The metabolic physiology of early stage *Argyrosomus japonicus* with insight into the potential effects of pCO_2 induced ocean acidification

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Abstract

Ocean acidification is a phenomenon associated with global change and anthropogenic CO_2 emissions that is changing the chemistry of seawater. These changes result in elevated pCO_2 and reduced pH in seawater and this is impacting marine organisms in various ways. Marine fishes are considered generally tolerant to conditions of ocean acidification; however, these assumptions are based on juvenile and adult fish tolerance and the larval stages have not been frequently assessed. Furthermore, it has been suggested that temperate species, particularly those with an estuarine association, may be tolerant to variable CO_2 and pH.

This study used an eco-physiological approach to understand how the early life stages of *Argyrosomus japonicus*, an estuarine dependent marine fisheries species found in warm-temperate regions, may be impacted by ocean acidification. The metabolic response of early stage larvae (hatching to early juvenile stage) was assessed under conditions of elevated pCO_2 and reduced pH in a controlled laboratory setting. Small volume static respirometry was used to determine the oxygen consumption rate of larvae raised in three pCO_2 treatments including a low (pCO_2 = 327.50 $\pm$ 80.07 µatm at pH 8.15), moderate (pCO_2 477.40 $\pm$ 59.46 µatm at pH 8.03) and high treatment (pCO_2 910.20 $\pm$ 136.45 µatm at pH 7.78). These treatment levels were relevant to the present (low) and projected conditions of ocean acidification for the years 2050 (moderate) and 2100 (high). Prior to experimentation with ocean acidification treatments, baseline metabolic rates and diurnal variation in oxygen consumption rates in early stage *A. japonicus* was determined.

Distinct ontogenetic structuring of metabolic rates was observed in early stage *A. japonicus,* with no cyclical fluctuations in metabolic rate occurring during the 24 hour photoperiodic cycle. Pre-flexion larvae showed no metabolic response to ocean acidification treatments; however post-flexion stage larvae showed metabolic depression of standard metabolic rate in the moderate (32.5%) and high (9.5%) pCO2 treatments (P = 0.02). Larvae raised in the high pCO_2 treatment also showed high levels of mortality with no individuals surviving past the post-flexion stage. Larvae raised in the moderate pCO_2 treatment were unaffected.

This study concluded that ocean acidification conditions expected for the end of the century will have significant impacts on the metabolism of early stage *A. japonicus,* which may result in reduced growth, retardation of skeletal development and ultimately survival as a result of increased mortality. Furthermore, the timing of reduced metabolic scope will significantly impact the recruitment ability of *A. japonicus* larvae into estuarine habitats. This could ultimately impact the sustainability of *A. japonicus* populations. Most importantly, this study highlighted the need to consider the combined effect of ontogeny and life-history strategy when assessing the vulnerability of species to ocean acidification.

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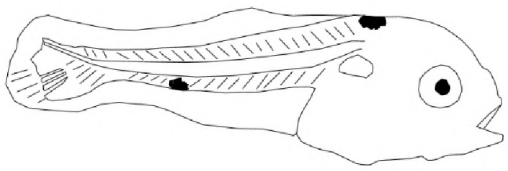

This thesis is dedicated to Silvia Edworthy

Mom, thank you for encouraging me to pursue this career.
You continue to guide me every day in your absence and I want nothing more than to become the woman you were.
Thank you for teaching me the true meaning of perseverance and strength.
I hope I am making you proud.

Chapter One

Ecophysiology and its role in climate change research with details on methodology

Global change and ocean acidification

Human influence is the primary driver of changes in atmospheric composition, specifically carbon dioxide concentration, which is ultimately the largest contributor to changes in global climate (Vitousek 1994). Increased burning of fossil fuels and land use change are the main contributors to increased concentrations of CO_2 in the atmosphere, which have increased exponentially (~ 40%) since the industrial revolution (Doney et al. 2009; Zeebe 2012; Field et al. 2014) from 280 to 387 ppm, with 50% of this increase having occurred in the last 30 years (Feely et al. 2009). At high concentrations, CO_2 in the atmosphere results in radiative forcing and general warming of the atmosphere (Doney et al. 2009; Serreze 2010; Zeebe 2012). This change in global climate has disrupted terrestrial and marine ecosystems and is expected to have severe consequences in the future as emissions of CO_2 continue unabated. It is therefore not surprising that climate change research is currently receiving considerable attention.

The fifth and most recent assessment report of the Intergovernmental Panel on Climate Change states with very high confidence that the physical ocean environment is changing as a result of anthropogenic CO_2 emissions (Hoegh-Guldberg et al. 2014). In the marine environment, climate change manifests itself by changes in seawater temperature, oxygen concentration, salinity and sea level (Howes et al. 2015). Global average sea surface temperatures have gradually increased by ~ 0.25 °C between 1971 and 2010 (Rhein et al. 2013). Ocean warming and stratification of surface waters have resulted in reduced oxygen availability due to the solubility effect (Howes et al. 2015) as well as the availability of inorganic nutrients in these areas (Rhein et al. 2013). Coastal areas appear to be more affected by hypoxia and stratification than the open ocean (Hoegh-Guldberg et al. 2014). A sea level rise of 3.0 ± 0.7 mm per year has also been documented to have occurred globally between 1993 and 2010 (Hay et al. 2015), which is considered to be a result of thermal expansion induced by warming as well as the melting of glaciers (Church et al. 2013).

Ocean acidification occurs as a secondary result of increasing atmospheric concentrations of carbon dioxide. Carbon dioxide from the atmosphere rapidly equilibrates with surface ocean water and causes a dramatic rise in seawater pCO_2 and a resultant decline in pH (Michaelidis et al. 2007; Doney et al. 2009; Feely et al. 2009; Rhein et al. 2013; Howes et al. 2015). This accumulation of CO_2 in seawater ultimately affects ocean chemistry through a series of complex equilibrium reactions (Figure 1.1) according to the equation:

$$CO_{2(atmos)} \leftrightarrow CO_{2\,(aq)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$

(Doney et al. 2009).

The outcome of these reactions includes an increase in dissolved CO_2 and bicarbonate ion (HCO_3^-) concentration as well as a reduction in carbonate ions (CO_3^{2-}) and pH ($-\log_{10}[H^+]$), resulting in more acidic conditions (Guinotte and Fabry 2008; Doney et al. 2009; Field et al. 2014; Hoegh-Guldberg et al. 2014). The ocean absorbs up to 25–30% of the CO_2 emitted by human activity, and since the industrial revolution average ocean pH has already declined by 0.1 units (Orr et al. 2005; Feely et al. 2009; Field et al. 2014; Hoegh-Guldberg et al. 2014). Even though this may seem an insignificant amount, considering that pH is measured on a log scale, it is actually a substantial decline (Doney et al. 2009). Furthermore, ocean pH is projected to decline by a further 0.3 units by the year 2100 resulting in a 150% increase in H^+ and a 50% decrease in CO_2^{3-} (Guinotte and Fabry 2008; Pörtner 2008; Doney et al. 2009; Feely et al. 2009). This amounts to a decline of pH by 0.0015 and 0.0024 annually under conditions of continued CO_2 emissions at the current rate (RCP emission scenario 8.5) (Field et al. 2014). These changes in carbonate chemistry also alter the concentration of related carbonate compounds, such as calcium carbonate ($CaCO_3$), calcite (Ω_{ca}) and aragonite making seawater corrosive (Ω_{ar}) (Doney et al. 2009; Zeebe 2012).

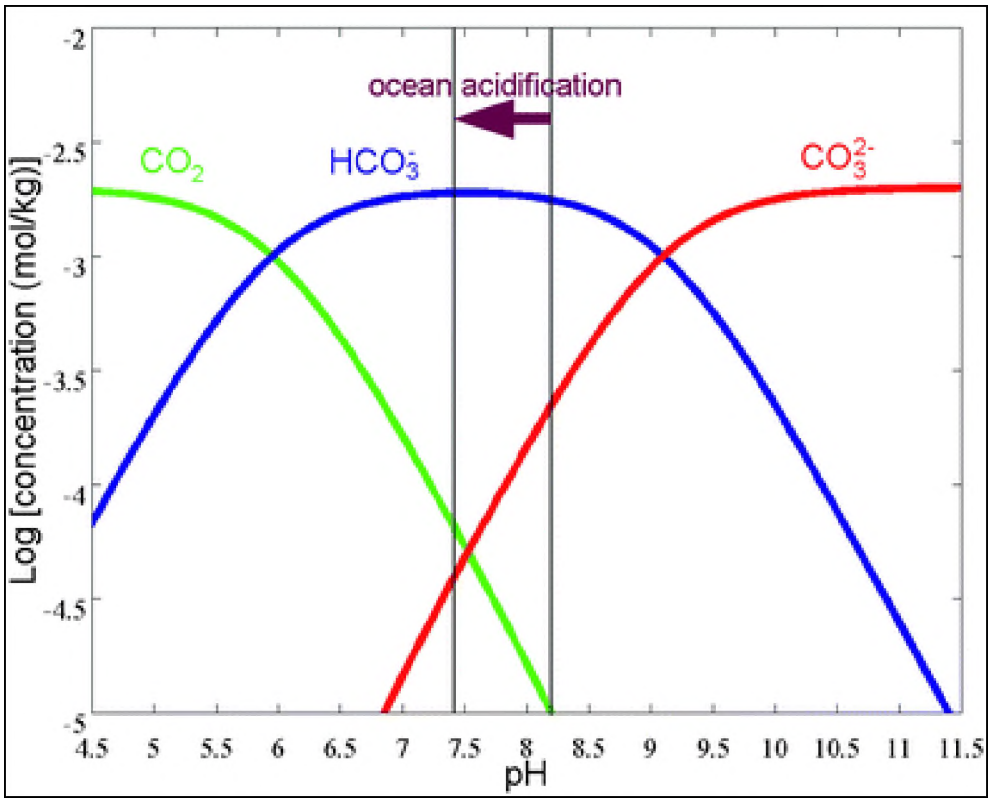

Figure 1.1: Concentrations for the reactions of carbon dioxide in seawater based on pH (Bjerrum plot) at DIC = 2100 µmol L^{-1}, salinity = 35 and temperature = 25 °C. The arrow shows the ocean acidification expected for the year 2100 under the business as usual emission scenario (RCP 8.5) (Hofmann and Schellnhuber 2010).

The effects of ocean acidification on marine organisms

Several recent studies have shown that ocean acidification is having considerable impacts on marine ecosystems (Orr et al. 2005; Guinotte and Fabry 2008; Kroeker et al. 2010). It has also been suggested that previous mass extinction events (Permian-Triassic) may have been partly driven by the effect of high CO_2 concentrations (Knoll et al. 1996; Pörtner et al. 2004; Knoll et al. 2007), which in turn suggests that our biodiversity may be at risk due to changes in ocean chemistry predicted for the future, particularly when faced with additional stressors associated with climate change.

Warming and hypoxia effects are by far the most frequently researched climate change stressors for marine organisms. Studies on numerous species have shown that organism performance and fitness is reduced outside their species-specific temperature optima due to physiological constraints (Pörtner 2001; Pörtner and Knust 2007). Many mobile organisms, such as fishes, are able to avoid unfavourable

temperatures in some cases by changes in distribution and latitudinal movements (Pörtner 2001; Pörtner 2010). However, there is no such refuge from ocean acidification, which occurs relatively uniformly across the global oceans, and it is therefore not possible for organisms to find refuge through spatial migrations (Doney et al. 2009; Sunday et al. 2014).

Changes in seawater CO_2 and pH, like changes in temperature, induce physiological performance constraints (Pörtner 2008). Interestingly, it appears that species responses to ocean acidification are highly variable among taxa. Some species have been classified as extremely vulnerable and face the risk of extinction, whereas others are able to tolerate both moderate and high levels of acidification (e.g. species adapted to surviving near hydrothermal vents or in intertidal areas) (Pörtner 2008). Although there are differences in tolerance among species, the general consensus is that ocean acidification poses a threat to most marine organisms (Dupont et al. 2010a; Kroeker et al. 2010). Changes in species composition and survival will ultimately have significant consequences on marine ecosystems (Dupont et al. 2010a). In light of this, there is a clear need to understand the species-specific responses (including all phases of their life history) to contribute to our understanding of the ecosystem level responses to ocean acidification.

A number of biological responses occur in marine organisms when faced with the changes in seawater chemistry associated with ocean acidification. A range of chemical stressors may be involved, which are variable depending on the process that is affected. For example, calcifying organisms are most affected by the reduced saturation state of calcium carbonate ($CaCO_3$) in seawater as a result of acidification (Gattuso et al. 1998; Riebesell et al. 2000), whereas fish are more affected by increasing concentrations of dissolved CO_2, which affects acid-base regulation and metabolism (Fabry et al. 2008).

To date, invertebrates, specifically calcifying organisms, have received the most research attention (Ishimatsu et al. 2008). Responses of invertebrates to ocean acidification include have been shown to include developmental delays (Stumpp et al. 2011), reduced calcification rates (Ries et al. 2009) and negative metabolic responses (Lannig et al. 2010; Stumpp et al. 2011), which may ultimately affect survival (e.g.

Dupont et al. 2008). These effects have been documented in a number of corals (Hoegh-Guldberg et al. 2007; Kleypas and Yates 2009), bivalves (Gazeau et al. 2007), plankton (Orr et al. 2005) and echinoderms (Dupont et al. 2010b; Stumpp et al. 2011). Few invertebrate species have shown high levels of tolerance to ocean acidification and are able to comfortably withstand the levels predicted for the near and distant future (Kroeker et al. 2010). Significantly fewer studies have assessed the effects of increased ocean acidification on marine vertebrates, especially fishes (Ishimatsu et al. 2008).

Studies that have assessed the effect of ocean acidification on fishes show variation in stress responses among species (e.g. Ishimatsu et al. 2005; Cattano et al. 2016). This is likely attributed to which biological or physiological process is affected, whether some form of adaption is taking place and what implications the stress has on fitness and survival (Kroeker et al. 2010). However, the general conclusion is that there is a strong likelihood that conditions of high CO_2 in the ocean may affect the physiological function and behaviour of fishes, at one or more stages in development, causing both subtle and potentially lethal consequences in the long term (Ishimatsu et al. 2005; Munday et al. 2009b; Munday et al. 2014). These consequences include reduced growth and survival (Baumann et al. 2012; Murray et al. 2016), changes in otolith development (Checkley et al. 2009; Munday et al. 2011), tissue damage (Frommel et al. 2012) and changes in sensory function and behaviour (Munday et al. 2009b; Dixson et al. 2010; Simpson et al. 2011).

Habitat, life history strategy and ontogeny are thought to influence species tolerances to ocean acidification (Ishimatsu et al. 2005; Cattano et al. 2016). For example, it has been suggested that temperate species are generally more tolerant to ocean acidification as a result of regular exposure to highly variable environments (Ishimatsu et al. 2005; Fabry et al. 2008; Munday et al. 2009a). In addition, highly active, migratory species (e.g. epipelagic fishes) are also suggested to show higher tolerance as a result of high metabolic rates and a capacity for anaerobic metabolism which may buffer the increased costs of acid-base regulation (Fabry et al. 2008). The influence of ontogeny on the tolerance of fishes to ocean acidification is relevant to this study as there is increasing evidence to suggest that the early developmental stages in fishes are likely to be more vulnerable to the effects of ocean acidification compared to the juvenile

and adult stages (Ishimatsu et al. 2004; Guinotte and Fabry 2008; Frommel et al. 2013; Pimentel et al. 2014a; Cattano et al. 2016). As such, the early life stages may present a CO_2 tolerance bottleneck, which will ultimately affect population survival.

Although changes in pH can often be managed over short-term exposure using adaptive responses, e.g., by passive buffering of extracellular pH or metabolic suppression (Fabry et al. 2008), these cannot be maintained in the long-term and may result in metabolic changes, defective development, reduced growth or impairment of some other process, and even mortality. Due to technical constraints (such as the small size and low metabolic rates), these responses are rarely measured in larval fishes and it is therefore difficult to determine whether fish larvae respond in the same way that invertebrate larvae do or if they are more comparable to juvenile fishes. Therefore, research on the effects of ocean acidification in early stages of fishes is critical to obtain a better understanding of its impacts on fishes.

Although preliminary studies have found that the early life stages of fishes may be extremely sensitive to conditions of high CO_2, the findings are not yet sufficient to draw convincing conclusions (Baumann et al. 2012). This is mainly due to the fact that most studies assessing the tolerance of early stage fish to acidification have used treatments that by far exceed the predicted rates of ocean acidification. For example, some studies have documented between 60–100 % mortality over short exposure periods (24–48 h) in the early stages of numerous fish species (Kikkawa et al. 2003; Hayashi et al. 2004; Ishimatsu et al. 2004; Ishimatsu et al. 2005; Fabry et al. 2008). However, the levels of these acidification treatments far exceeded the bounds predicted for the end of the century (Hayashi et al. 2004). More studies are beginning to assess more relevant pCO_2 levels predicted for the end of the century. For example, a recent study found that Atlantic cod survival is significantly reduced in the first 25 days post-hatch (Stiasny et al. 2016), suggesting that high early mortality rates are a realistic expectation.

The role of eco-physiology in climate change research

While patterns of change are important for predictive science, the understanding of the processes driving change is necessary in order to make predictions at various levels of biological organisation (Horodysky et al. 2015). Eco-physiological research

provides a suitable tool for linking changes in environmental conditions to organism fitness and behaviour (Horodysky et al. 2015), thereby providing a mechanistic understanding of the effects of climate variables on individuals (Pörtner and Knust 2007). This information can ultimately be related to the patterns of change from species, to populations and to ecosystems.

Pörtner et al. (2004) highlighted the importance of assessing an organism's physiological response to environmental stressors in order to determine the impacts of climate change on individuals. Physiological studies can be used to gain an understanding of the changes in fitness, or performance parameters such as behaviour, reproduction or movement, which are relevant at a population level (Horodysky et al. 2015). Ultimately, this understanding can also assist in predicting population and ecosystem responses to change (Horodysky et al. 2015).

Measurements of metabolic rate (oxygen consumption rate) have become a frequently used tool in assessing the biological response of organisms to climate change (Clark et al. 2013). This is because the overall animal performance and fitness is related to that animal's physiology (Clark et al. 2013). In addition an organism's physiology is related to, and influenced by, their environment. Therefore, it is expected that species evolve in order to maximise their physiological performance under certain environmental conditions (Clark et al. 2013). When exposed to non-optimal conditions, fish will respond with a decrease in physiological performance, and this in turn may negatively affect growth, development, fitness or reproduction (Clark et al. 2013).

When an organism is exposed to increased CO_2 concentrations in their environment, CO_2 diffuses more rapidly across body tissues and accumulates internally. The CO_2 reacts with body fluids and reduces the internal pH of the organism (Fabry et al. 2008). Organisms can use a number of methods to manage this internal pH balance, however, these mechanisms are generally limited to those species adapted to manage CO_2 accumulation from respiratory processes, and therefore they are not efficient at managing extreme changes in CO_2 (Fabry et al. 2008). The mechanisms of managing internal pH imbalances include: passive buffering of internal CO_2; active ion transport and exchange across membranes; active CO_2 transport out of the body; and metabolic suppression to conserve metabolic energy (Fabry et al. 2008). All but the final

mechanism require metabolic energy to enforce and maintain, thereby potentially shifting energy allocations among different maintenance processes. Therefore, the management of internal pH often comes at a metabolic cost, which then reduces the energy available for other important survival processes, such as growth or reproduction. Comparisons of the metabolic rate of individuals exposed to different pH levels can therefore show if energy is allocated to acid-base regulation.

Climate change and ocean acidification in South Africa

Worldwide, it is predicted that coastal areas are especially sensitive to the impacts of global change, including ocean acidification (Harley et al. 2006). The South African coastal environment provides an ideal platform to investigate global climate change (Whitfield et al. 2016). This is attributed to the biogeographical diversity of the coastline, with which no other global coastline of a similar size can compare (Whitfield et al. 2016). The coastline spans a total length of 3650 km and supports numerous habitat types ranging from rocky shoreline (27%) sandy beach areas (42%) and areas of mixed shoreline (31%) (Bally et al. 1984). There are also a total of 250 functional estuaries along the coast of South Africa, the majority of which occur along the south (123) and east (117) coasts (Turpie et al. 2002). The South African coastline is divided into three major biogeographic regions including the subtropical region on the east coast, the warm temperate region on the south coast and the cool temperate region on the west coast (Potts et al. 2015). This variety in biogeography occurs as a result of the confluence of two major ocean currents, the warm Agulhas current on the east and the cool Benguela current on the west coast (Hutchings et al. 2002; Hutchings et al. 2009). The extent of biogeographical and species diversity of this coastline puts it at particular risk to environmental change.

Climate change is likely to affect the South African coastal and nearshore zones by changes in sea surface temperature, frequency of upwelling events, current strength, rainfall, pH and rising sea levels (Potts et al. 2015). Changes in the distribution and abundance of various biotic groups within South African waters have already been recorded, and these are primarily related to changes in temperature (James et al. 2013, Potts et al. 2015, Whitfield et al. 2016). Regional changes in seawater chemistry as a result of ocean acidification in South African coastal areas have not been quantified or assessed in much detail and information on regional acidification is

limited to the globally modelled scenarios (Figure 1.2). The limited data available for carbonate chemistry in South Africa suggest that inshore areas, particularly along the south of the west coast, are particularly vulnerable to ocean acidification (de Villiers and Tsanwani 2014).

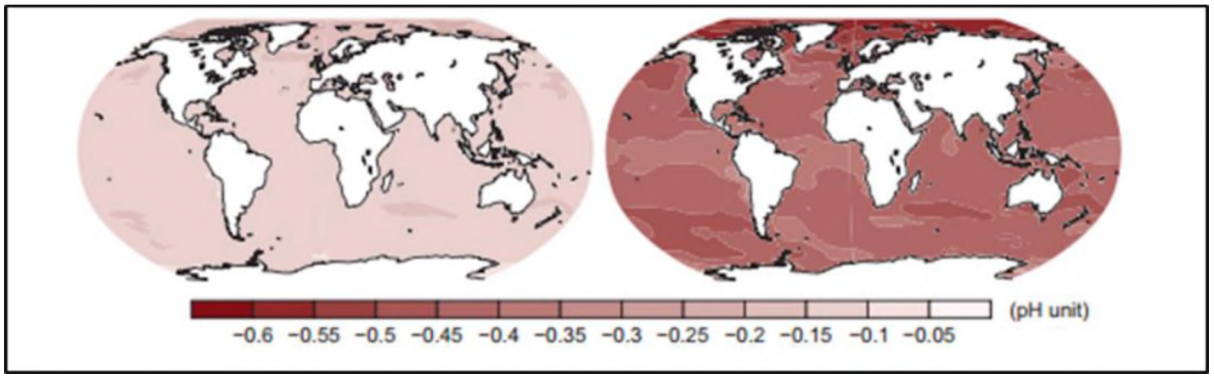

Figure 1.2: Mean change in surface ocean pH (produced by a CMIP5 multi-model) for emission scenario RCP 8.5 from the period 1986 – 2005 to 2081 – 2200 (IPCC, 2013)

The effects of climate change and ocean acidification, in addition to harvesting and fishing pressure, may have detrimental effects on coastal marine resource populations. The impacts that these pose on marine resources is particularly relevant in a country like South Africa where many communities are still dependent on subsistence fishing and harvesting for their livelihoods. It has been recorded that 83% of the total catch in the South African recreational and commercial (seine and gillnet) fisheries are comprised of individuals of estuarine dependent species with these species representing 50% of all the species caught (Lamberth and Turpie 2009). This highlights the importance of prioritising research on estuarine dependent species, particularly those important in fisheries, to determine the impacts of climate change on vulnerable communities.

The response of fishes to climate change in South Africa has been found to vary depending on their life history and behaviour (Potts et al. 2015). One of the more complex life history styles is that of the estuarine dependent group. Estuarine dependent marine species are species with a marine origin that depend on estuarine areas as nursery habitats in specific stages of their life cycle in order to survive and complete their life cycle (Whitfield 1994). Estuarine environments provide benefits such as shelter, increased food availability, suitable physico-chemical conditions and reduced predation risk to these species (e.g. Able 1999; Whitfield 1999; Kandjou and

Kaiser 2014; Davidson et al. 2016). South African estuarine dependent marine species are generally important in coastal fisheries (Potts et al. 2015).

Argyrosomus japonicus: a model species

Argyrosomus japonicus is a large sciaenid that occurs in nearshore coastal waters and estuaries of the Indian and eastern Pacific oceans (Griffiths 1996; Fitzgibbon et al. 2007). As adults, *Argyrosomus japonicus* can attain a large size of up to 1.8 m (75 kg) (Griffiths and Heemstra 1995) and are a popular species in commercial and recreational fisheries in South Africa and Australia (Griffiths 1996; Brouwer et al. 1997; Silberschneider and Gray 2008), as well as in the subsistence fishery in South Africa (Brouwer et al. 1997; Childs and Fennessy 2013).

Argyrosomus japonicus has a wide distribution along the South African coastline, occurring along the warm-temperate south and subtropical east coasts (Whitfield 1998). Adult *A. japonicus* spawn at sea, and the larvae (< 20 mm TL) occur in nearshore pelagic coastal regions and surf zones (Gray and McDonall 1993; Griffiths 1996; Silberschneider and Gray 2008). Upon reaching a size of approximately 20 mm TL (early juvenile) they begin to recruit into estuaries (Beckley 1990; Gray and McDonall 1993; Griffiths 1996; Whitfield 1998). The occurrence of *A. japonicus* in estuaries as juveniles (< 150 mm TL) is thought to be obligatory and these early juveniles are completely dependent on estuarine nurseries (Cowley et al. 2008). Early development and growth in estuaries is rapid, with juveniles attaining approximately 35 cm total length (TL) within their first year of life (Silberschneider and Gray, 2008).

Argyrosomus japonicus juveniles have been found to be more abundant in turbid estuaries with a high freshwater discharge (Marais 1988; Gray and McDonall 1993; Taylor et al. 2006). Griffiths (1996) suggested that turbid systems are the preferred nursery habitat for *Argyrosomus japonicus* as early juveniles are absent in non-turbid estuaries such as the Swartvlei and Knysna estuaries. A recent study investigating the role of turbidity in the recruitment of post-flexion *A. japonicus* found that post-flexion larvae are significantly attracted to turbid water, suggesting that they may follow turbidity plumes when recruiting into estuaries (Wilsnagh 2016). Many late juvenile and early adult *A. japonicus* (> 300 mm TL) continue to utilise estuarine areas, particularly the lower reaches before returning to sea as adults (Griffiths 1996; Cowley

et al. 2008). The occurrence of adults in estuaries has been linked more to the availability of prey (Griffiths 1997a). Early juvenile (< 50 mm TL) *A. japonicus* feed on mysids and copepods, after which the diet gradually shifts to teleost fishes around a size of ~ 100 mm TL (Griffiths 1997a). Adults in the marine environment feed on a number of species but teleost fish contribute the largest proportion to their diet (Griffiths 1997b).

Argyrosomus japonicus is a popular angling species in areas throughout its range of distribution (Lenanton and Potter 1987; Gray and McDonall 1993) and is one of the most frequently caught species in the recreational fishery in South Africa (Mann et al. 2002; Pradervand 2004). Due to its large size and late maturity, this species is particularly susceptible to the impacts of fishing (Ferguson et al. 2014). *Argyrosomus japonicus* is showing major stock declines in both Australia (Silberschneider et al. 2009) and South Africa (Mirimin et al. 2016). The South African population has been described as collapsed (Griffiths 1997c) and the abundance of remaining wild adult populations is alarmingly low (Mirimin et al. 2016). The decline has been attributed to recruitment overfishing as mainly immature juveniles (< 1 000 mm TL) are targeted (Pradervand 2004) when they aggregate in estuarine environments (Griffiths 1997c) and removed before they attain sexual maturity. Despite the alarming stock status of *A. japonicus* in South Africa, management regulations remain suboptimal. In addition, there is little enforcement and compliance success to the regulations that do exist (Mirimin et al. 2016). Due to its popularity as an angling species, Whitfield and Cowley (2010) suggest that this species should be considered a flagship species representing the health of South African estuaries.

Recently, aquaculture of Sciaenids has increased in popularity to provide food resources and to offset the demand on wild stocks imposed by fisheries (Silberschneider and Gray 2008). *Argyrosomus japonicus* was identified as a suitable candidate for aquaculture as it is fast growing and is in high demand as a valuable food fish in South Africa (Daniel 2004; Collett et al. 2008). There has been a recent effort to develop aquaculture protocols to successfully rear this species (Daniel 2004; Collett et al. 2008; Collett et al. 2011; Musson and Kaiser 2014) and a few aquaculture companies in South Africa have succeeded in efficient breeding and farming of this species.

The life history strategy of this species, combined with its recent successful rearing in aquaculture in South Africa, contributed to the selection of this species as a model for assessing the physiological impacts of ocean acidification on the marine larvae of estuarine dependent species. There is no information on the response of marine spawning, estuarine dependent fishes to the impacts of ocean acidification.

As juveniles, *A. japonicus* have been observed in large numbers in turbid, freshwater rich estuarine systems and in the upper reaches of marine dominated estuaries. Based on this occurence it is clear that they are tolerant of low levels of salinity (Ter Morshuizen et al. 1996; Cowley et al. 2008). As these freshwater dominated areas normally have low pH levels compared with the marine environment, it is likely that juveniles of this species will be tolerant to the impact of ocean acidification. However, the larvae of this species occur in nearshore coastal waters, which are subject to incremental acidification over time. Since these larvae are not likely to have developed sufficient acid-base regulation systems early on in development, these coastal-occurring life stages may be more vulnerable to the effects of acidification than the juveniles and adult stages. This could result in a bottleneck in development and survival to the juvenile and adult stages, eventually impacting *A. japonicus* populations in estuaries and surrounding coastal areas.

Overall aims and objectives

The overarching aim of this study was to examine the impacts of ocean acidification on the metabolism of the early marine life stages of the estuarine dependent, *Argyrosomus japonicus.* Baseline metabolism data were collected for the early life stages of the species in order to describe the metabolism for the larval stages. This was followed by an experiment designed to compare the metabolic response of early life stages exposed to present and future predicted pH levels for 2050 and 2100.

Chapter Two

Baseline metabolic rates and 24 hour variation in oxygen consumption in the early development of Argyrosomus japonicus

Introduction

Metabolism can be quantified by the energy consuming activities of an organism (Nelson 2016) and is used as an indication of how organisms partition energy resources to activities that allow them to survive, grow and reproduce (Post and Lee 1996). The metabolic profile, which is a composition of the various metabolic rates of a species, therefore gives an indication of the efficiency of energy intake, transformation and allocation (Fry 1971; Brown et al. 2004). McKenzie et al. (2016) suggested that an organism's physiology determines its ability to successfully survive under specific environmental conditions. As a result, physiological condition is a reflection of the performance and fitness of an organism (Pörtner 2010). When combined with information on changing environmental conditions, physiological information can provide insight into species and community level responses (Pörtner and Farrell 2008). These kinds of data have served numerous ecological applications including resource management, conservation (McKenzie et al. 2016) and climate change assessments (Pörtner and Farrell 2008). With the prediction of the impacts of climate change at the forefront of global research priorities, there has been a recent interest in eco-physiological studies that assess the metabolic response of species to increasingly sub-optimal environmental conditions (Clark et al. 2013).

In fishes, energy consuming activities include swimming, growth, reproduction and excretion, and large energy investments are made to accommodate these activities (Brown et al. 2004; Clark et al. 2013). Changes in physical ocean conditions are likely to affect these activities through metabolic pathways, which may compromise the fitness of individuals, populations and communities and may eventually lead to large scale changes in ecosystems (Pörtner and Farrell 2008). Understanding metabolic rates and metabolic profiles in marine organisms is therefore critical to assist with the prediction of their response to a rapidly changing climate.

There are four types of metabolic rates that can be determined using various respirometry methods. These include the standard metabolic rate (SMR), routine metabolic rate (RMR), active metabolic rate (AMR), and maximum metabolic rate (MMR) (Fry 1971). Each of these is related to the activity of the organism during oxygen consumption measurements. For the purposes of this study, these will be explained with reference to individual fish as described by Fry (1971). Standard metabolic rate refers to the metabolic rate of a post-absorptive, acclimated, undisturbed, resting individual during the period of their circadian rhythm when they show the lowest oxygen consumption rate. This measurement translates to the minimum amount of energy required for survival (Nelson and Chabot 2011) and includes energetic processes such as biosynthesis of macromolecules, ion transport across membranes (e.g. osmoregulation) and other internal, life sustaining processes, which are independent of growth, activity or reproduction (Nelson and Chabot 2011). Normal physiological functioning is impaired below this metabolic rate. The RMR is the mean metabolic rate of a fish in a resting state but exhibiting random swimming activity as would be common for a fish acting in a natural way. The AMR is the average metabolic rate of an active fish swimming at a sustained, constant speed. Finally, MMR is the maximum rate of metabolism of a fish that is exercised to exhaustion prior to measuring oxygen consumption. Metabolic scope is calculated as the difference between AMR and SMR (Fry 1971) and represents the amount of energy available to the individual for activities over and above the energy required to maintain survival processes. This energy is apportioned to activities such as growth, feeding, reproduction and behavioural activities (Priede, 1985) and ultimately determines the survival and competitive ability of organisms and their ability to respond to changes in their environment (Killen et al. 2007; Clark et al. 2013). It is important to understand the eco-physiology of an organism in order to link physiology with ecological processes (Killen et al. 2010; Clark et al. 2013) such as life history strategy, distribution, habitat use and behaviour.

The larval stages of fishes are usually subject to high mortality, which has been attributed to a narrow metabolic scope in the early stages of development (Killen et al. 2007). For example, a study on juvenile *Argyrosomus japonicus* showed a greater increase in oxygen consumption rate when small individuals were exposed to increased temperature than larger individuals (Pirozzi and Booth 2009). These

changes in physiology can ultimately affect the adult population through changes in recruitment success when conditions are not favourable. Metabolic scope is therefore a useful measure to identify potential survival bottlenecks during ontogenetic development, because the life stages with the lowest metabolic scope are likely to be more vulnerable to adverse conditions due to a reduced energetic capacity for acclimation (physiological or behavioural). Apart from improving our understanding of fundamental physiological principles and the potential impacts of a changing climate, information on larval fish physiology can be used to determine the mechanistic drivers of species, population and community level responses to the environmental and anthropogenic pressures (Burggren and Blank 2009; Horodysky et al. 2015). In the case of larvae, a physiological approach is specifically useful when determining constraints limiting recruitment into adult populations, particularly in environments that are predicted to change in the future (Killen et al. 2007).This is because metabolic scope may highlight energy bottlenecks during early development.

Fishes are known to show endogenous fluctuations in metabolic rate over the 24 hour photoperiodic cycle, and this generally reflects their diurnal or nocturnal activity patterns (Marais 1978; Du Preez et al. 1986; Deacon and Hecht 1996). Therefore, the oxygen consumption of an individual may vary in the 24 hour cycle, without any change in the external environment, and it is important to determine the optimal time for measuring the baseline metabolic rates of SMR and AMR. This will also ensure a standardised measure of oxygen consumption and provide reference levels of metabolism before treatment conditions are assessed.

Detailed metabolic information on *A. japonicus*, particularly on the early life stages, remains limited (Fitzgibbon et al. 2007; Pirozzi and Booth 2009). There has been no previous assessment of metabolic rates in the larval stages or the fluctuations in metabolic rate during the photoperiodic cycle for this species. Such metabolic information will be useful for future studies as it will provide a reference to which time of day metabolic measurements should be taken (Kandjou and Kaiser 2014). Furthermore, there is a paucity of research that addresses the physiology of any species throughout ontogenetic development (Post and Lee 1996; Killen et al. 2007). The aims of this chapter were to 1) determine the baseline metabolic rates throughout the early development of *A. japonicus* in order to determine the reference metabolic

rates and metabolic scope for this species and 2) determine the 24 hour fluctuations in oxygen consumption of this species. It was hypothesised that there will be an increase in metabolic rates (RMR, SMR, AMR, metabolic scope) with development in the early stages. It is proposed that this increase will be gradual during the pre-flexion stages due to lack of swimming ability and will then increase faster in the post-flexion stages when the individuals become more mobile. Due to the life history strategy of this species, it is also expected that metabolic rate will be higher in the day for the early stages due to their reliance on vision for feeding and estuarine recruitment.

Materials and methods

1. Experimental animals

Experimental fish were obtained from a single, induced spawning event from a wild broodstock (F1 generation) at the PureOcean aquaculture facility in East London, South Africa. The larvae were first hatched in darkness in three hatching cones after which the hatched larvae were moved to the 8 000 L cylindrical grow-out tanks with a stocking density of 30 larvae per litre. All experimental individuals were reared using standard aquaculture protocols (Table 2.1) in a single 8 000 L tank. The light cycle was kept at 16 L: 8 D in order to replicate optimal summer conditions in temperate South Africa, where these summer spawning species are regularly found in high abundance. Larvae were fed with rotifers *Brachionus plicatilis* enriched with *Tetraselmis* spp. and de-capsulated *Artemia franciscana* in the early stages and later weaned onto a pelleted diet (Skretting Gemma Wean 200–1 000 µm) at 18 DAH (days after hatch) as per aquaculture protocol to ensure optimal survival and growth. Rotifers and artemia were provided in excess and maintained at an estimated concentration of two individuals per ml, which was checked regularly throughout the day to ensure consistent availability of food throughout the entire photoperiodic cycle. Rotifer feeding was terminated at 13 DAH and *Artemia franciscana* at 24 DAH. Water clarity was maintained at just below 1 m Secchi disk reading with green algae (*Tetraselmis* spp.) to ensure optimum feeding conditions for the larvae until 24 DAH. Once weaned onto the pelleted diet, the larvae were fed hourly throughout the photoperiod using a belt feeder. Pellet size was increased according to growth of larvae as per standard feeding protocols of *A. japonicus* aquaculture.

Table 2.1: Water quality parameters maintained during the study period based on standard aquaculture protocol for early stage *Argyrosomus japonicus*

Parameter	Value	Units
Temperature	24 ± 1.0	ºC
Salinity	35	PSU
pH	8.15 ± 0.5	-
Dissolved oxygen	8.3 ± 1.0	$mg.l^{-1}$

2. Oxygen consumption measurements

Static respirometry was used to undertake 24 hour oxygen consumption measurements throughout development to include each life-stage of *Argyrosomus japonicus,* from hatching through to the settlement stage (0–27 DAH) (Table 2.2). Care was taken to include developmental milestones such as hatching, loss of the yolk sac, gas bladder formation, notochord flexion, first feeding and settlement (Table 2.2). Respirometry protocol was carefully designed according to recommendations made by Clark et al. (2013), Chabot et al. (2016) and Peck and Moyano (2016). Respirometry equipment was validated by assessing suitable respirometer volumes, oxygen ingress and drift associated with bacterial respiration prior to experimentation. Small volume static respirometry chambers (24 chamber microplates, Loligo systems), with volumes ranging from 200 µl, 750 µl and 4 ml, were used to assess oxygen consumption rates in pre-flexion larvae using a 24 channel microplate reader (SDR 436, PreSense) housed in a water bath that maintained temperature at 25 ± 0.05 ºC. One individual larva was placed in each chamber and 20 larvae were measured for oxygen consumption per trial. For each trial, four blank seawater chambers were included to account for bacterial respiration.

For post-flexion larvae, larger respirometers with volumes of 5 ml and 15 ml were constructed from glass and stainless steel tubing and were placed in a dark-sided water bath that maintained water temperature at 25 ± 0.05°C under conditions that minimised disturbance. The larger volume static respirometers were used to reduce stress as the post-flexion larvae were more mobile. For oxygen measurement in the larger respirometers, water was circulated from the chambers through flow-through cells using a peristaltic pump; the oxygen concentration ($mg.l^{-1}$) was recorded using red flash dye technology and readings were transmitted via a fiber-optic cable

connected to a Firesting oxygen reader (Pyroscience e.K., Aachen, Germany). The flow of water re-circulating in a loop through the flow-through cells ensured adequate mixing of water in the chambers and maintained uniform oxygen distribution within the chambers. The large volume respirometry system consisted of four chambers per trial, of which three of the chambers contained individual fish and one blank chamber was included per respirometry trial. The seawater used in the respirometry trials was treated with 12.5% sodium hypochlorite (neutralised with thiosulfate) and ultraviolet light sterilisation to minimise the background respiration rates. The use of sodium hypochlorite is common practice in aquaculture and is used to reduce bacterial growth in seawater systems.

Nine randomly selected individuals were used to assess oxygen consumption per life stage (Table 2.2). Selected individuals were fasted for approximately 6 hours prior to each measurement by placing them in beakers filled with clean seawater. These purging/ acclimation chambers were housed in the water bath used for respirometry for approximately one to four hours to ensure the individuals used for respirometry were in a resting, post-absorptive state and acclimated to the measurement temperature to ensure the accurate determination of SMR (fasting, respirometer size and acclimation time depended on the size and activity of the individuals). Individuals were then placed in the respirometry chambers and oxygen consumption was measured for approximately one hour (depending on individual consumption rate) three times a day, at 08h00; 15h00 and 22h00 over a total period of 15 hours. This resulted in one measurement period for the morning, afternoon and night for each cycle.

Measurement time and chamber volume were adjusted to avoid a reduction in the oxygen concentration below 80% saturation. This was done to ensure that hypoxia induced stress and oxygen ingress were avoided. Oxygen concentration was measured in the chambers every 60 seconds during each one hour measurement period. After all measurements were completed, fish were removed from the chamber, placed on filter paper (membrane filter, 0.2µm) and excess water was removed using suction from a vacuum pump. Fish were then weighed to the nearest 0.0001 mg. Due to their small size, it was not possible to weigh pre-flexion larvae and individual weights could only be determined from 13 DAH. For smaller individuals, a sample of 3–5 larvae

were weighed at a time and an average weight was calculated. Due to the inaccuracy of weighing smaller larvae, oxygen consumption was expressed as μmol O_2. $indv^{-1}.h^{-1}$ and mass specific oxygen consumption ($mgO_2.g^{-1}.h^{-1}$) was only calculated for larger individuals (13 DAH onwards) and therefore analysed separately. These measurement protocols were repeated over three consecutive days per life stage to avoid the influence of development on metabolic rate as development in *A. japonicus* occurs rapidly in the early stages.

Table 2.2: Developmental stages of *Argyrosomus japonicus* larvae from 0–27 DAH throughout the study period

DAH	Life-stage	Description
0 – 3	Hatchling	From hatching to complete absorption of yolk sac ± 1.3 mm TL
3 – 6	Early pre-flexion	From yolk sac absorption to the start of notochord flexion. Gas bladder forms during this stage and larvae begin active feeding on rotifers
10 – 12	Late pre-flexion	Begin feeding on *Artemia franciscana*
14 – 16	Flexion	Completion of notochord development and development of fin elements
20 – 22	Post-flexion	Increased swimming activity and feeding on pelleted diet
26 – 27	Settlement	Completion of metamorphosis; appear and behave like fully fledged young fish

2.1 Calculations of baseline metabolic rates

The first five to ten minutes of the recorded measurements were excluded from the calculations to account for larval acclimation to the chambers. Any oxygen concentration readings that were below 80% saturation of normal seawater (5.3 mg L^{-1}) were also excluded from the calculations. A least squares linear regression of oxygen concentration over time was performed on the oxygen consumption data. Residual analysis was first applied to determine whether the data were suitable for use in a linear regression model. The regression model was used to calculate ΔO_2 and the following equation was used to calculate MO_2 (oxygen consumption in mgO_2. $indv^{-1}.h^{-1}$) independent of mass for each life stage: $MO_2 = \frac{[\Delta O_2] \times vol}{t}$ where, $\Delta[O_2]$ is the decrease of oxygen concentration in the water(Δ mg O_2), t is the total recording time (h), vol is the volume of the respirometer (l). The average oxygen consumption values for the blank chambers were then subtracted from the final calculated oxygen

consumption values for each individual to account for background respiration in the seawater.

For flexion, post-flexion and settlement larvae (13 DAH and older), mass specific oxygen consumption ($mgO_2.g^{-1}.h^{-1}$) was calculated using: $MO_2 = \frac{[\Delta O_2] \times vol/M}{t}$ where M is the wet mass of an individual larva weighed to the nearest 0.0001 g. Mass-independent and mass-dependent oxygen consumption rates were analysed separately.

Standard (SMR) and active (AMR) metabolic rate were determined from the oxygen consumption rate by using the 5% and 95% percentile of all data obtained during the 15 hour measurement period (Reid et al. 2012; Kandjou and Kaiser 2014) for each life stage separately. The RMR was calculated by averaging all remaining metabolic rates obtained in the 18 hour measurement period (Kandjou and Kaiser, 2014). Metabolic scope was then calculated using the following equation:

$$Metabolic\ scope\ (MS) = AMR - SMR.$$

All oxygen consumption data were analysed descriptively without the inclusion of statistical testing due to the constraint of all larvae being contained in a single tank during the rearing process and therefore not allowing for sufficient replication. Data were assessed for normality using visual distribution fitting and a Kolmogorov–Smirnov test at a significance level of $P = 0.05$. According to these tests, oxygen consumption data for each life stage were normally distributed ($P > 0.05$).

Results

1. Baseline metabolic rates

The RMR increased consistently throughout development barring a stable period during the pre-flexion stages (3–6 DAH) and a slight reduction during flexion (12–15 DAH) (Figure 2.1). RMR increased more rapidly from flexion during the later life stages (20–27 DAH). There appeared to be more individual variation in the early stages (hatchling, early pre-flexion and flexion) evidenced by coefficient of variation (CV) values being around 80% (Table 2.3). CV values decreased in the post-flexion stages (< 60%) and both SD and SE peaking during the settlement phase (Table 2.3).

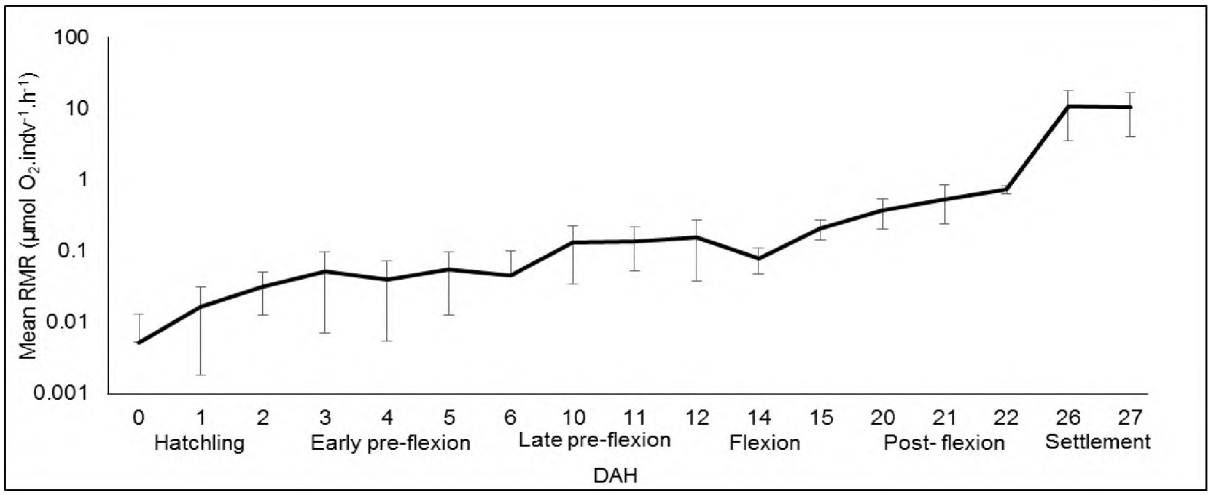

Figure 2.1: Routine metabolic rate (RMR) on a logarithmic scale throughout the early development of *Argyrosomus japonicus* (hatching to settlement stage). RMR is the mean metabolic rate (µmol O_2. $indv^{-1}.h^{-1}$ ± SD)

Both SMR and AMR remained relatively stable in early development. However, there was a dramatic increase in AMR during the settlement stage (22 DAH) when compared with the SMR (Figure 2.2). While the SMR increased gradually during the earlier life stages (0–22 DAH), the AMR peaked during the late pre-flexion stage (6–11 DAH), declined during the flexion stage (12–5 DAH) and increased rapidly after flexion (20–27 DAH, Figure 2.2).

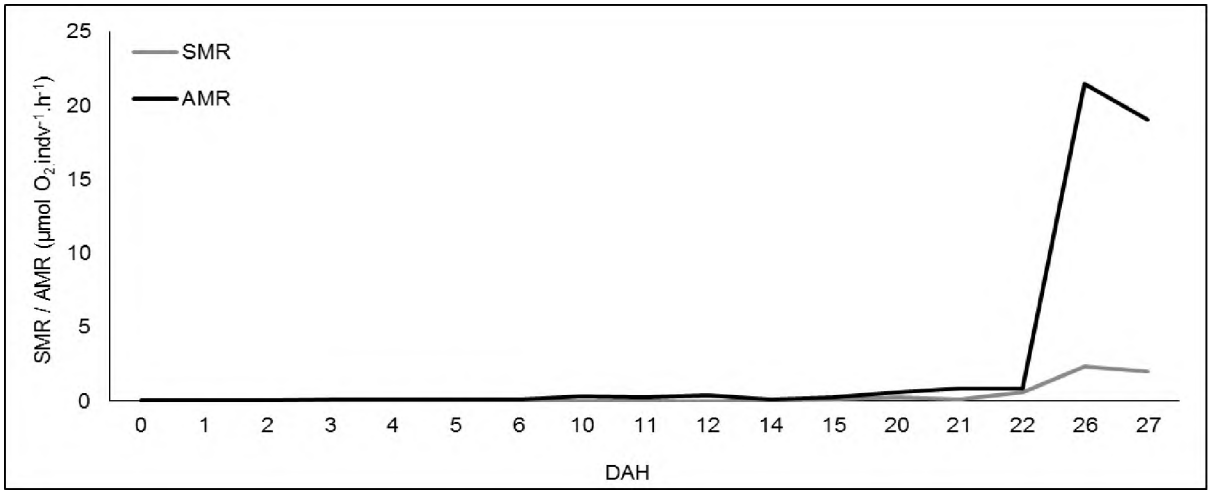

Figure 2.2: Standard and active metabolic rate throughout the development of *Argyrosomus japonicus* (hatching to settlement stage) as determined by the 5 and 95 percentiles of metabolic rates (µmol O_2. $indv^{-1}.h^{-1}$)

Metabolic scope was low during the early stages (0–6 DAH) and increased between 6 and 12 DAH. There was a distinct reduction in metabolic scope (~ 0.30 µmol O_2.indv^{-1}.h^{-1}) on day 14, which coincided with the beginning of flexion (Figure 2.3). This was attributed to a rapid decline in the AMR and a simultaneous increase in the SMR (Figure 2.3). The metabolic scope was highest following flexion (Figure 2.3).

The mean mass of larvae increased by 0.012 g from flexion to post-flexion and more rapidly by 0.088 g from post-flexion to settlement (Table 2.4). The mean mass specific metabolic rate declined slightly from the flexion to post flexion phase. However, it increased by more than five times by the settlement phase (Table 2.4). There was a significant, positive linear relationship (y = 0.3227x + 0.0011) between mass corrected standard metabolic rate and body mass (r^2 = 0.955; *P* = 0.00014) (Figure 2.4).

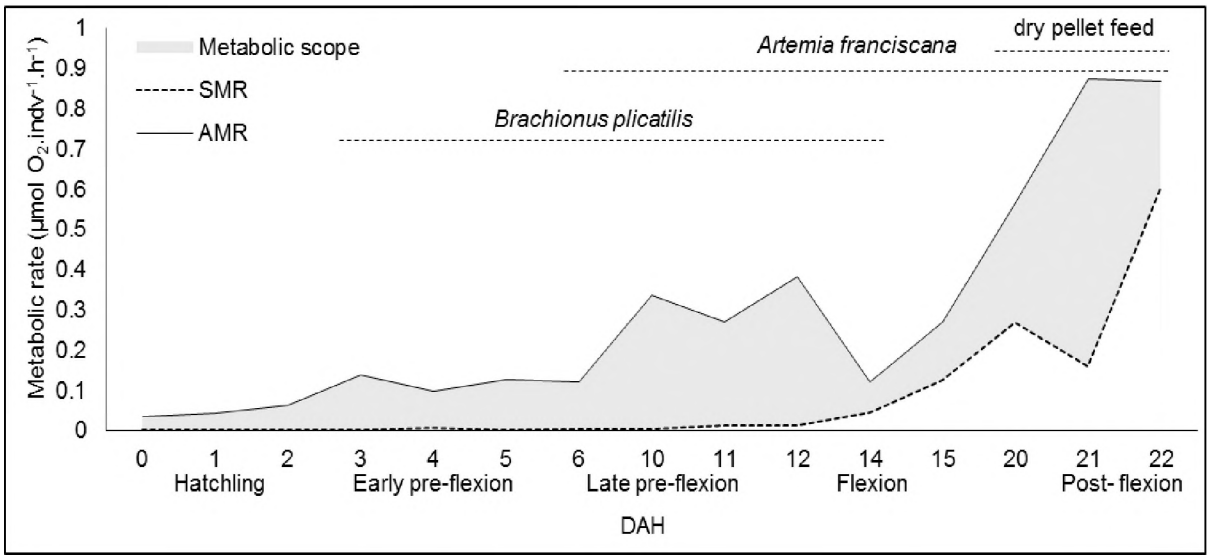

Figure 2.3: Standard metabolic rate, active metabolic rate and metabolic scope throughout the development of *Argyrosomus japonicus* (hatching to post-flexion stage) as determined by the 5 and 95 percentile of metabolic rate (µmol O_2. indv^{-1}.h^{-1}) including details on the time of introduction of different feed types

Table 2.3: Descriptive statistics for metabolic rates for each life stage in early stage *Argyrosomus japonicus* (µmol O_2.indv^{-1}.h^{-1})

Life stage	DAH	n	Mean	Median	Min	Max	SD	SE	CV (%)
Hatchling	0 – 2	98	0.022	0.018	0.00003	0.066	0.019	0.002	87.19
Early pre-flexion	3 – 6	105	0.049	0.041	0.00061	0.203	0.041	0.004	83.30
Late pre-flexion	10 - 12	99	0.139	0.121	0.00035	0.409	0.088	0.010	63.46
Flexion	14 - 16	13	0.132	0.127	0.00716	0.383	0.108	0.023	82.00
Post-flexion	20 - 22	21	0.590	0.647	0.15980	0.873	0.241	0.053	40.82
Settlement	26 - 27	13	10.471	8.338	2.00659	21.463	6.470	1.795	61.78

Table 2.4: Mass specific (wet weight) baseline metabolic rates for flexion, post-flexion and settlement stage *Argyrosomus japonicus* (mg O_2.g^{-1}.h^{-1})

Life stage	DAH	n	Mass (g)	Mean	Median	Min	Max	SD	SE
Flexion	14 - 16	12	0.0026	1.446	1.398	1.1106	2.0381	0.293	0.084
Post-flexion	20 - 22	21	0.0149	1.390	1.060	0.3902	6.6537	1.295	0.282
Settlement	26 - 27	13	0.1031	5.516	4.813	2.6707	11.673	2.610	0.724

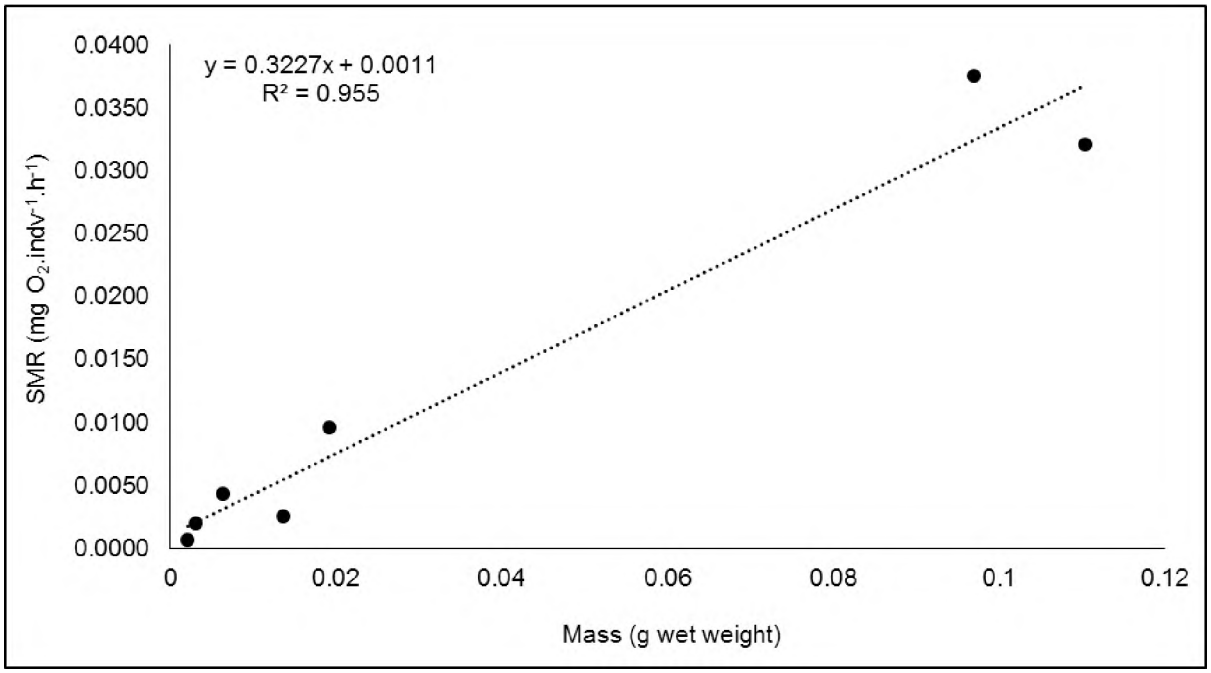

Figure 2.4: Mass corrected standard metabolic rate (mg O_2.g^{-1}.h^{-1}) of flexion, post-flexion and settlement stage *Argyrosomus japonicus* in relation to body mass (as g wet weight)

2. Diurnal metabolic rates

Average oxygen consumption was similar in the morning, afternoon and night for fish in the early life stages (from hatching to post flexion) (Figure 2.5). However, average oxygen consumption during the settlement stage was highest in the morning (12.78 µmol O_2.indv^{-1}.h^{-1}), followed by the afternoon (10.08 µmol O_2.indv^{-1}.h^{-1}) and night (5.87 µmol O_2.indv^{-1}.h^{-1}) (Figure 2.5). This resulted in a significant increase in the SMR during the daytime measurements in settlement stage *A. japonicus*.

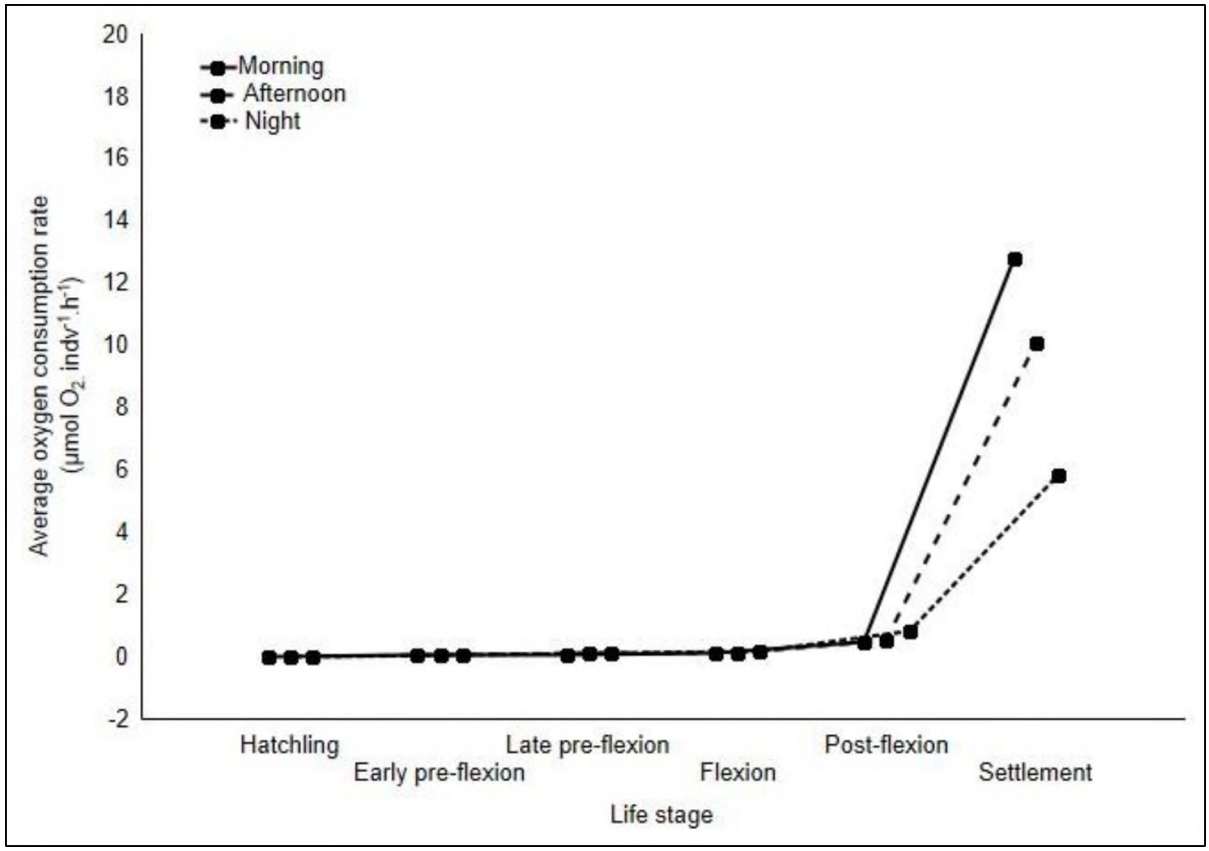

Figure 2.5: Average oxygen consumption rate of *Argyrosomus japonicus* larvae at different life stages during different times of the 24 hour photoperiod (µmol O_2. indv^{-1}.h^{-1})

Discussion

Existing research on the metabolism of *Argyrosomus japonicus* is limited to the larger juvenile stages (60–1 000 g) of Australian populations (Fitzgibbon et al. 2007; Pirozzi and Booth 2009) in order to determine the energetic requirements of juveniles for aquaculture. There have been no assessments of the changes in metabolic rates with ontogenetic development, or diurnal fluctuations in metabolism for this species.

Results from this study showed that there is distinct metabolic structuring during the early life stages of *A. japonicus* development, with the flexion (15 DAH) stage showing a significantly reduced metabolic scope. There was no variation in metabolic rates with photoperiod in the early life stages; however, metabolic rate was highest during daylight hours in settlement stage larvae.

The RMR (which is the average metabolic rate incorporating both AMR and SMR) increased with age and this is likely a result of the increased activity (represented by AMR) and energetic demands (represented by SMR) associated with rapid development in the early stages (Peck and Moyano 2016). A similar pattern was observed in the RMR of larval striped mullet (*Mugil cephalus*) during early development (Walsh et al. 1989). In *A. japonicus*, the initial increase in RMR appears to be most rapid after hatching, after which it increases at a more gradual rate in the later stages (early pre-flexion to flexion). In the early stages of development (prior to flexion), changes in metabolism were the least pronounced.

Interestingly, there appeared to be a slight decline in RMR during flexion, corresponding with a large decline in the active metabolic rate (AMR). Because AMR decreases rapidly during the flexion stage and SMR continues to increase there is a reduced metabolic scope during this stage. This is similar to the findings for gilthead seabream (*Sparus aurata*) (Parra and Yúfera 2001), winter flounder (*Pseudopleuronectes americanus*) (Laurence 1975) and olive flounder (*Paralichthys olivaceus*) (Kurokura et al. 1995), which showed reduced metabolic rate during metamorphosis. Similarly, Killen et al. (2007) found that shorthorn sculpin (*Myoxocephalus scorpius*) had the lowest metabolic scope just after metamorphosis. It has been suggested that there is an increase in energetic demand during periods of rapid morphological development (von Herbing and Boutilier 1996; Killen et al. 2007) and that larval fish potentially manage this demand by reducing activity during this time (Parra and Yúfera 2001).

The reduced metabolic scope observed during flexion suggests that energy bottlenecks (depressions in metabolic scope) occur during periods when energy is being allocated, specifically to development, thereby limiting the energy available for other energetically costly activities. Such activities could include the maintenance of

homeostasis in changing environmental conditions (Killen et al. 2007). The flexion stage is frequently reported as the period of highest mortality in aquaculture of *A. japonicus* (Grant L., *pers. comm.*, April 2016), which most likely occurs as a result of these bottlenecks. The recognition of energy bottlenecks is necessary as it allows for the successful identification of key life stages that should be considered when assessing vulnerability to changes in environmental conditions. This response may be species specific. For example, the highest metabolic rate for the larvae of red sea bream (*Pagrus major*) was observed during metamorphosis, suggesting that they have high energy availability during flexion (Ishibashi et al. 2005). This suggests that species-specific information is required to determine the timing of energy bottlenecks during early development. To do this the measurement of metabolic scope, which represents the energy available for adaptation, is critical as life stages with a limited metabolic scope often exhibit high mortality rates (Bailey and Houde 1989) and recruitment bottlenecks during unfavourable environmental conditions (Killen et al. 2007).

The metabolic scope of *A. japonicus* increased after flexion, with the highest values observed at settlement. Killen et al. (2007) found that juveniles of three marine species (ocean pout, *Macrozoarces americanus*; lumpfish, *Cyclopterus lumpus*; and shorthorn sculpin, *Myoxocephalus scorpius*) had a considerably higher metabolic scope than the larval stages. They attributed this to a rapid increase in AMR during the early juvenile phase when individuals begin swimming actively. In the case of many estuarine dependent marine species, this also coincides with the time that individuals begin recruiting into estuaries (e.g. Griffiths 1996; Cowley et al. 2008). The elevated metabolic scope associated with this developmental stage in *A. japonicus* could facilitate the physical demands required to navigate to estuaries and the tolerance that is required to withstand the highly fluctuating estuarine environments.

It is possible that changes in metabolic rate in early-stage *A. japonicus* reflect changes in activity of fish during early development (von Herbing and Boutilier 1996). Although this study did not specifically measure activity of the individual due to difficulty of observing the small larvae in the respirometry chambers, the link between metabolic rate and swimming has been made in a study by von Herbing and Boutilier (1996) on larger Atlantic cod larvae. They found a positive relationship between metabolic rate

and swimming activity, although this was only observed in the life stages following flexion (von Herbing and Boutilier 1996). This relationship was attributed to feeding activity and the onset of morphological development during flexion (von Herbing and Boutilier 1996).

In this study, the AMR seemed to respond to the addition of different food types. It is possible that this could be attributed to the different activity patterns required to capture different prey types. This is further supported by the views of Burggren and Blank (2009) who found that the onset of feeding increased both metabolic rate and swimming activity in larvae. It is likely that the larvae are able to consume and capture different prey items during development due to their increased metabolic capacity in conjunction with better developed swimming apparatus.

In addition to determining the reference metabolic levels of early stage *A. japonicus*, the relationship between metabolic rate and mass was also explored. The dependence of metabolic rate on organism mass is fairly well understood and the scaling of metabolic rate with mass seems to be relatively universal among all animal species and this phenomenon is termed "allometric law" (Giguere et al. 1988). However, the scaling of metabolic rates with body mass in fish is poorly understood (Killen et al. 2007). Studies that have attempted to assess the mass specific metabolism in larval fishes have reported vast differences in mass dependence (Post and Lee 1996), and it has been suggested that the relationship between body mass and metabolic rate in fish larvae may vary among species (Burggren and Blank 2009). In this study, there was a positive linear relationship between SMR and body mass. Similar results have been described in the early stages of other marine fish species such as the short spined sea scorpion, *Myoxocephalus scorpius,* lumpfish *Cyclopterus lumpus* and eelpout, *Macrozoarces americanus* in a metabolic study by Killen et al. (2007) and also in the eggs and larvae of bay anchovy, *Anchoa mitchilli*, sea bream, *Archosargus rhomboidalis* and lined sole, *Achiris lineatus* (Houde and Schekter 1983). However, Killen et al (2007) stressed that the lack of standardised methods limits our current understanding of mass-dependent metabolic rates in larval fish.

This study also assessed the variation in metabolic rate within the 24 hour photoperiod and provided the first comprehensive assessment of the diurnal rhythmic changes in metabolic rate throughout the early development of *Argyrosomus japonicus*. There

were no clear changes in metabolic rate during the morning, afternoon and night in the early stages (hatching to post-flexion). This suggests that no cyclical changes in activity occur in response to day/night rhythms. This result is contradictory to the findings suggested for numerous adult marine fish species. Black sea mullet (*Liza saliens*) (Shekk 1986), flounder (*Paralichthys olivaceus*) (Liu et al. 1997), Mediterranean yellowtail (*Seriola dumerili*) (De la Gándara et al. 2002), white steenbras (*Lithognathus lithognathus*) (Kandjou and Kaiser 2014) and the estuarine dependent spotted grunter (*Pomadasys commersonnii*) (Radull et al. 2002) have all shown diurnal changes in metabolic rate. However, it is important to consider that all these studies have only been conducted on the adult stages of fish and no study has previously identified diurnal rhythms in metabolism in the larval stages.

Metabolic rate was highest during daylight hours in settlement stage larvae. Because measurements of metabolic rates reflect activity, this finding suggests that settlement stage *A. japonicus* are more active during daylight than at night. The variation in metabolic rate in settlement stage *A. japonicus* among time of day is potentially explained by the findings in a study by Ballagh (2011) which suggests that optimal feeding of early juvenile (10–50 mm TL) *A. japonicus* (fed on a pelleted diet) occurred when all sensory functions were available in daylight conditions in *A. japonicus*. It is also thought that settlement stage larvae may be attracted to a turbidity plume, which is likely identified using vision, when recruiting into estuaries (Wilsnaugh 2016). The measurement of diurnal variation in metabolism in this study was done with the intention to determine at which time of day metabolic measurements should be taken. The results imply that, because activity does not change over the 24 hour photoperiod in the early stages of *A. japonicus* raised in a controlled laboratory environment, metabolic measurements are not restricted to any specific time of day, barring in the settlement stage when readings should be taken at night in order to avoid the over-estimation of SMR.

Conclusion

The results from this study provide a detailed description of the metabolic structure of *Argyrosomus japonicus* throughout early ontogenetic development. This contributes to our limited understanding of metabolism in fish larvae and provides insight into the

physiological bottlenecks in development that may render the species vulnerable to environmental change. The study also provided the first insight into the diurnal metabolic fluctuations for the early life stages of the species, which is required to optimise studies examining the metabolic structure of the species. Future research should aim to examine the metabolic structure through the life stages of the species. This baseline data are essential if further population level questions are to be understood.

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Chapter Three

The effects of CO_2 induced seawater acidification on metabolism in early stage Argyrosomus japonicus

Introduction

Ocean acidification occurs as a result of increasing atmospheric CO_2, which results in a direct increase in dissolved CO_2 in seawater as the ocean absorbs this gas to remain at equilibrium with the atmosphere (Henry's Law) (Pörtner et al. 2004). Recent research suggests that some fish species are able to compensate for oxygen demands by managing acid-base regulation (Michaelidis et al. 2007; Munday et al. 2009a). However, this compensation may come at an energetic cost, affecting physiological functions such as metabolic rates (Michaelidis et al. 2007; Melzner et al. 2009b). Increases in the metabolic rate of fishes exposed to acidification have been attributed to an increase in energetic requirements to maintain internal acid-base balance as blood pH declines. A decline in internal pH and increase in CO_2 reduces the ability of blood to retain O_2 and is defined as the Bohr effect (Crocker and Cech Jr 1997; Ishimatsu et al. 2005; Michaelidis et al. 2007). This results in increased oxygen demands to which organisms have to respond physiologically in order to maintain functionality. As a result, many organisms attempt to regulate internal pH in order to avoid physiological stress. Acid-base regulation, however, is limited by energy availability, a concept similar to the oxygen and capacity limited thermal tolerance model (OCLTT) as described by Pörtner H-O (2010). As a result, shifts in metabolic energy allocation occur in order to maintain internal acid-base balance. The re-allocation of energy from various physiological sources differentially affects the energy available for survival, growth and additional activities (Michaelidis et al. 2007).

Several studies have found that increased concentrations of CO_2 in seawater directly affect the physiology and metabolism of fish species; however, tolerance to high pCO_2 appears to be species specific, with species showing either an increase or decrease in metabolic scope in response to acidification (e.g. Munday et al. 2009a, Michaelidis et al. 2007, Gräns et al. 2014). Due to the contrasting responses to changing pH, species specific research on the physiological response to changes in environmental

conditions as a result of climate change is necessary (Rijnsdorp et al. 2009; Ern and Esbaugh 2016).

The early developmental life stages of fish have been identified as the most vulnerable to ocean acidification (Ishimatsu et al. 2005; Munday et al. 2009a; Rijnsdorp et al. 2009; Pörtner and Peck 2010; Ern and Esbaugh 2016), with adult and juvenile fish showing higher tolerance to high treatments of pCO_2 than larvae (Stiasny et al. 2016). The tolerance of the adult and juvenile fish to ocean acidification has been attributed, in part, to their relatively high metabolic tolerance to increased CO_2 concentrations in seawater (Kikkawa et al. 2003; Cattano et al. 2016; Murray et al. 2016) when compared to the egg, larval and juvenile stages (Ishimatsu et al. 2005; Killen et al. 2007; Guinotte and Fabry 2008; Murray et al. 2016). Based on this, several authors have concluded that the vulnerability of the early life stages can be attributed to a less developed acid-base regulation system (Melzner et al. 2009b; Frommel et al. 2013; Murray et al. 2016; Stiasny et al. 2016). Larval sensitivity to ocean acidification is also common in many invertebrate species which, more often than not, show negative responses in growth and survival in the larval stages (Kroeker et al. 2010).

It is thought that estuarine-associated species may be physiologically tolerant to pH fluctuations due to the variable pH conditions in estuarine environments (Ringwood and Keppler 2002). Indeed, Lonthair et al. (2017) found that red drum (*Sciaenops ocellatus*), which has an egg and larval estuarine phase, were tolerant of ocean acidification. No studies have been conducted on the early life stages of estuarine dependent species, and this presents a major gap in our understanding as the early life stages occupy the marine environment.

The aim of this study was to compare the metabolic response of early life stages of the estuarine dependent *Argyrosomus japonicus* exposed to ambient levels of pH and those predicted for 2050 and 2100. This type of research has never been undertaken in South Africa and will provide the first indication of how a popular fishery resource will be affected by ocean acidification. It was hypothesised that:

1) Increased pCO_2 in seawater, and the resultant decline in pH, would alter the metabolic structure of larval and early juvenile *A. japonicus* through a reduction in metabolic scope.

2) This reduction in metabolic scope would be caused by an elevation in standard metabolic rate.
3) These differences would be significant in the flexion life stage, which was shown to have the lowest metabolic scope (Chapter 2).

Materials and methods

1. Experimental animals

Fertilised *Argyrosomus japonicus* eggs from a first generation, wild, spawning broodstock were obtained from the PureOcean aquaculture facility in East London (Eastern Cape, South Africa). The fertilized eggs were stocked directly into nine 800 L static systems at a stocking density of 15 larvae per litre. To do this, fertilized eggs were mixed to ensure a uniform distribution in a five litre bucket and the density (by volume) was estimated from 20 replicate 2 ml samples. The seawater in each tank was treated with 12.5% Sodium Hypochlorite neutralized with 3.6 g Thiosulfate as well as activated carbon to prevent bacterial and algal fouling and to remove any toxic contamination before the initiation of the experiment. This practice is standard for *Argyrosomus japonicus* aquaculture in South Africa. Ammonia and nitrite levels were measured daily using a Palintest Photometer (Benchtop Kit 7100) and relevant tablet reagents. Water quality was maintained at desired levels (ammonia and nitrite < 1 $mg.l^{-1}$ NH_4 and NO_2) by siphoning out debris from the bottom of the tanks and replacing 10–20% of the seawater every two days or when necessary.

Larvae were fed according to feeding protocols that have been developed for the optimal growth of *Argyrosomus japonicus* larvae in aquaculture. Larvae were fed with rotifers *Brachionus plicatilis* enriched with dead algal paste consisting of the species *Nanno chloropsis* (Nanno 3600, Reed Mariculture inc.). Dead algal cells were used to avoid pH fluctuations related to respiration that would be unrelated to the treatment of CO_2. Extra *Nanno chloropsis* (± 1 ml/ day to achieve an estimated concentration of 1 ml per million rotifers) was also added to maintain desired turbidity in the tanks to ensure a dark background, which ensures optimum feeding conditions for the larvae (Chesney 2005; Shields and Lupatsch 2012). The larvae were weaned onto a diet of capsulated *Artemia franciscana* at 6 DAH (days after hatch) and then onto a pelleted diet (Skretting GEMMA Micro, 1 mm) at 18 DAH. *Brachionus plicatilis* and hatched

Artemia franciscana were provided in excess and maintained at an estimated concentration of 2–5 individuals per ml, which was checked at regular intervals throughout the day to ensure consistent availability of food. Rotifer feeding was terminated at 13 DAH and *Artemia* spp. at 24 DAH. Once weaned onto the pelleted diet, the larvae were fed regularly throughout the day (every two hours).

2. Experimental treatments

The nine rearing tanks were allocated one of three different pH treatments, with three replicate tanks per treatment in a randomised block design (Figure 3.1). The three CO_2 treatments were based on the IPCC business as usual emission scenario RCP 8.5 (Riahi et al. 2011) and included a low (pH 8.15, untreated seawater), moderate CO_2 treatment level, predicted for the year 2050 (pH 8.03) and a high CO_2 treatment level, predicted for the year 2100 (pH 7.78) (Table 3.1).The pH treatments in each tank were maintained by trickling regulated amounts of CO_2 from a single CO_2 cylinder. The inflow of CO_2 was controlled using pH regulators, which measured pH in each tank with a regularly calibrated glass pH electrode (TUNZE) and regulated the inflow of CO_2 through a solenoid valve (TUNZE 7070/2, Germany). Temperature and pH (total scale) were measured three times daily (Aqualytic AL15 multi-parameter meter) and total alkalinity was measured every three days using an automated titration system (Hannah instruments HI84531 mini-titrator) using an endpoint titration method to pH 8.30 (phenolphthalein) and 4.5 (bromcresol green-methyl red). Seawater pCO_2 concentration and associated saturation states of Ω Ca and Ω Ar were determined using the program CO_2SYS (Lewis and Wallace 1998) on the basis of measured input parameters, pH and total alkalinity (set of constants of Merbach et al. 1973, refitted by Dickson and Millero 1987); $KHSO_4$ by Dickson; Total pH scale ($mol.kg^{-1}$-H_2O).

Salinity was maintained at 35 (seawater) and temperature at ~23.50 ± 0.50 °C (mean ± SD), which are the optimal rearing conditions for *A. japonicus.* Dissolved oxygen was maintained at 6.6 $mg.l^{-1}$ to ensure 100% saturation in the seawater. Turbidity was maintained at just under 1 m Secchi disk reading to optimise the feeding of the larvae. Photoperiod was maintained at 16 L: 8D using an automatic timer in order to replicate the photoperiod experienced by the larvae at the time of spawning in their natural environment.

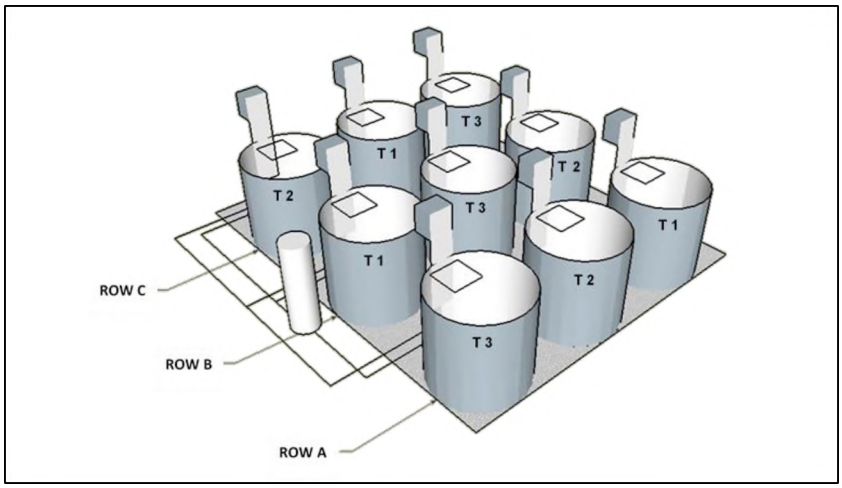


Figure 3.1. Schematic diagram of randomised block experimental design indicating the pCO_2 treatments in each tank. T1 = low at 8.15 pH; T2 = moderate at 8.03 pH; T3 = high at 7.78 pH.

3. Metabolic measurements

Static respirometry was used to determine the oxygen consumption rate in the *A. japonicus* larvae throughout early development. One individual larva was placed in each respirometry chamber and oxygen uptake was measured. Small volume respirometry trials measured six larvae from each treatment per trial and included measurements of six blank chambers to account for drift (24 chambers per trial). These chambers were used from hatching until the flexion stage. In the larger volume respirometers one larva per treatment and one blank chamber were measured (four respirometry chambers per trial). Volume of the respirometry chambers and duration of the trials were adjusted based on the rate of oxygen uptake.

Seawater used for respirometry trials was sterilised using sodium hypochlorite (neutralised with thiosulfate) and UV sterilisation prior to each trial. Larvae were allowed to acclimate to the seawater and measurement temperatures for 2–6 hours (depending on size), also allowing for complete gut evacuation. Two to three trials were conducted per day over three consecutive days per life stage. Most trials lasted between 40 and 60 minutes, and oxygen concentration readings were taken every 30 seconds. Details on the respirometry methods are given in Chapter Two.

After each trial, the larvae were removed from each chamber and euthanised with an overdose of 2-phenoxyethanol and stored in 5% formalin solution for later measurements. The larvae were categorised by life stage and measured to the nearest 0.001 mm total length (TL) using a stereomicroscope at 36X magnification with a digital camera (BestScope BS 3040, 5 MP camera).

4. Statistical analysis

Physico-chemical data did not meet the assumptions of normality and therefore physico-chemical variability was compared among the three pCO_2 treatments using a Kruskal–Wallis test.

Data on body length was found to show heteroscedasticity and was therefore log transformed prior to regression analysis. Data on body length were compared among treatments using analysis of covariance (ANCOVA) with age (DAH) as a covariate. A Tukey post-hoc test was used to identify which treatment combinations contributed to the differences observed.

Metabolic rate was calculated from the oxygen consumption data. Readings taken in the first five minutes and any readings below 80% oxygen saturation (< 5.3 mg L^{-1}) of saturated seawater were removed before analysis to account for acclimation to the respirometry chamber and stress induced by oxygen limitation respectively. A least squares linear regression of oxygen concentration over time was calculated. Residual analysis was applied to ensure that the data met the assumptions of a regression analysis. The regression model was used to calculate ΔO_2 and the following equation was used to calculate oxygen consumption (mgO_2. $indv^{-1}$. h^{-1}) independent of mass for each life stage: $MO_2 = \frac{[\Delta O_2] \times vol}{t}$, where, $\Delta[O_2]$ is the linear decline of oxygen concentration in the water(Δ mg O_2), t is the total recording time (h), vol is the volume of the respirometer (L). The average oxygen consumption values for the blank chambers were then subtracted from the final calculated oxygen consumption values for each individual to account for background respiration in the seawater.

Standard (SMR) and active (AMR) metabolic rate were determined from the calculated oxygen consumption rate by using the 5% and 95% percentile (Reid et al. 2012;

Kandjou and Kaiser 2014) of all data obtained during each measurement period per treatment. The RMR was calculated by averaging all remaining metabolic rates obtained in the 18 hour measurement period (Kandjou and Kaiser, 2014). Metabolic scope was then calculated using the following equation: $Metabolic\ scope\ (MS) = AMR - SMR.$

Data for each life stage were analysed separately. Data were assessed for normality and homogeneity of variance using Shapiro–Wilk and Levene's tests at a significance level of $P = 0.05$. Analysis of co-variance (ANCOVA) was used to assess the effect of pCO_2 treatments on metabolic rate with total length (mm) as a covariate. All assumptions of ANCOVA were tested prior to analysis in order to ensure the data was appropriate for this analysis. All analyses were conducted using Statistica 13 (Dell Inc. 2015).

Results

1. Physico-chemical conditions

Treatment levels of pCO_2 and values for associated carbonate system species were calculated based on seawater pH, the mean and standard deviation of treatment parameters are displayed in Table 3.1. Although temperature generally fluctuated around a mean of 23.50 ± 0.05°C, it declined by ± 2 °C from 8–11 DAH in the high and low treatments and by ± 1 °C in the moderate treatment and then increased again and declined by ± 1 °C again in all treatments at day 26 DAH (Figure 3.2). However, analysis of variance (ANOVA) revealed no significant difference in temperature among treatments (n = 125; $H = 1.01$; $P = 0.60$). Total alkalinity also did not differ significantly among treatments whereas pH (n = 125; $H = 92.21$; $P < 0.01$) (Figure 3.3), and therefore pCO_2 (n = 125; $H = 91.73$; $P < 0.01$) and associated parameters (Ω Ca and Ω Ar), did differ significantly among treatments ($P < 0.05$).

Table 3.1. The pCO_2 treatment conditions and associated speciation values of the carbonate system maintained throughout the duration of the study. The pCO_2 values were calculated from pH (total scale) and A_T using the program CO_2SYS. All values are displayed as means ±S.D. Parameters that differed significantly among treatment indicated in **bold*** ($P < 0.05$).

	P	**Low**	**Moderate**	**High**
Temperature (°C)	0.6	23.57 ± 0.70	23.65 ± 0.53	23.49 ± 0.74
pH	**< 0.01***	8.16 ± 0.08	8.01 ± 0.04	7.78 ± 0.06
T_A (µmol $HCO3^-.kg^{-1}SW$)	0.08	2561.30 ± 190.35	2484.70 ± 133.68	2494.10 ± 124.33
pCO_2 (µatm)	**< 0.01***	327.50 ± 80.07	477.40 ± 59.46	910.20 ± 136.45
Ω Ca	**< 0.01***	5.33 ± 0.97	3.88 ± 0.41	2.42 ± 0.27
Ω Ar	**< 0.01***	3.50 ± 0.64	2.54 ± 0.27	1.59 ± 0.28

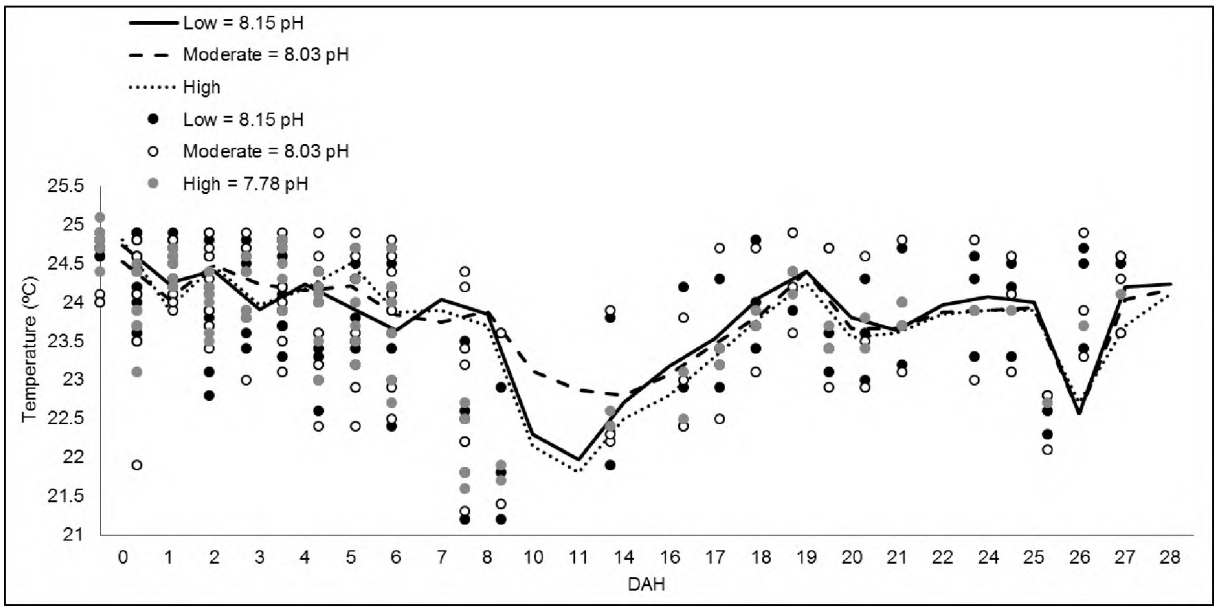


Figure 3.2. Mean temperature of each treatment throughout the duration of the study (DAH = days after hatch).

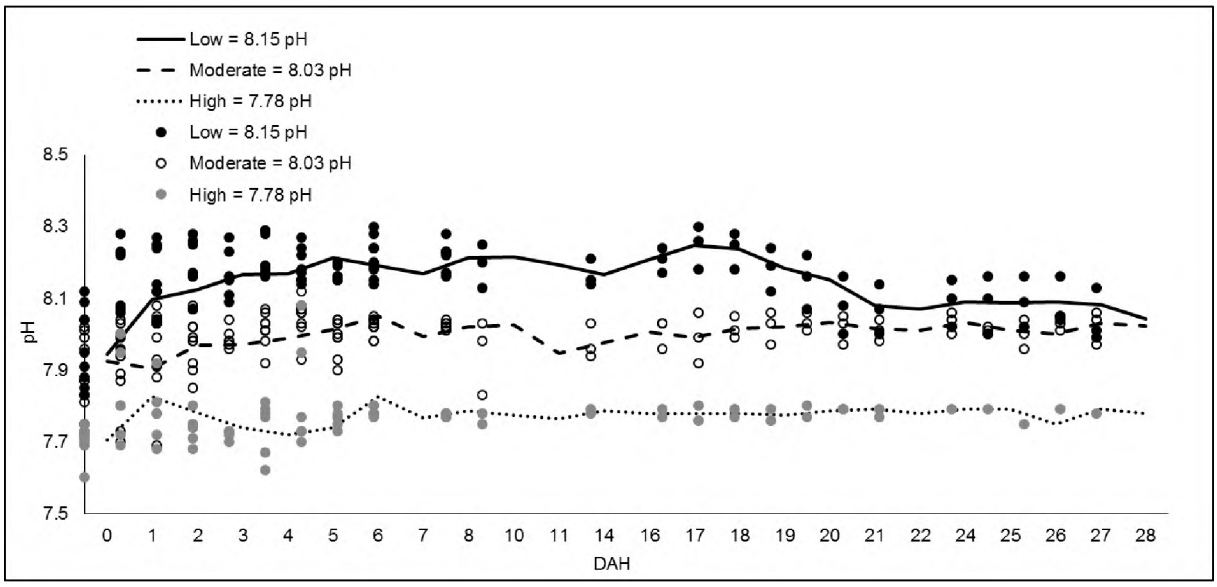


Figure 3.3. Mean pH of each treatment throughout the duration of the study (DAH = days after hatch).

2. Body length of larvae raised under different pCO_2 treatments

There was a linear relationship between larval size (TL) and age (DAH) in all three pCO_2 treatments ($R^2 > 0.9$) (Figure 3.4). There was a significant effect of age and treatment on total length (ANCOVA - $P < 0.01$). A similar growth rate occured in the low ($y = 0.024x + 0.35$; $R^2 = 0.96$) and moderate ($y = 0.025x + 0.34$; $R^2 = 0.95$) pCO_2 treatment larvae and these growth rates were higher compared to larvae from the high ($y = 0.018x + 0.36$; $R^2 = 0.93$) pCO_2 treatment (Figure 3.4). This finding is confirmed by the Tukey post-hoc test, which revealed that the high pCO_2 treatment differed significantly from both the low treatment and the moderate pCO_2 treatment ($P < 0.01$). There was no difference between the growth rate in the low and moderate pCO_2 treatment larvae. A larger variation in size was observed in the later life stages (22–28 DAH) compared to the earlier life stages (0–22 DAH), where length was more uniform suggesting that size disparity increases in the later developmental stages (Figure 3.4). No larvae were available for measurement from 22–28 DAH in the high pCO_2 treatment, as there were no survivors in this treatment from 22 DAH (Figure 3.4).

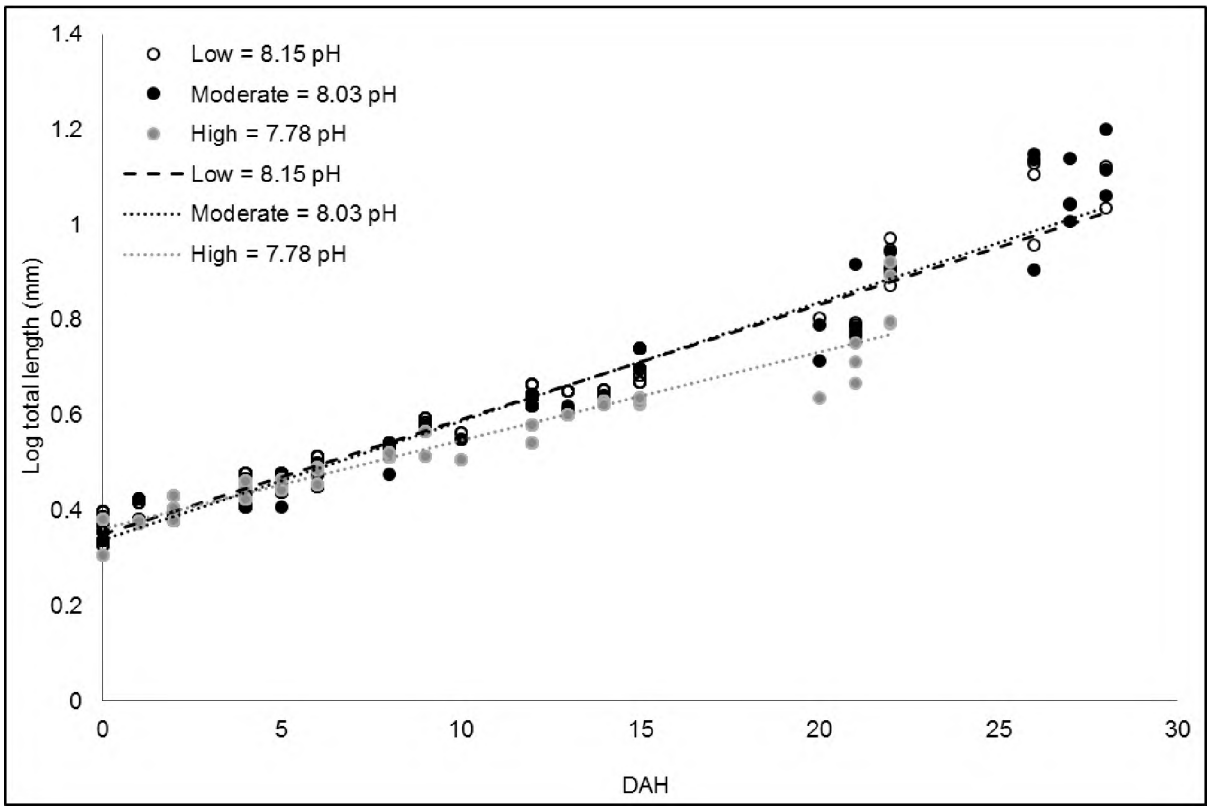


Figure 3.4. Total length (TL) of early stage *A. japonicus* throughout early development (0–28 DAH).

3. Effect of pCO_2 treatments on metabolic rate

Metabolic rates in early stage *Argyrosomus japonicus* raised under all three pCO_2 treatments showed similar metabolic structuring as the larvae studied in Chapter Two (Figure 2.3). Metabolic scope was reduced during flexion in all three pCO_2 treatments, with treatment having no significant effect on metabolic scope during this life stage (15 DAH). The SMR and AMR increased rapidly in the post-flexion stages (Figures 3.6 and 3.7). There was increasing variability in RMR with age, with low variability in the early life stages (CV < 13%) and the highest variability occurring in the post-flexion (CV = 18 -23%) and settlement stages (CV = 14 – 20%) (Table 3.2).

Table 3.2. Descriptive statistics of RMR (μmol O_2.indv^{-1}.h^{-1}) for each life stage from hatching to settlement (0–28 DAH)

Life stage	DAH	Treatment	n	Mean	Min	Max	SD	SE	CV (%)
Hatchlings	0 - 2	Low	41	2.43	2.13	2.62	0.12	0.02	4.94
		Moderate	40	2.41	2.17	2.65	0.15	0.02	6.22
		High	40	2.41	2.03	2.70	0.20	0.03	8.30
Early pre-flexion	3 - 6	Low	41	2.93	2.73	3.26	0.17	0.03	5.80
		Moderate	44	2.88	2.56	3.16	0.20	0.03	6.94
		High	48	2.88	2.66	3.10	0.13	0.02	4.51
Late pre-flexion	10 - 12	Low	45	3.84	3.27	4.61	0.46	0.07	11.98
		Moderate	45	3.74	3.00	4.32	0.34	0.05	9.09
		High	50	3.42	3.22	3.80	0.21	0.03	6.14
Flexion	14 - 16	Low	27	4.56	4.25	4.84	0.18	0.03	3.95
		Moderate	29	4.59	4.08	5.50	0.57	0.11	12.42
		High	29	4.15	4.00	4.33	0.13	0.03	3.13
Post flexion	20 - 22	Low	8	7.32	5.96	9.36	1.34	0.47	18.31
		Moderate	9	7.13	5.17	8.79	1.30	0.43	18.23
		High	8	6.05	4.33	8.35	1.43	0.51	23.64
Settlement	26 - 28	Low	6	11.73	9.04	13.44	1.71	0.70	14.58
		Moderate	9	12.35	8.03	15.89	2.39	0.80	19.35
		High	-	-	-	-	-	-	-

There was no significant effect of pCO_2 treatment on RMR, AMR and metabolic scope in any of the life stages (ANCOVA, $P > 0.05$) (Table 3.3). The RMR was also similar between the treatments (Figure 3.5). There was no significant effect of treatment on the SMR up to the flexion stage (Table 3.3). However, there was a significant effect of treatment on SMR in the post-flexion stage ($P = 0.02$), with a 32.5% and 9.5% reduction in the moderate and high pCO_2 treatments, respectively, when compared with the low (Figure 3.6).

Table 3.3. Analysis of Covariance results (P) for metabolic rates of larvae raised under the three pCO_2 treatment conditions. RMR = routine metabolic rate; SMR = Standard metabolic rate; AMR = active metabolic rate; DAH = days after hatch. Significant values in ***bold**.

Life stage	Metabolic rate	TL (mm)	Treatment
Hatchling	RMR	0.15	0.79
	SMR	0.58	0.87
	AMR	0.15	0.64
	Metabolic scope	0.29	0.79
Early pre-flexion	RMR	***0.01**	0.90
	SMR	0.11	0.63
	AMR	0.15	0.80
	Metabolic scope	0.37	0.82
Late pre-flexion	RMR	0.06	0.94
	SMR	0.66	0.92
	AMR	***0.02**	0.50
	Metabolic scope	***0.02**	0.43
Flexion	RMR	1.00	0.86
	SMR	0.79	0.80
	AMR	0.71	0.97
	Metabolic scope	0.71	0.97
Post-flexion	RMR	**0.03**	0.28
	SMR	***<0.01**	***<0.01**
	AMR	0.47	0.89
	Metabolic scope	0.33	0.46

The AMR did not differ significantly among treatment in any of the life stages; however, when compared with the low and medium treatment, there was no peak in metabolic rate in the high pCO_2 treatment at 21 DAH (Figure 3.7). The metabolic scope was 64% lower on 21 DAH when compared to the larvae in the low treatment (Figure 3.8). Interestingly, there was a mass mortality of larvae in the high pCO_2 treatment on 21 DAH, and all larvae had died by 22 DAH.

The larvae in the moderate treatment had the highest metabolic scope at 21 DAH (Figure 3.8). These larvae also grew the fastest, although there was no significant difference in the growth between the larvae from the low and the medium treatment

(Figure 3.4). In contrast, the larvae raised in the high pCO_2 treatment showed the lowest metabolic scope at 21 DAH (Figure 3.8) and these larvae showed significantly reduced growth (Figure 3.4).

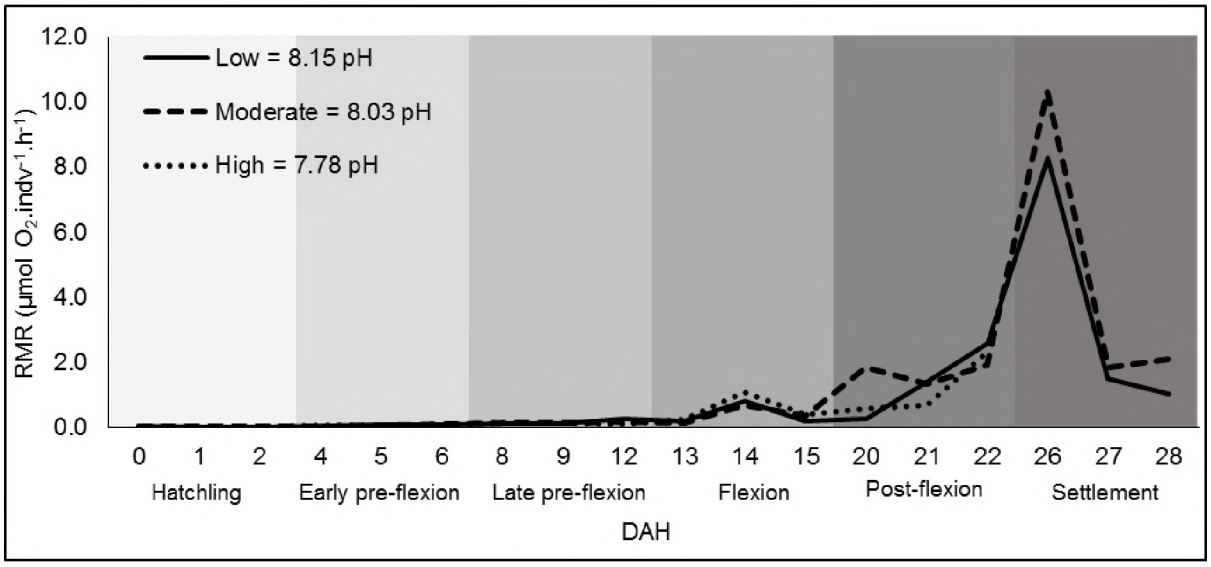


Figure 3.5. Routine metabolic rate for larvae raised in each pCO_2 treatment from age 0–22 DAH (days after hatch)

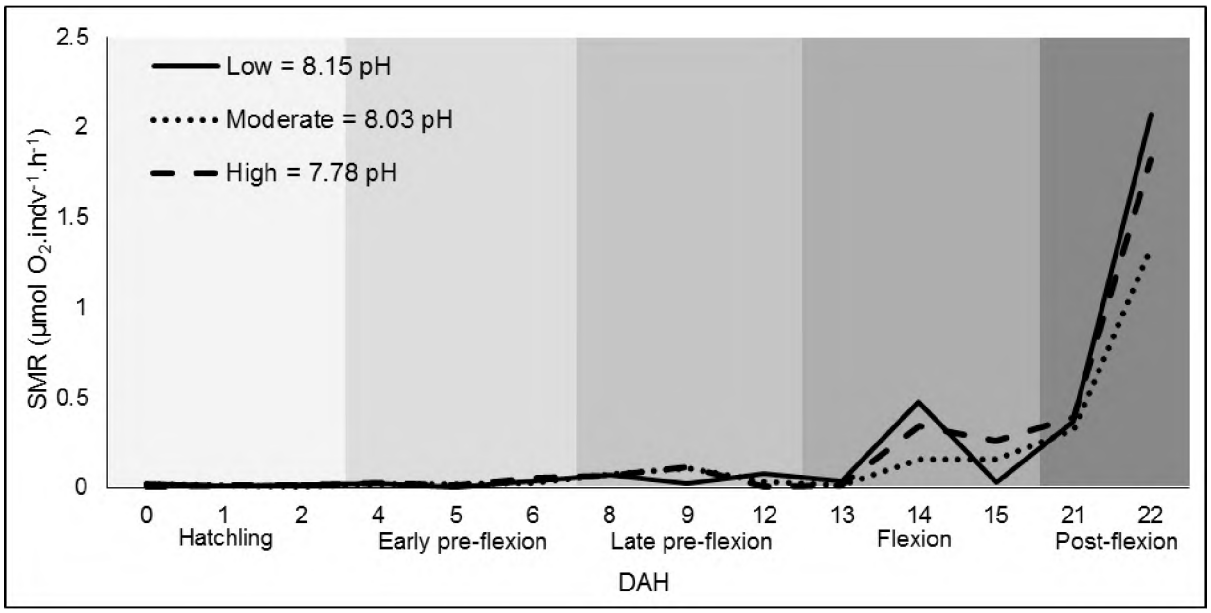


Figure 3.6. Standard metabolic rate for larvae raised in each pCO_2 treatment from age 0–22 DAH (days after hatch)

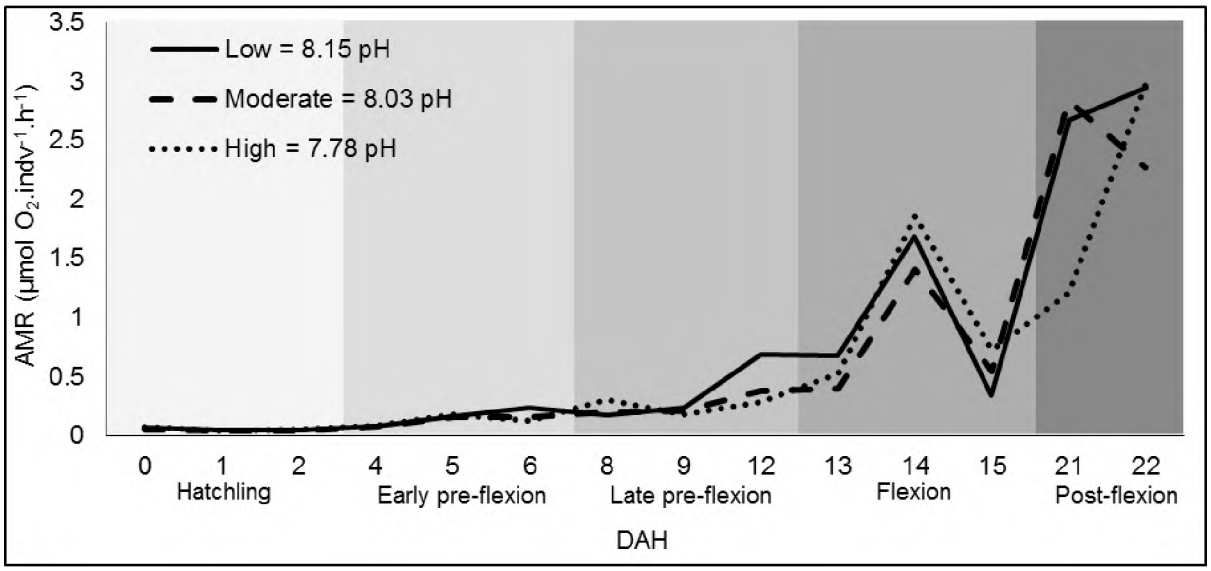


Figure 3.7. Active metabolic rate for larvae raised in each pCO_2 treatment from age 0–22 DAH (days after hatch)

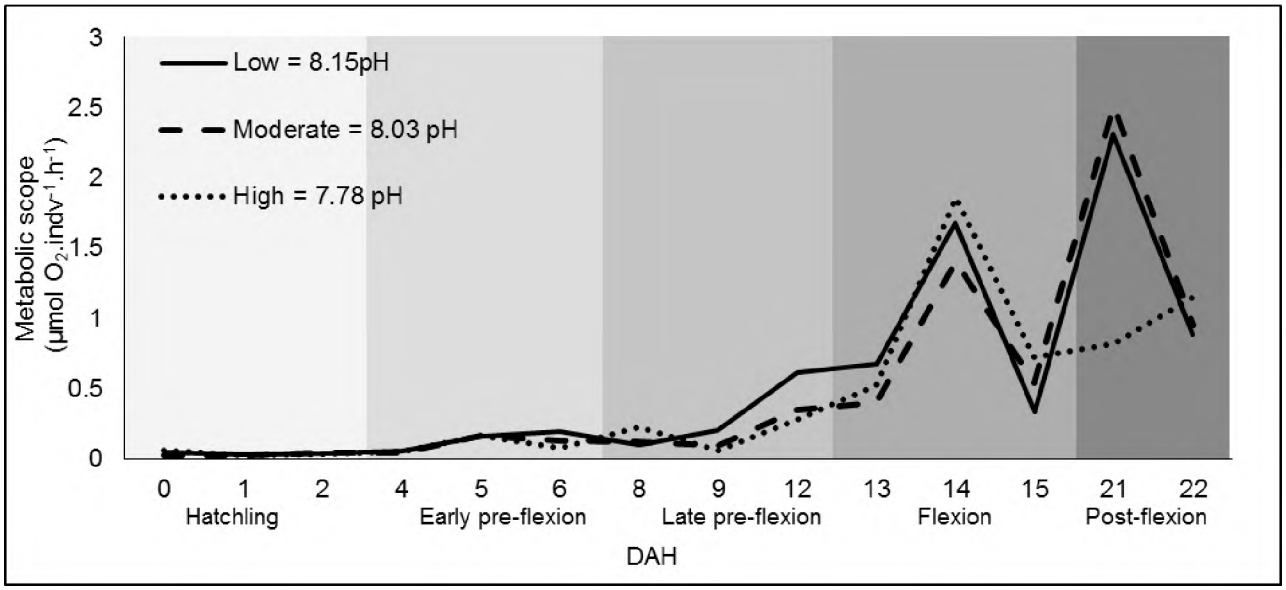


Figure 3.8. Metabolic scope for larvae raised in each pCO_2 treatment from age 0–22 DAH (days after hatch)

Discussion

The findings from this study suggest that metabolic energy allocation in *A. japonicus* may be impacted with increased acidification following flexion and this may potentially impact growth and survival. However, this effect was only evident in the high pCO_2 treatment (~ 910 µatm), which reflects the levels expected for the end of the century. The moderate pCO_2 treatment (~ 480 µatm) expected for the year 2050 did not

significantly affect larval *A. japonicus* growth and survival or metabolic rate. Interestingly, the larvae raised in the moderate treatment had the highest metabolic scope and faster growth rates suggesting they are tolerant to the level of ocean acidification expected in the year 2050.

The effect of ocean acidification treatments was not uniform among the life-stages. Elevated seawater pCO_2 had little effect on the overall metabolic structure of *Argyrosomus japonicus* in the earliest life stages (preceding flexion). In contrast, larvae from other fish species seem to show sensitivity to elevated pCO_2 early on in their development, resulting in impaired growth and development. For example, the embryos and pre-flexion larvae of Atlantic herring (*Clupea harengus*) (Franke and Clemmesen 2011), Atlantic cod (*Gadus morhua*) (Frommel et al. 2012), dolphinfish (*Coryphaena hippurus*) (Pimentel et al. 2014a) and the Mediterranean wrasse (*Symphodus ocellatus*) (Cattano et al. 2016) were negatively impacted by increased pCO_2 levels. Only two similar studies found that the CO_2 tolerance of two marine fish species, Japanese sillago (*Sillago japonica*) and red seabream (*Pagrus major*), decreased from the pre to post-flexion and juvenile stages (Kikkawa et al. 2003; Ishimatsu et al. 2004). These authors could not determine why sensitivity changed with life-stage, however, they hypothesised that the reduced tolerance in the post-flexion stages is a result of the larger surface area for gas exchange facilitated by the development of the gill lamellae, whereas gas diffusion in early stage larvae is limited by diffusion across body surfaces (Ishimatsu et al. 2004). It is possible that this may be the case for the larvae of *A. japonicus* and that the metabolic effects of increased pCO_2 are only evident when internal concentrations reach a level where they require active acid-base regulation.

Metabolic scope, AMR and SMR did not differ significantly among treatments in the pre-flexion life-stages. The comparatively low and stable SMR and AMR during early developmental stages (egg – pre-flexion) of *A. japonicus* suggests that they have low energetic demands. In addition, the similarity in metabolic structure from 0–15 DAH, between the three pCO_2 treatments suggests that acidification does not have a metabolic impact on these stages. Despite this, there appeared to be at least one energy bottleneck during these early phases. The metabolic scope of *A. japonicus* larvae declined during the period of notochord flexion (15 DAH) in the baseline

experiment and again in all three treatments. It is possible that the decline in metabolic scope signals the termination of feeding, which occurs during metamorphosis, when additional energy is thought to be allotted towards development (Parra and Yúfera 2001). However, it appears that low energetic demands prior to this developmental milestone in fish larvae buffer the metabolic costs required to maintain acid-base balance in conditions of high pCO_2.

In contrast to the early life stages, metabolic structure began to differ among treatments in the post-flexion life stages. Although there was no statistically significant change in the average metabolic scope for each of the life stages among the three treatments, there was a large reduction in metabolic scope on Day 21 in the larvae reared in the high pCO_2 treatment (7.78 pH), with a 67% reduction in scope between the moderate and high treatment and a 64% reduction in scope between the low and high treatment. In the context of metabolic rates in small fish larvae, these are large changes. Metabolic scope specifically has not previously been assessed in the early larval stages of fish in response to ocean acidification. However, the juveniles of two species of cardinalfish (*Ostorhinchus doederleini* and *O. cyanosoma*) from the Great Barrier reef that were exposed to pCO_2 levels for 2100, also showed reduced metabolic scope of up to 30% (Munday et al. 2009a). These authors suggest that the vulnerability of these coral reef species to ocean acidification is a result of being adapted to stable tropical marine environments, unlike species that are adapted to environments that show large natural variations in pCO_2 (Munday et al. 2009a). These findings on coral reef fish highlight that reduced aerobic scope ultimately affects individual performance and therefore could have significant impacts on the success of marine fishes by reducing their capacity for aerobic activity (Munday et al. 2009a).

Metabolic scope is determined by the difference in AMR and SMR. The decline in metabolic scope during the post-flexion stage in the high pCO_2 treatment in this study was caused by a decline in AMR, which represents the energy available for activity over and above survival processes. Munday et al. (2009a) found that both species of cardinalfish studied showed an increase in SMR resulting in reduced metabolic scope and this was attributed to some energetic costs of acid-base regulation. One of the species studied, *Ostorhinchus cyanosoma*, also showed a decrease in maximum

metabolic rate. This resulted in a greater decrease in metabolic scope, compared to *O. doederleini,* making this species especially sensitive to ocean acidification.

The larvae from the low and moderate treatments showed a significant peak in AMR at 21 DAH, which was absent in the high pCO_2 treatment. This peak in AMR in the low and moderate treatment can most likely be attributed to the energy dedicated to swimming activity, which is facilitated by the development of the notochord and fins during flexion. Interestingly a concurrent study found significantly reduced skeletal development at this stage (Erasmus in prep.). This suggests that fish in the high pCO_2 treatment lack the skeletal structures necessary to initiate active swimming and explains the absence of a peak in AMR.

The peak in AMR also coincided with significant changes in SMR. The SMR was significantly lower in the high pCO_2 treatments in post-flexion larvae, suggesting potential metabolic depression. This result was unexpected as previous research on juvenile cardinalfish and red drum exposed to acidification treatments (Munday et al. 2009a; Ern and Esbaugh 2016) has suggested that the SMR should be elevated or remain constant in fishes due to the increased energetic expenditure required for acid-base regulation in high pCO_2 treatments. This is due to the fact that acid-base regulation is a survival process that is achieved through ion transport mechanisms. This process requires additional energy, particularly in species that are not well adapted to regulate internal pH (Pörtner et al. 2004) to allow them to meet their oxygen demands (Munday et al. 2009a; Melzner et al. 2009b). Few studies have examined the SMR response of fish larvae to changes in pCO_2 concentration, despite the general consensus in literature that fish larvae have a poorer acid-base regulation efficiency than juveniles and adults (Ishimatsu et al. 2004; Melzner et al. 2009b; Stiasny et al. 2016). The larval stages may not be able to regulate pH as efficiently due to lack of specialised ion-regulation apparatus prior to the formation of gills (Falk-Petersen 2005; Stiasny et al. 2016) and may therefore display metabolic depression of SMR as a response in order to conserve energy (Melzner et al. 2009b).

Metabolic depression is a physiological phenomenon frequently documented in the larval stages of invertebrates (e.g. Pörtner et al. 2004; Michaelidis et al. 2005; Melzner et al. 2009b) and has been suggested to be a mechanism to conserve energy as a

result of poor acid-base regulation (evidenced by low extracellular pH) (Pörtner et al. 2004; Melzner et al. 2009b). This has been observed in the larvae of sipunculids (Reipschläger and Pörtner 1996), mussels (Michaelidis et al. 2005), sea urchins (Stumpp et al. 2011) and corals (Nakamura et al. 2011). The reduced SMR observed in fish larvae raised in the high pCO_2 treatment in this study suggest that larval *A. japonicus* may respond similarly to the impact of elevated pCO_2.

Both metabolic depression and reduced metabolic scope are thought to result in decreased growth, development and survival of marine species (e.g. Melzner et al. 2009b). Many studies have documented these negative effects of end of century pCO_2 levels on larval fish (Baumann et al. 2012; Kim et al. 2015); however, only a few studies have tried to link survival, growth and development to physiological processes (Seibel and Walsh 2003; Michaelidis et al. 2007; Pimentel et al. 2014b). Gräns et al. (2014) found a negative response in growth of juvenile Atlantic halibut (*Hippoglossus hippoglossus*) to ocean acidification. The results from this study suggest that there is a potential link between metabolic scope and growth rate in *A. japonicus,* as shifts in metabolic scope appear to correlate with the timing of developmental delays in larvae raised in the high pCO_2 treatment (Figure 3.9).

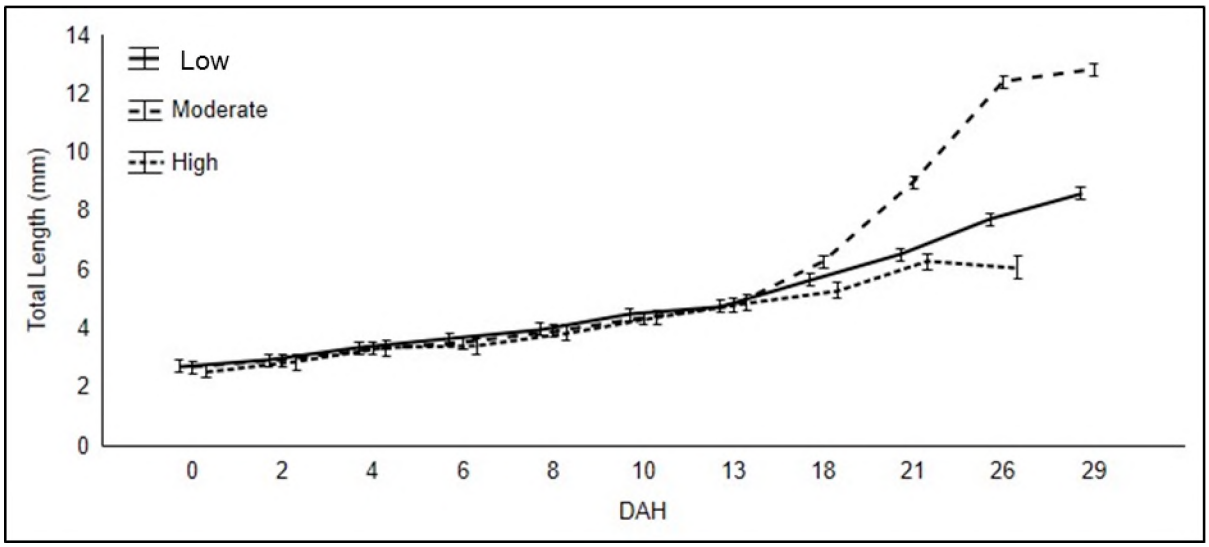


Figure 3.9: Total length of larvae raised in each pCO_2 treatment from a concurrent study by Erasmus et al. 2016 (in prep) (mean ± SE).

There is evidence to suggest that ocean acidification influences the development of skeletal structures in fish. For example it has been found that bone density was

reduced in larval olive flounder (*Paralichthys olivaceus*) (Kim et al. 2015) and skeletal deformities increased in frequency in early stage sole (*Solea senegalensis*) (Pimentel et al. 2014b) with increasing CO_2 in seawater. This study also revealed a potential link between physiology and development of *A. japonicus*. For example, changes in SMR occurred during the period following notochord flexion. This may be an indication of the high energetic demands of completing metamorphosis prior to estuarine recruitment, which occurs at approximately 20 mm TL (Griffiths 1996), such that even small changes in metabolic structure may be detrimental to development during this time. This was supported by the findings of a concurrent study on the development of skeletal and morphological structures of *A. japonicus* (Erasmus et al., in prep.). There was rapid skeletal development on 21 DAH (Erasmus et al. in prep.), which is likely to require a large portion of metabolic energy, and this process was retarded in the larvae reared in the high pCO_2 treatment, in addition to their AMR being significantly reduced at 21 DAH.

Mortality has been documented in larval Atlantic cod (*Gadus morhua*) (Stiasny et al. 2016) and Atlantic silverside (*Menidia menidia*) (Baumann et al. 2012) under high pCO_2 treatments (1100 µatm). Changes in metabolic structure of *A. japonicus* post-flexion larvae at 21 DAH coincided with an observed mass mortality of individuals in the high (~ 910 ppm) pCO_2 treatment. This suggests that changes in metabolism may impact the survival of post-flexion *A. japonicus* in high pCO_2 waters predicted for the end of the century. Fish with reduced metabolic scope likely did not have sufficient energy available to maintain some survival process under the stress of acidification. However, the exact reason for the high mortality rates observed in this study were not clear.

The timing of these developmental and energetic limitations could potentially be detrimental to this species as these changes occur specifically in the flexion stage, which coincides with the stage at which the larvae of this species begin to recruit into estuaries (from 20 mm TL, Griffiths 1996). This phase is likely to have high energy costs related to the swimming effort required to recruit into estuaries as well as to physiologically manage the variable conditions in these dynamic systems (such as salinity, pH and temperature). The reduction in SMR at this stage may ultimately result in less energy being available for basic survival functions. The reduction in AMR and

metabolic scope may make this species even more sensitive to acidification, as less energy will be available for rapid swimming against tidal and estuarine currents. High mortality during recruitment has been identified as one of the main reasons for population collapses of *A. japonicus* (Griffiths 1996), with ocean acidification potentially posing an additional threat to this process.

Conclusion

The findings of this study suggest that the tolerance of *A. japonicus* larvae to the current rate of ocean acidification is likely to continue for the next 50 years; however, by the end of the century, high pCO_2 levels may have significant consequences for the survival of this species. It appears that this tolerance is potentially linked to metabolic physiology and differs among life-stages during early development. This study provides the first evidence that estuarine dependent species, with a marine larval phase, may be sensitive to ocean acidification in their early life stages. This highlights the importance of including all life stages and species with contrasting life-history strategies in assessments of species tolerance to ocean acidification. Furthermore, energetic limitations will likely limit development and recruitment ability of post-flexion larvae as well as reducing their ability to tolerate fluctuating estuarine environments. Regardless, if larvae are unable to breach the post-flexion energy bottleneck induced by acidification the recruitment of the species to adult populations may be compromised.

Chapter Four

General discussion and conclusion

The use of metabolic rate measurements (oxygen consumption rate) has become a frequently used tool in addressing biological responses to climate change (Clark et al. 2013). Increased concentrations of CO_2, associated with acidification, have been found to directly affect the physiology and metabolism of marine fish species, with significant reductions in metabolic scope documented in the juveniles of two tropical coral reef species (Munday et al. 2009a) and warm-temperate adult gilthead seabream (Michaelidis et al. 2007). In contrast, other species, such as the temperate juvenile Atlantic halibut (*Hippoglossus hippoglossus*) (Gräns et al. 2014) as well as other species of coral reef fish (Rummer et al. 2013), have shown an increase in metabolic scope in response to acidification. Changes in organism physiology are strongly associated with the vulnerability of species to stressors such as ocean acidification. Although previous research has furthered our understanding of the sensitivity of marine fish to ocean acidification, there are still large gaps in our understanding, with little known about the response of the larval stage of fish species or how life-history may influence sensitivity to acidification (particularly when life-history strategy changes with ontogenetic development). This study looks at the response of larval *A. japonicus* to ocean acidification and how vulnerability is influenced by life-history strategy. This is the first assessment of an estuarine dependent marine species with a pelagic marine egg and larval phase.

Ontogenetic and life-history considerations for vulnerability

Various authors have suggested that the early larval stages of marine organisms are particularly vulnerable to environmental change when compared to juveniles and adults (Reipschläger and Pörtner 1996; Ishimatsu et al. 2004; Frommel et al. 2013). This vulnerability has been extensively assessed in invertebrate larvae (e.g. Dupont et al. 2008; Kurihara 2008; Stumpp et al. 2011) but much less frequently in fish larvae (Wittmann and Pörtner 2013). The few studies that have assessed the larval stages of marine fish have shown reduced growth and survival (Baumann et al. 2012; Murray et al. 2016), changes in otolith development (Checkley et al. 2009; Munday et al.

2011), and tissue damage (Frommel et al. 2012). Only one study (Murray et al. 2016) considered the entire ontogenetic development of the early life-stage, and this lack of information presents a large research gap in our understanding of how ocean acidification will impact populations. This study, which assessed the complete early development of *A. japonicus*, showed that vulnerability varied among life-stages and identified metabolic bottlenecks. Understanding and identifying these bottlenecks is essential if we are to understand how species will respond to ocean acidification in the future. For example, ontogenetic bottlenecks early in development have been identified in the thermal tolerance of larval sole (*Solea solea*) from the North Sea (Rijnsdorp et al. 2009) (Figure 4.1).

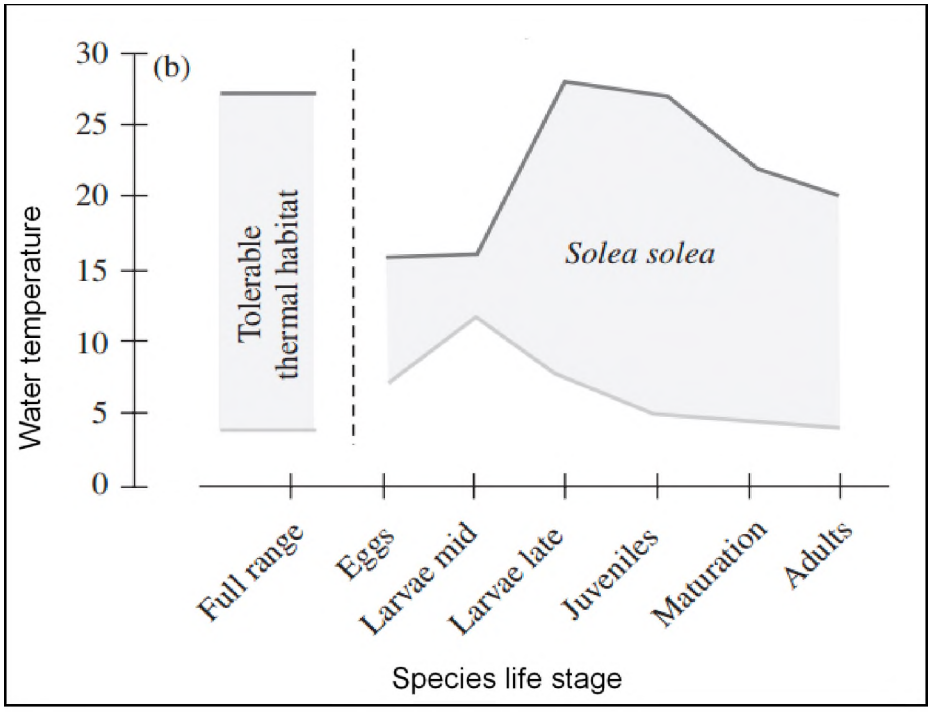


Figure 4.1: Ontogenetic changes in thermal tolerance in sole (*Solea solea*) from the North Sea (Rijnsdorp et al. 2009) indicating the increased thermal sensitivity in the earlier life stages (Pörtner and Peck 2010).

The vulnerability of *Argyrosomus japonicus* to ocean acidification during early development is due to their association with the pelagic marine environment during this stage. This species has a warm-temperate distribution and is wholly dependent on estuaries in the juvenile stages, however, during early development (prior to settlement) they have a pelagic marine phase (Beckley 1990; Gray and McDonall

1993; Griffiths 1996; Whitfield 1998). Recruitment into estuaries in *A. japonicus* occurs in the post-flexion, settlement stage (20 mm TL, ± 30 DAH (days after hatch)) (Griffiths 1996). This coincides with the time when this species showed the highest sensitivity to the effects of ocean acidification, suggesting that this stressor will induce a recruitment bottleneck to estuarine populations. Different strategies of recruitment into estuaries are apparent among marine fish species (Boehlert and Mundy 1988).

A recent study on the estuarine dependent red drum (*Sciaenops occelatus*) from America, found that this species appears to be tolerant to ocean acidification showing resilience in survival, growth and behaviour (Lonthair et al. 2017). Based on their results Lonthair et al. (2017) concluded that estuarine-associated species may be less sensitive to the effect of ocean acidification than marine species with no estuarine association. While *Sciaenops occelatus* and *A. japonicus* are from the same family (Sciaenidae), the fundamental difference between the two species lies in their early developmental life-history strategy. *Sciaenops occelatus* broadcast spawn in estuarine channels and the embryos are carried into estuaries prior to hatching (Holt et al. 1983; Lonthair et al. 2017). Once hatched the larvae continue to develop in estuarine areas, and this is obligatory, after which they migrate to adult marine habitats (Holt et al. 1983). Estuary pH is correlated to changes in salinity, photosynthetic cycles and dissolved oxygen (Ringwood and Keppler 2002) as well as the pH of the marine environment resulting in strong pH gradients in estuarine environments (James et al. 2013). Because *A. japonicus* are not exposed to the variable pH environment of estuaries during the most sensitive egg and larval stages, this may explain their vulnerability to ocean acidification. This highlights the importance of considering the variability in life-history strategy and developmental stage when determining the vulnerability of species to ocean acidification.

The levels of pCO_2 and pH are also considered to be more variable in temperate environments and, as such, authors working on tropical species have suggested that temperate species may be more tolerant to ocean acidification than tropical marine species due to the variable nature of their habitats (Fabry et al. 2008; Munday et al. 2009a). This resilience seems to be more evident in the juvenile and adult life-history stages. For example, juvenile wolfish (*Anarhichas minor*) (Foss et al. 2003) and salmon (*Salmo salar*) (Fivelstad et al. 2003) did not show reduced growth performance

when exposed to high levels of CO_2 (5900 μatm) for prolonged periods. Melzner et al. (2009a) also found that juvenile Atlantic cod (*Gadus morhua*) did not show alterations in swimming performance, active metabolism or metabolic scope when exposed to similar levels of CO_2 (3000 to 5900 μatm) (Melzner et al. 2009a). *Argyrosomus japonicus* appeared to respond similarly to the abovementioned species when exposed to the moderate pCO_2 levels showing some resilience to the 8.03 pH treatment. This could be due to the fact that this pH level may be intermittently experienced in coastal areas where these larvae occur, thereby falling within their range of tolerance. However, results for this study found that the post-flexion larvae of *A. japonicus* may not be as resilient to the effects of acidification once they surpass this naturally occurring range (7.78 pH) as the juvenile and adult phases of other temperate species. This is evidenced by the reduced metabolic scope, metabolic depression and high levels of mortality observed in *A. japonicus* post-flexion larvae under ocean acidification treatments predicted for the end of the century.

Synergistic pressures and vulnerability of *Argyrosomus japonicus*

Coastal areas have been identified as areas of particular concern due to the pressures associated with anthropogenic climate change (Harley et al. 2006). This concern is based on the various abiotic changes occurring in these areas as a result of natural variability in combination with human activities (Figure 4.2) as well as the resultant ecological responses (Harley et al. 2006). These changes are threatening the social, economic and ecological resources provided by coastal regions (Harley et al. 2006). Understanding the ecological effects of climate change on ecosystems is complex as 1) not all species respond in the same way to factors of change, with some species being more tolerant than others and 2) variables of change are not independent from one another and generally species face a combination of multiple stressors related to environmental change and anthropogenic impacts.

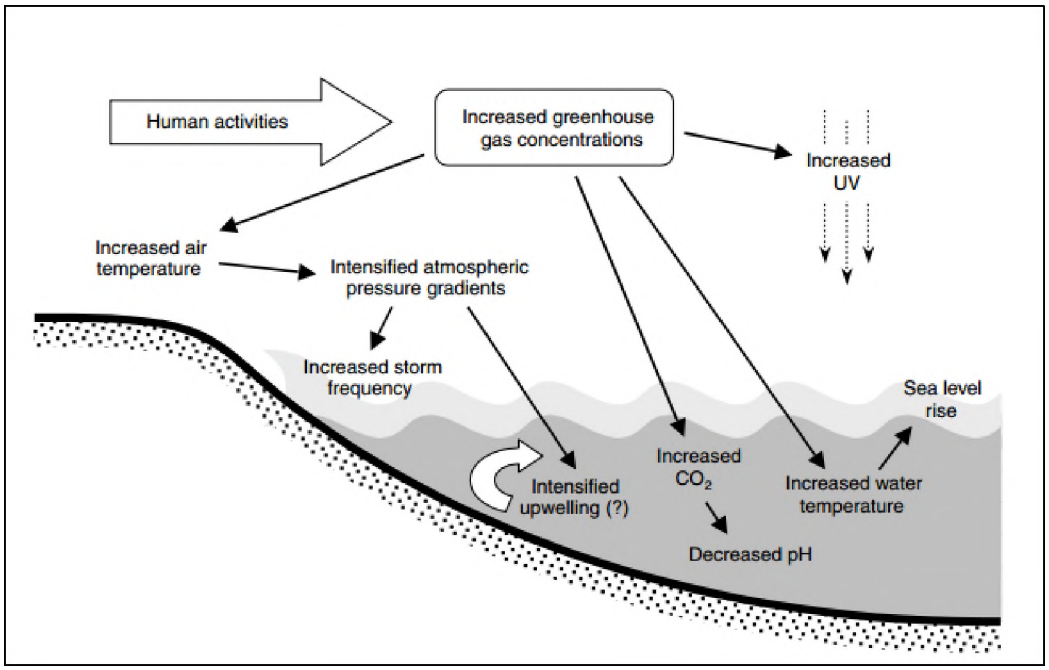


Figure 4.2: The abiotic changes influencing coastal ecosystems as a result of human activity (Harley et al. 2006)

From the results of this research it becomes evident that the pressures of ocean acidification faced by *A. japonicus* are not consistent throughout their ontogenetic development, and that this is related to their changing life-history strategies as they develop from larvae to adults (Figure 4.3). Griffiths (1996) divided the life-cycle of *A. japonicus* into four phases, each with a differing habitat association, namely: 1) eggs and larvae which occur in the nearshore, marine pelagic environment, 2) early juveniles which occur in the upper reaches of estuaries, 3) juveniles which occur in both the upper and lower reaches of estuaries and the surf zone and 4) adults which occur in the nearshore marine environments and surf zones but also frequent estuaries. It is well documented that the later three phases (juveniles and adults) are vulnerable to the influence of human activities, particularly fishing (Griffiths 1996). As a result, stocks of this species are targeted heavily during their juvenile phase as well as in the fecund adult stages, significantly reducing the abundance of individuals recruiting and surviving to an age where they themselves can reproduce (Ferguson et al. 2014). This has resulted in the collapse of the *A. japonicus* fishery in South Africa (Griffiths 1996; Mirimin et al. 2016) as a result of a combined effect of recruitment and growth overfishing (Childs et al. 2015). Australian populations of *A. japonicus* have been similarly affected (Ferguson et al. 2014). Fishing is regarded as one of the most

serious anthropogenic pressures facing fishes and more recently it has been suggested that overfishing may increase the vulnerability of some species to variability in climate and marine environments, thus amplifying the threat to populations (Hsieh et al. 2005; Harley et al. 2006; Hsieh et al. 2008). This is attributed to reduced spatial heterogeneity as a result of modified age structures and constriction of their distribution (Hsieh et al. 2008). This change in spatial heteregoneity renders heavily exploited species more vulnerable to environmental change (Hsieh et al. 2008). In addition, climate change is modifying the impacts of fishing by the addition of pressures that change fish community structure, distribution and production (MacNeil et al. 2010).

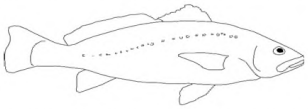
Habitat	*Coastal nearshore*	*Estuarine*	*Estuarine & marine*
Pressures	**Ocean acidification** Other climate change drivers Naturally variable physico-chemistry	Variable estuarine physico-chemistry Fishing	Fishing Natural physico-chemical variables
			
Developmental stage	Larvae	Juvenile	Adult

Figure 4.3: Diagrammatic representation of the synergistic pressures faced by *A. japonicus* throughout ontogenetic development and changing life-history strategy

In addition to the pressures of fishing, marine species with an estuarine life-phase are also significantly impacted by other anthropogenically induced abiotic changes facing estuaries, such as freshwater abstraction, habitat destruction, pollution, land use change and other climate change drivers (warming, sea level rise and storms, changes in rainfall and salinity) (Whitfield 1992; James et al. 2013). These synergistic pressures compromise the nursery function of estuaries for *A. japonicus* and other estuarine dependent species. The combined effects of multiple stressors is likely to have significant impacts on *A. japonicus* populations surviving to the adult stages. This highlights the necessity of recognising the complex of pressures faced by species in various stages of development as these pressures may vary in type and intensity with developmental stage, habitat occurrence and life-history strategy (Figure 4.3).

Reduced growth, survival and recruitment to juvenile and adult populations are particularly concerning for species that are ecologically, economically and socially important. For most fish species, these benefits are often interrelated as it is usually the large, predatory fish that play key ecological roles that are targeted in fisheries. *Argyrosomus japonicus* is an excellent example of such a species that serves a key ecological role, both in estuaries and coastal habitats, and is also a popular fishery species. *Argyrosomus japonicus* is a piscivorous predator in estuaries and surf zones (Griffiths 1997c). As a result, reductions in biomass of this species can have indirect effects on populations of other species that they interact with, thereby leading to alterations in shallow water food webs. In addition to its ecological relevance as a predator, *Argyrosomus japonicus* is also valued in the South African and Australian capture fisheries (Griffiths 1997c; Silberschneider and Gray 2008). *A. japonicus* is one of the most important species fished in estuaries throughout South Africa (Lamberth and Turpie 2003) and is highly sought after in the commercial, recreational and subsistence fisheries (Brouwer et al. 1997; Childs and Fennessy 2013). The influence that overfishing has had on this species is well understood; however, the impacts of climate change variables and the combined effects of these pressures has not been assessed in much detail. The continuing pressures of fishing and climate change at the current rate is likely to have detrimental consequences for the persistence of this species, particularly considering the high mortality rate of larvae under treatments of ocean acidification predicted for the end of the century as revealed in this study.

The *A. japonicus* stock is deemed to have collapsed in South Africa (DAFF 2014) and is overfished in Australia (Silberschneider and Gray 2008) and it is therefore not surprising that aquaculture of this species has increased in popularity in both South Africa (Bolton et al. 2013) and Australia (Silberschneider and Gray 2008). It is anticipated that the aquaculture industry of *A. japonicus* is likely to continue to expand to larger scale productions (Mirimin et al. 2016). Highly variable pH in aquaculture systems has to be managed accordingly in order to maintain productivity, and this is already a challenge in hatchery systems resulting in larger size disparities and increased cannibalism (Grant L., *pers. comm.*, April 2016). The additional pressure of ocean acidification facing these systems may require further assessment in order to negate the effect it might have on *A. japonicus* larvae farmed in hatcheries.

Limitations of this study and recommendations for future research

This research was the first known attempt to assess the combined effect of life-history strategy and developmental stage on the vulnerability of a warm-temperate marine fish species with an obligatory estuarine phase to ocean acidification. Furthermore, this study is the first known attempt at completing a comprehensive experimental ocean acidification study on a temperate marine fish species in Africa, thereby setting a valuable benchmark for future research in this field. The results from this study highlighted the definite need for continuous global and local research in order to unravel the effects that ocean acidification may have, in combination with other pressures, on both individual species and their ecosystems.

Understanding the physiological implications of ocean acidification, particularly in early stage fishes, should be a priority as this area is significantly lacking in understanding. However, physiological endpoints and energetics are complex to measure and understand. As an example, the results from this study revealed metabolic shifts in larvae exposed to high ocean acidification treatments, however, the consequence and mechanisms behind this require further investigation. This revealed the need for understanding acid-base regulation mechanisms in fish larvae and how this process develops with growth and development. This information, together with a comprehensive understanding of larval energy budgets can reveal significant information on how acid-base regulation is energetically compensated when facing pressures during early development. Describing these processes will provide a valuable addition to understanding the effects of ocean acidification and other drivers on early stage fishes.

Although it is useful to understand the effects of single drivers of environmental change on organisms as a baseline, following this it would be beneficial to assess how exposure to one factor may affect the sensitivity to another. For example, there is evidence to suggest that exposure to ocean acidification amplifies the effect of increasing ocean temperatures on marine organisms (O'Donnell et al. 2009; Lannig et al. 2010). This will be particularly important when assessing dynamic environments such as coastal zones and estuaries. A logical progression of research following this study would be to assess how exposure to ocean acidification in the early life stages may affect the vulnerability of later stage juveniles to variability in environmental

parameters, such as salinity, temperature and pH experienced in estuaries. For example, Ern and Esbaugh (2016) examined the cost of acid-base regulation and osmoregulation on the overall energy budget in red drum (*S. ocellatus*) under the stress of both acidification and hypoxia. In addition they quantified the cost of osmoregulation in order to determine whether exposure to CO_2 and hypoxia would affect osmoregulation in variable estuarine environments (Ern and Esbaugh 2016). This approach is useful to determine the trade-offs of various physiological processes when organisms are exposed to varying environmental conditions.

Another relationship that should potentially be explored for fish species is how food availability may impact the sensitivity of organisms to ocean acidification. Such integrated research has already been conducted on invertebrate species (e.g. Melzner et al. 2011; Kroeker et al. 2014), which have shown amplified negative effects as a result of increased energy demands under acidic conditions.

Although, in most laboratory based studies, it is near impossible to measure all processes and endpoints due to the complexity of maintaining and controlling multiple variables, it is useful to first identify the response of organisms to single variables in a controlled setting in order to understand specific mechanisms before attempting more complex, ecological approaches (Figure 4.4).

For *A. japonicus* specifically, following this laboratory based research it would be beneficial to assess the effects that exposure to ocean acidification may have on behaviours such as feeding, cannibalism and response to stimuli. This could provide useful knowledge to assist in inferring the effects that ocean acidification might have in a natural setting, where larvae compete for food, interact with other animals and respond to stimuli to recruit into estuarine nurseries.

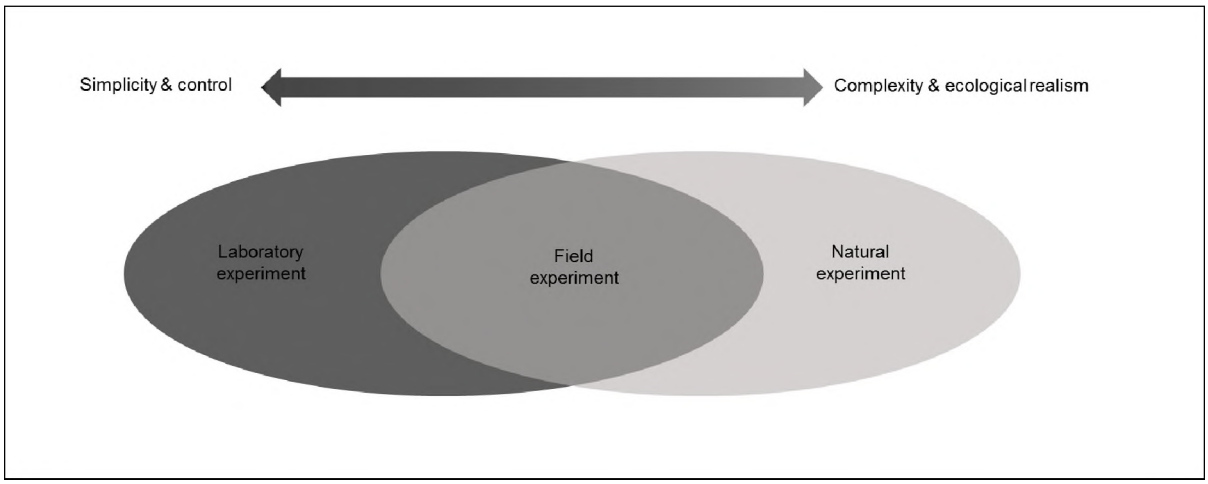


Figure 4.4: The level of complexity of three ecophysiology research approaches associated with assessing the effects of environmental drivers on marine organisms (Spicer 2014).

Another interesting result revealed by this study is that the effects of ocean acidification may not be significant at levels predicted for the next 50 years as opposed to the apparently lethal effects of treatments representing the next 100 years. This suggests that there may be a "tipping point" in the tolerance of the early life stages of these species to the effects of ocean acidification. Establishing these tipping points in tolerance will be useful to estimate realistic time-frames for the management of this species under these conditions. Non-linear relationships between pH and response variable in other studies have been suggested to be indicative of the presence of tipping points in tolerance (Ries et al. 2009; Kroeker et al. 2010). A common example of a tipping point in a marine environment is the relationship between coral survival and water temperature. These organisms, generally have a narrow thermal tolerance and any deviation from these during extreme weather events can result in mass mortality (Laurance et al. 2011).

The complexity of marine organisms and their relationship with the environment calls for a broader approach to climate change research. Global ocean acidification research is tending towards a multi-driver, ecosystem approach in order to understand the complex effect of ocean acidification on marine systems. This involves including the interactions of various climate change drivers on a host of interacting species in order to try and infer the impact of global change on entire ecosystems. This type of research will provide a valuable addition to the currently popular single species

studies. Priority ecosystems should also be identified for future research. For example, coastal areas are generally subject to the effects of human influence (Harley et al. 2006) as well as contributing significant resources for human benefit (Harley et al. 2006; Costanza et al. 2014).

Although ocean acidification research has increased in popularity, there has been very little research on this subject in Africa and presents a major research gap. This study, in addition to contributing to the global understanding of ocean acidification, provides a particularly good benchmark to continue ocean acidification research in Africa. It is recommended that this research is continued in various fields in Africa in order to understand the impacts it may have on our highly valued coastal ecosystems. It is suggested that this includes an effort to conduct long term monitoring of *in situ,* real-time acidification, and to include multiple drivers and species. If this approach is taken, South Africa will soon contribute significantly to the global picture of the effects of this phenomenon.

Final conclusion

This study revealed that ocean acidification has the potential to significantly impact an estuarine dependent marine species, *Argyrosomus japonicus.* The metabolic physiology of *A. japonicus* was negatively influenced by ocean acidification predicted for the end of the century, particularly during the energy bottlenecks associated with the post-flexion stage. These results suggest that this species may not survive the predicted levels of ocean acidification for the future. However, further research, which includes other species and drivers is necessary to contribute to our understanding of how ocean acidification will impact our valuable marine ecosystems.

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