumann et al. 2012, Chambers et al. 2014). Initial fish ocean acidification research suggests that seawater acidified by elevated pCO_2 rather than HCl, exerted more severe effects on egg and larval fish survival (e.g. Ishimatsu et al. 2004, Kikkawa et al. 2004). This led to the assumption that negative effects observed on the early stages of fishes may stem from reduced calcium carbonate ($CaCO_3$) availability in future acidified conditions, rather than reduced pH alone (Orr et al. 2005, Ishimatsu et al. 2008, Findlay et al. 2011). Consequently, calcifying marine organisms are considered the most vulnerable to ocean acidification, based on the assumption that calcification will decrease as a result of reduced carbonate ion concentrations (Findlay et al. 2011). Reduced $CaCO_3$ results from rapidly increasing hydrogen ion concentrations, which bind carbonate to form bicarbonate (HCO_3^-), thereby reducing the carbonate pool for $CaCO_3$, and its polymorphs, calcite and aragonite (Fabry et al. 2008, Munday et al. 2008). Reduced $CaCO_3$ saturation states have been suggested to affect the calcification of marine organisms (Gazeau et al. 2007, Fabry et al. 2008, Wood et al. 2008).

Here we show that reduced $CaCO_3$ saturation states may not be directly responsible for retarded skeletogenesis, growth, development, and reduced survival of fishes. Calcium carbonate is readily available in the aquatic environment (Gilbert 2000) and early stages of fishes are capable of accumulating calcium from the aquatic environment even when under-saturated (Gilbert 2000, Pörtner et al. 2004). Wood et al. (2003) suggest that hypercapnia inhibited the net uptake of calcium (Ca^{2+}) ions and stimulated Ca^{2+} efflux, reducing Ca^{2+} availability for skeletogenesis among freshwater stingrays (*Potamotrygon* spp.); consequently, it is possible that increasing Ca^{+} dissolution may induce a steeper assimilation gradient, and concomitant higher calcium assimilation cost in marine fishes (Langdon et al. 2003, Wood et al. 2008). Calcium is primarily assimilated through chloride cells at the cost of ATP^{+} (Laurent and Perry 1991). In this case, skeletal development is not impacted by limited calcium ions, but instead by the elevated energetic demand for calcium carbonate assimilation in hypercapnic environments. The increased energetic cost for Ca^{2+} assimilation, in addition to elevated acid-base regulation costs, may limit the energy available for skeletal development and growth at times of high energy demand, as was observed at metamorphosis. Consequently, while decreased calcium availability in the future ocean is unlikely to impact directly on fish skeletogenesis, growth and survival, increased assimilation costs might add to the metabolic stressors in acidified conditions, in addition to acid-base regulation costs.

An alternative explanation for the reduction in growth and skeletogenesis observed in the high pCO_2 treatment may relate to the increased acid-base regulation cost (as discussed in Chapter 2 and 3). The findings of this study, combined with the results of the parallel metabolic study (Edworthy 2017), suggest that the increased energy used for acid-base regulation in high pCO_2 limits the energy available for growth and skeletal development. However, the effects of this limited energy become evident only at times of high energy demand, i.e. metamorphosis (Figure 4.2). This suggests that the increase in acid-base regulation, which is required in heightened pCO_2 will come with an increased metabolic cost. In most cases, this cost includes a shift in energy away from physiological processes, such as growth (e.g. Baumann et al. 2012), muscle development (e.g. Wood et al. 2008) or digestion (Heuer and Grosell 2016) towards internal environment regulation (Pörtner 2010). For example, Heuer and Grosell (2016) found that Gulf toadfish (*Opsanus beta*) are capable of upregulating metabolism in heightened pCO_2 at a cost of ATP^{+} which is expected to result in energy trade-offs from other essential physiological processes. This trade-off in energy away from development to acid-base

regulation, is supported by Heuer and Grosell (2016) who suggest similar energy trade-offs in elevated pCO_2.

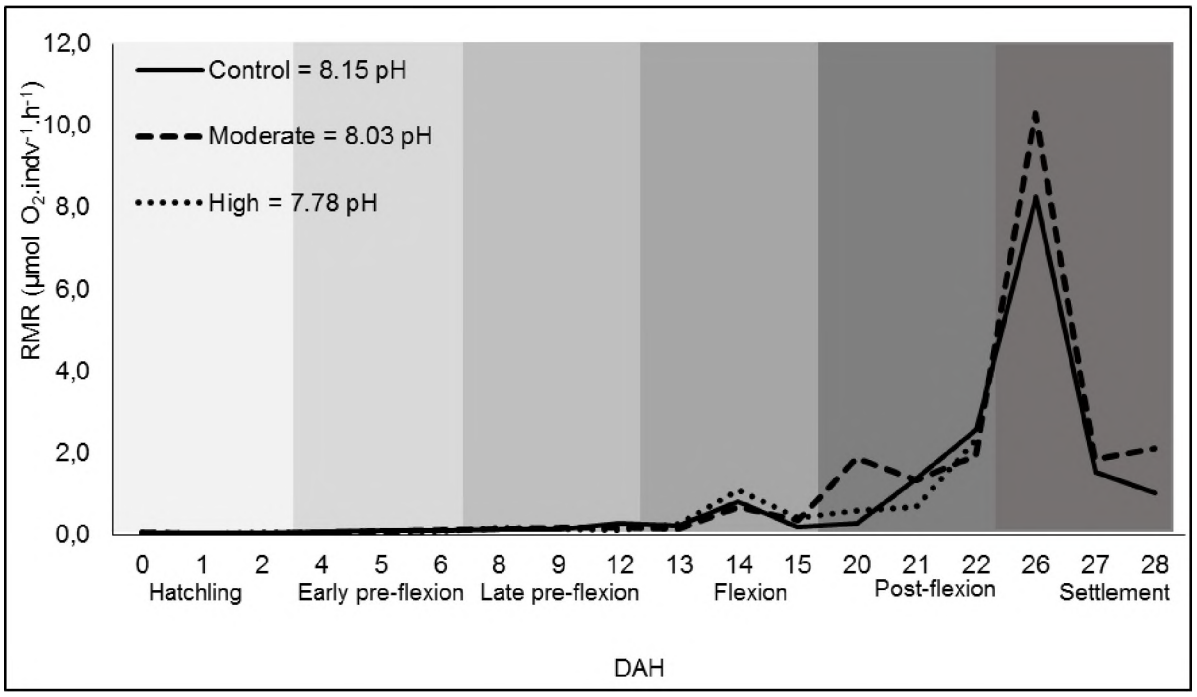


Figure 4.2. The mean heightened routine metabolic rate at metamorphosis (21 to 26 dph) for *A. japonicus* reared in current (control), year 2050 (intermediate) and high (year 2100) pCO_2 treatment up to 29 days post-hatch (Edworthy 2017).

Fishes are known to have well-developed acid-base regulation systems (Pörtner et al. 2004), to such a degree that fishes are considered the most unlikely of all marine organisms to be affected by ocean acidification (Fabry et al. 2008). This acid-base regulation is primarily attributed to the gill, considered by some studies to have evolved primarily for acid-base regulation (Baker et al. 2015). This may explain the timing of the reduction in metabolic scope (Edworthy 2017), which coincided with metamorphosis and the onset of gill development from 21 dph (Figure 4.3). Consequently, reduced growth and skeletogenesis may be a consequence of the energy required for acid-base regulation, which is thought to become active along with the formation of gill filaments and secondary lamellae (Frommel et al. 2012). A shift in energy allocation away from growth and ossification towards acid-base regulation with the onset of fully developed gills may account for the reduced development observed in the high pCO_2 treatment. Further research is needed to understand the link between gill development, growth and skeletogenesis in high pCO_2.

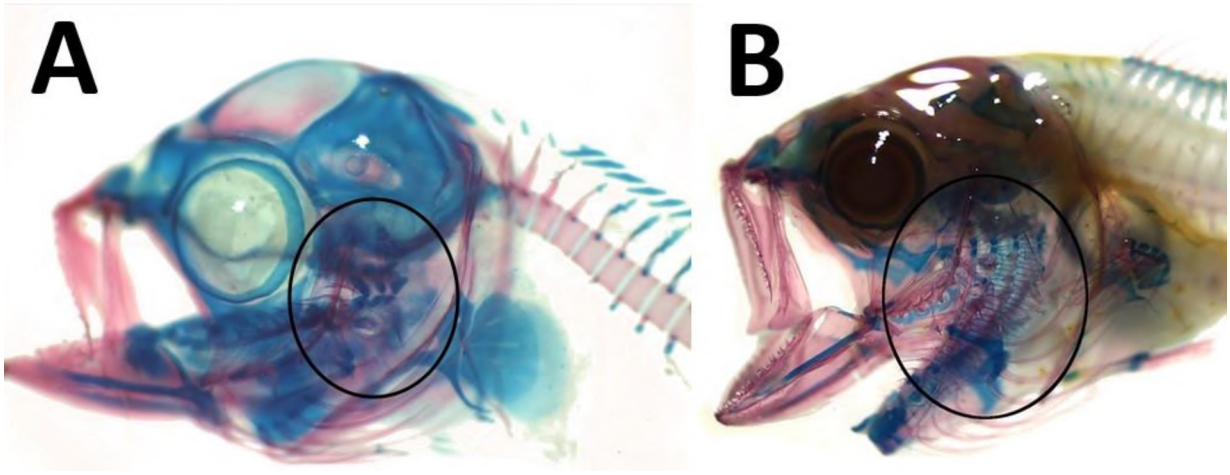


Figure 4.3. Gill skeletal structure development in non-acidic double-stained *A. japonicus* larvae at metamorphosis. A. Specimen at 21 days post-hatch, gill arches not fully developed and branchial rays absent. B. Specimen on 26 days post-hatch, four developed gill arches and branchial rays present.

Few studies have addressed the trade-off between the metabolic cost of acid-base regulation and early development in fishes subjected to high pCO_2 levels. However, Baumann et al. (2012) suggest that poor growth and survival among estuarine silverside (*Menidia beryllina*) in elevated pCO_2 (~ 1000 µatm) may be related to underdeveloped acid-base regulation during early development and, even if these organisms are capable of elevating acid-base regulation, that this will lead to higher metabolic costs. Rosa et al. (2014) attributed the reduced survival of bamboo sharks, *Chiloscyllium punctatum,* in elevated pCO_2 conditions to compromised acid-base regulation and a depressed metabolic scope. Interestingly, the results of the concurrent metabolic study on *A. japonicus* supports this theory about changes in metabolic scope. Edworthy (2017) found no differences in the metabolic scope of *A. japonicus* between 0 and 21 dph. However, the metabolic scope of individuals in the high pCO_2 treatment was significantly depressed from 21 dph (Edworthy 2017), supporting the conjecture that poor growth and skeletogenesis in elevated CO_2 may be correlated to the metabolism.

An alternative explanation is proposed by Chambers et al. (2014), who attribute reduced skeletal development in the head, in high pCO_2, to a high vulnerability during metamorphosis and the subsequent influence of elevated pCO_2 on the thyroid hormone. However, no further explanation is given. Thyroid hormone (TH) plays a critical role in the regulation of osmatic and metabolic balances (Peter 2013). Fishes in stressful conditions stimulate the thyroid to increase TH production, which ultimately increases fish metabolism (Peter 2013).

Consequently, increased TH production may result in faster skeletal development and growth. However, a study by Boeuf et al. (1989) has shown that, should TH be indirectly impacted on by an environmental stressor, such as elevated pCO_2, the role of TH in ionic and osmotic regulation is impaired, which would ultimately reduce metabolic scope and may also explain the observed reductions in growth and development. This could explain the increased growth, development and skeletal development in the year 2050 pCO_2 treatment.

Individuals in the intermediate pCO_2 treatment had the fastest growth, development and skeletogenesis. This is not intuitive as one would expect that the cost of acid-base regulation would reduce the energy that can be allocated to growth, development and skeletogenesis. The combined findings of increased metabolic rate in high pCO_2 and the faster development in the medium pCO_2 treatment suggest that fishes may upregulate metabolism in high pCO_2 to meet the heightened energy demand incurred by increased acid-base regulation costs. This conjecture is supported by Wood et al. (2008) who suggest that organisms can increase their metabolic rate to deal with heightened pCO_2; however, prolonged exposure to these unfavourable conditions will ultimately result in adverse costs or energy expenditure trade-offs (Wood et al. 2008, Heuer and Grosell 2016). These energy trade-offs may eventually lead to reduced performance, lower feeding success and, eventually, impaired fitness and survival (Wood et al. 2008, Heuer and Grosell 2016). This heightened metabolic state could result in more energy available for skeletogenesis and growth, which should ultimately favour these organisms and support increased survival, as evident. However, this increase in growth and skeletogenesis may not necessarily be advantageous. Several studies have observed adverse impacts of elevated growth or development in elevated pCO_2 (e.g. Wood et al. 2008, Chambers et al. 2014, Rossi et al. 2015). For example, Chambers et al. (2014) concluded that summer flounder (*Paralichthys dentatus*) reared in elevated pCO_2 (1860 µatm) grew faster and initiated metamorphosis earlier, but with fewer energy reserves, they were subject to 50% higher mortality. Wood et al. (2008) found that exposing the ophiuroid brittle star, *Amphiura filiformis,* to end-of-century CO_2 levels for 40 days resulted in significantly increased calcification and increased metabolism, but that this came at a notable cost to muscle development.

The contrasting results between the intermediate and high pCO_2 treatments in this study suggest that *A. japonicus* have well-defined upper and lower pCO_2 limits, or pejus limits. Pejus limits refer to a point at which physiological performance ceases to be optimal and where individuals can no longer perform optimally without adverse energy trade-offs (Pörtner 2010, Heuer and

Grosell 2016). Future studies should aim to identify the pejus pCO_2 limits of species of interest to better predict their response to hypercapnia. In the case of *A. japonicus* this would entail a larger number of carbon dioxide treatments between the intermediate and high pCO_2 chosen for this study, to clearly define the pejus pCO_2 levels for the species.

The principal finding of this study may be that the estuarine-dependant *A. japonicus* could be sensitive to pCO_2 levels predicted by the end of the century. Whether this vulnerability is restricted to this species, life stage, or species with similar life history characteristics remains unclear. Rossi et al. (2015) propose that the vulnerability of fishes to ocean acidification may be species specific. Similarly, the impacts of ocean acidification are likely to be life-stage dependant, with the early life stages being vulnerable to ocean acidification (Baumann et al. 2012, Chambers et al. 2014, Rossi et al. 2015) and adults less so (Ishimatsu et al. 2008). Results from this study indicate vulnerability during energetically demanding developmental stages, i.e. metamorphosis. This life-stage dependant vulnerability is also suggested for some invertebrates (e.g. Kurihara 2008).

The life stage at which *A. japonicus* was most impacted by elevated pCO_2 was metamorphosis, which coincides with recruitment into estuaries. Recruitment into estuaries in *A. japonicus* occurs from approximately 20 mm TL (Griffiths 1996) and, as such, reduced survival, growth, and skeletogenesis at this stage may disrupt recruitment to juvenile habitats. Recruitment to appropriate settlement habitat is a crucial stage in the life cycle of many fish species (Rossi et al. 2015). Rossi et al. (2015) found that exposure of the catadromous species barramundi (*Lates calcarifer*) to elevated pCO_2 may affect the attraction of settlement stage individuals to sounds from their settlement habitat. Similarly, exposure to elevated pCO_2 has been found to disrupt the olfactory mechanism by which marine fish (orange clownfish, *Amphiprion percula*) locate suitable habitat (Munday et al. 2009). As olfaction is thought to play a major role in the recruitment process of estuary-dependent species into estuaries (Munday et al. 2009), this type of disruption may have major consequences for fishes with this life history strategy. This area is therefore worthy of further research attention. Furthermore, although it was initially assumed that species with an estuarine association may have an intrinsic tolerance to elevated pCO_2, studies have shown that some species, such as the estuarine-resident inland silverside (*Menidia berryllina*), and the estuarine-associated marine species, the summer flounder (*Paralichthys dentatus*), may be vulnerable to the effects of increased pCO_2 during their early life (Baumann et al. 2012, Chambers et al. 2014).

Even though the results of this study indicate that ocean acidification may have adverse implications for the future of *A. japonicus* populations, the scope for adaptation of species to rapidly changing ocean chemistry is unknown (Fabry et al. 2008, Munday et al. 2008, Dixson et al. 2010). Although this study did not attempt to estimate adaptability of *A. japonicus* to elevated pCO_2, the lack of survivors in the high pCO_2 treatment, reduced growth, impaired skeletal development and relatively long generation times (~ 9 years) (Bernatzeder et al. 2010) suggest limited opportunity for adaption. Frommel et al. (2012) suggest that the long generation times of Atlantic cod (*Gadus morhua*), combined with the added anthropogenic stressors of fishing, pollution, and increasing temperature reduce the chances of genetic adaptation for this species. Research on smaller-bodied, fast-growing species, such as the Atlantic silverside (*Menidia menidia*) suggests that parental exposure to elevated pCO_2 may mediate the impacts on larval fish (Murray et al. 2014). Dixson et al. (2010) similarly suggest a limited capacity for clownfish *Amphiprion percula* to adapt to elevated pCO_2 (~ 1000 uatm). However, even if fishes are capable of adaption to end-of-century CO_2 levels they would still have to endure elevated metabolic demand or subsequent energetic trade-offs away from important processes towards acid-base regulation (Baumann et al. 2012, Heuer and Grosell 2016). This energy trade-off will have consequences for fish fitness in the future ocean, regardless of adaptability. Further research is required to comprehend how these changes in trade-offs in metabolic energy may affect transgenerational acclimation to elevated pCO_2.

Limitations and considerations for future research

Although the findings of this study suggest early vulnerability, there are certain weaknesses in the study that could not be avoided. This study was a lab-based study without the additional stressors expected in the marine environment, such as reduced food and predation. Food availability has been shown to play a pivotal role in an organism's ability to tolerate elevated pCO_2 (Thomsen et al. 2013). However, many prey species may also be negatively impacted by ocean acidification (Orr et al. 2005, Doney et al. 2009, Makarow et al. 2009). Furthermore, increased susceptibility to predators in elevated pCO_2 may increase mortality. For example, Dixson et al. (2010) showed the importance of testing the effect of elevated pCO_2 on predator avoidance, where orange clownfish *Amphiprion percula* larvae exposed to elevated pCO_2 were attracted to predators (rather than showing an avoidance response).

This study also tested the effect of elevated pCO_2 in isolation from other climate-related stressors, including ocean warming. Pimentel et al. (2014) found that flatfish (*Solea senegalensis*) larvae exposed to both hypercapnia and warming, showed a greater incidence of deformities and oversized otoliths than larvae exposed to acidification only, while a later study (Pimentel et al. 2016) on two teleost fishes, *Sparus aurata* and *Argyrosomus regius,* exposed to the combined effects of near-future predicted ocean warming (+4 °C) and acidification (~ 400 µatm) elicited decreased hatching success and survival in both species. Pimentel et al. (2016) also observed reduced growth rates among *S. aurata,* and that both species spent less time swimming and catching prey. Therefore, it is likely that the concomitant impacts of warming and acidification will adversely impact the fitness (growth rate, skeletogenesis, and survival) of *A. japonicus* in the future (Figure 4.4). Fishes functioning near the "pejus" or upper limit of their "pCO_2 window" (predicted to be ~ 800 µatm in this study) may display increased fitness in levels of pCO_2 predicted for the year 2050 (~ 550 µatm) (Figure 4.4), but the introduction of other climate change-related stressors (e.g. increased temperature and/or decreased food availability) may shift the optimum fitness window in favour of lower pCO_2 concentrations and result in a lower tolerance to elevated pCO_2 (see dotted line, Figure 4.4). It may be possible for evolutionary adaptation to increase the pCO_2 tolerance of fishes over multiple generations (as indicated by the red arrow, Figure 4.4); however, this covariance between maternal conditioning and heritable tolerance to future CO_2 levels remains unclear (Donelson et al. 2012, Welch and Munday 2017).

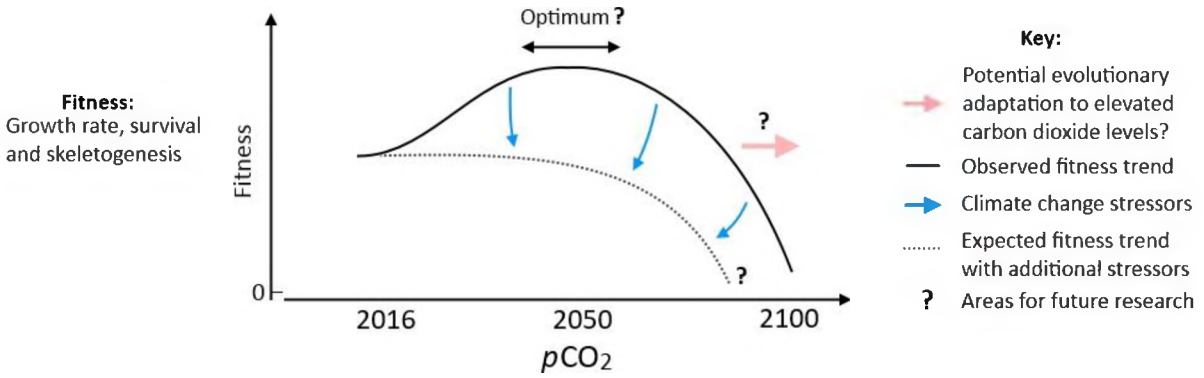


Figure 4.4. Hypothetical "pejus" curve and other potential stressors associated with climate change for *A. japonicus*. Fitness (depicted as growth rate, survival and skeletogenesis) of *A. japonicus* in relation to increasing pCO_2.

Another potential weakness in this study and other studies is the focus on a single species. As such, the ecosystem level response to future climate scenarios remains poorly understood. In addition, the duration of the study may have been too brief. While most studies extend for up to three weeks post-hatch (e.g. Baumann et al. 2012, Bignami et al. 2013), this 29-day study was still too short to understand the long-term effects of elevated pCO_2 on *A. japonicus*, given that metamorphosis was not fully completed in all of the treatments by the end of the study. Although eggs were immediately placed into their respective treatments in this study, the study design failed to take into account the impact of increased pCO_2 on the broodstock. Therefore, future studies should aim to maintain the broodstock in the levels tested to examine the potential selection drivers of elevated pCO_2.

Future research on larval fish skeletogenesis, should include understanding the process of mineralisation of the calcium phosphate skeleton, as well as the susceptibility of bone proteins to changing ocean chemistry. Bone proteins are fundamental building blocks for skeletal development and are closely linked with environmental conditions and metabolism (Cambier et al. 2009, Lall and Lewis-McCrea 2007, Sroga et al. 2011). It may also be of interest to investigate whether cartilaginous species are more sensitive to the effects of ocean acidification than bony fishes (osteichthyes), as relative ossification appeared to be more sensitive than relative chondrification in this study. Interestingly, there is a large expansion in the cartilaginous species post the Permian mass extinction event (Koot 2013) and Chondrichthyan fishes appear less affected than their bony cousins in the five mass extinctions over the last 400 million years (Kriwet et al. 2008). If the Permian mass extinction was indeed caused by an ocean acidification event, this suggests that the Chondrichtyes might be less vulnerable to the

effects of our changing ocean chemistry. Finally, future studies should aim to link growth and development with physiological processes, such as respiration, osmoregulation and thermoregulation, to fully comprehend the challenges larval fishes face in our future oceans.

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