An eco-physiological investigation of fisheries-induced evolution: comparing the resilience of larvae from exploited and unexploited commercial reef fish populations to projected ocean acidification

Dissertation submitted in fulfilment of the requirements for the degree of

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Ichthyology and Fisheries Science

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By

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Supervisors:

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Preface

This thesis is an original work and consists of a general introduction (Chapter 1); a description of the study sites, species profile, and details of early development (Chapter 2); three research chapters (Chapters 3, 4, and 5), and a general discussion (Chapter 6). The research chapters are written as stand-alone scientific publications and, as such, will contain overlap in descriptions of specimen collection and rearing. Repetition was reduced by describing the spawning and rearing of larvae and the rearing facility in Chapter 2.

Additional outputs completed during this thesis include:

Manuscripts published:

- **Muller C**, Childs AR, James NC & Potts WM. 2020. Effects of experimental ocean acidification on the larval morphology and metabolism of a temperate sparid, Chrysoblephus laticeps. *Oceans*, 2(1): 26–40.
- **Muller C**, Childs AR, Duncan MI, *et al*. 2020. Implantation, orientation, and validation of a commercially produced heart-rate logger for use in a perciform teleost fish. *Conservation Physiology*, 8(1).
- Allison C, **Muller C**, Childs AR *et al*. 2021. When cooling is worse than warming: investigations into the thermal tolerance of an endemic reef fish, Boopsoidea inornata. *African Journal of Marine Science*, 43(2): 1–11

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The research project, of which this thesis is a part, received research ethics approval from Rhodes University Animal Ethics Committee under the project title ~~"F~~isheries-induced evolution on fish thermal physiology – implications for climate change adaptation," Approval No: DIFS152018; for period January 2018 – December 2020.

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Declaration

I, Cuen Muller, hereby declare that the work described in this thesis was carried out in the Department of Ichthyology and Fisheries Science, Rhodes University, under the supervision of Professor WM Potts, Dr NC James and Dr AR Childs. The components of this thesis comprise original work by the author and have not been submitted to any other university.

Signed: 

Date: 27/10/2021

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Abstract

It is now accepted that anthropogenic-induced climate change is resulting in unprecedented rates of change to marine environments. Marine organisms are being challenged by rapidly increasing temperatures, acidification, expansion of oxygen dead zones, and higher frequencies and magnitudes of extreme weather events. Exploited fish populations are also undergoing selective harvesting. Certain traits, such as large size, fast growth, and/or bold/active behaviours, are being actively targeted and removed from the population gene pool. This selective removal of individuals may compromise the capacity of fish populations to resist or recover from environmental disturbances and reduce their ability to adapt to a changing environment as many of these traits are heritable. As most marine fishes' embryonic and larval stages represent the period when individuals are most sensitive to environmental disturbances, they are a critical bottleneck to population persistence in the face of exploitation and climate change. This thesis aimed to quantify and compare the metabolic physiology, growth, and development of an exploited and endemic sparid, the roman seabream *Chrysoblephus laticeps*, during the early larval stages under 1) ocean acidification conditions expected by the year 2100 and 2) from populations experiencing dissimilar rates of exploitation.

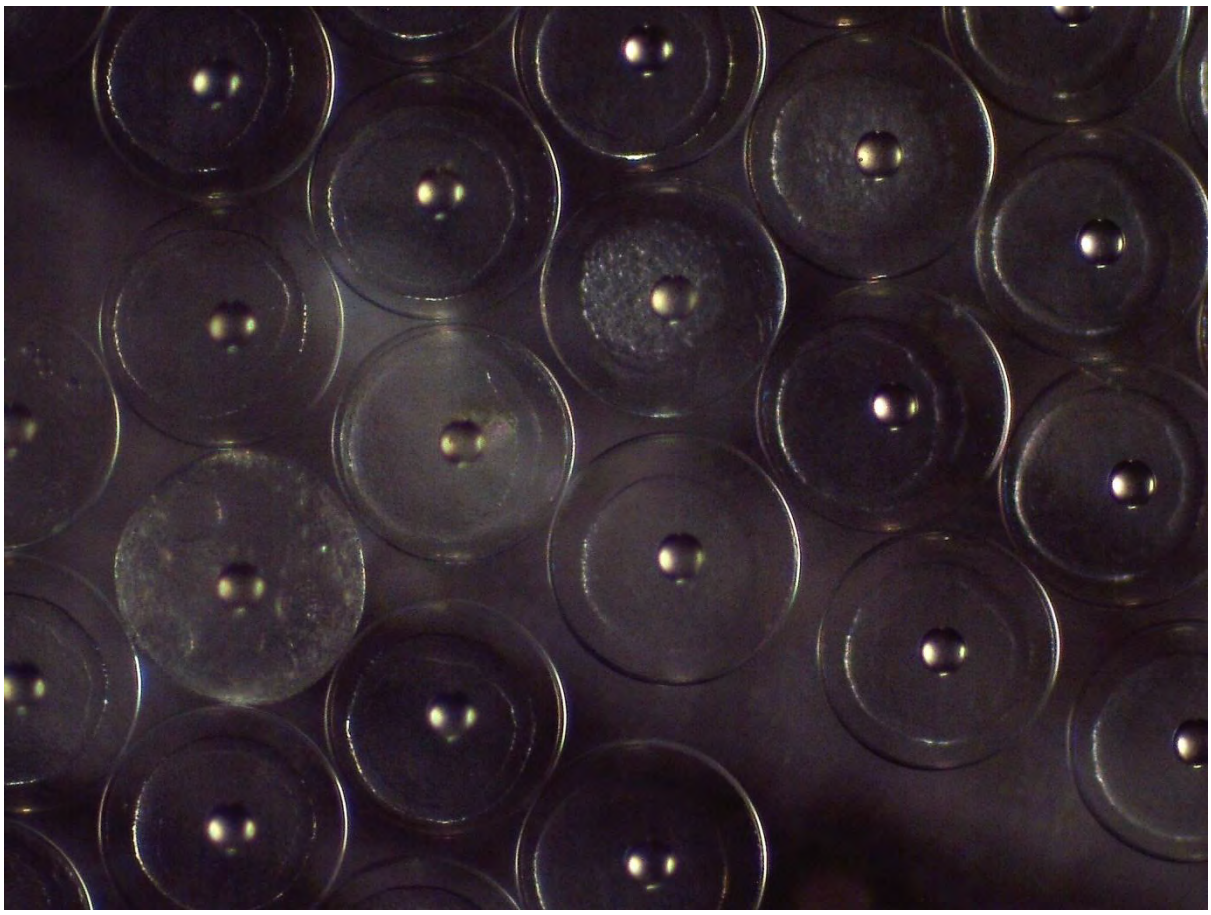
To quantify and compare the physiology of larvae, adult *C. laticeps* from an exploited population were captured and field-spawned. Fertilised eggs were placed into either control/present-day conditions (pH = 8.03, pCO_2 ≈ 420 µatm) or high-pCO_2/hypercapnic treatment conditions (pH = 7.63, pCO_2 ≈ 1400 µatm). The metabolic physiology of individual larvae was determined using a novel rolling-regression technique on static respirometry data. Here, estimates of the minimum and maximum oxygen consumption rates (VO_2) could be determined with high test-retest reliability. The very early developmental stages (yolk-sac stage) appeared resilient to high pCO_2 conditions despite being exposed to treatment conditions throughout the embryonic stage. Preflexion larvae showed sensitivity to treatment conditions by exhibiting reduced metabolic and growth rates, consistent with metabolic depression, associated with environmental stress. However, by the onset of flexion, which coincides with gill development, acid-base regulation, and muscle differentiation, metabolic and growth rates of treatment larvae were significantly greater than that of controls. This suggests that acid-base regulation imposes a high cost to maintain internal pH homeostasis. Importantly, these elevated metabolic costs were likely mediated through increased feeding rates in experimental conditions where food was *ad libitum*. In natural conditions, where food availability may be varied, high pCO_2 conditions could be associated with higher mortality rates.

Based on evidence that protected/unexploited populations are more genetically diverse and are composed of individuals representing a greater range of metabolic phenotypes, offspring were collected from a protected population experiencing otherwise similar environmental conditions to the exploited population. Metabolic rates of control larvae were generally similar to those of the exploited population. However, minimum rates of VO_2 were typically higher for larvae from the protected population at comparable life stages. Preflexion treatment larvae from the protected population did not appear to undergo a period of reduced metabolism or growth compared to their control counterparts. While metabolic rates at the onset of flexion were significantly higher for treatment larvae, this was not associated with growth differences. Growth over-compensation following periods of growth depression is often associated with deleterious effects, such as organ damage and body or developmental malformations. This suggests somewhat improved resilience to ocean acidification conditions.

This thesis found evidence that larval *C. laticeps* are sensitive to ocean acidification conditions expected by 2100. When this stressor is combined with increasing thermal variability, changing current coastal regimes, and heterogeneous food availability, also expected to occur by 2100, ocean acidification may compromise the population persistence of this species. However, an energetics approach to stress-tolerance suggests that larvae from the protected population may inherently show greater resilience to climate change-related environmental stressors. Evidence that exploitation affects the resilience of fish larvae to climate change highlights the need for an evolutionary approach to fisheries management and the importance of spatial protection in maintaining larger and more resilient populations while providing the raw material essential for adaptation.

Chapter 1

General introduction: the rationale behind this study



Eggs of the roman seabream *Chrysoblephus laticeps*, approximately 12 hour‘s post-fertilization

1.1 Conceptual background – climate change and fisheries exploitation

Marine ecosystems are increasingly being impacted by rapidly changing sea temperatures, acidification, hypoxia, and greater frequencies and magnitudes of extreme weather events (Hoegh-Guldberg and Bruno, 2010; Poloczanska *et al.*, 2013; Thompson *et al.*, 2013). Meanwhile, excessive exploitation of marine resources negatively impacts communities, ecological processes, and entire ecosystems, in some cases reducing their continued productivity (Agardy, 2000; Colloca *et al.*, 2017). As fishing is a selective process that targets specific traits, life stages, or populations, contemporary fishing has been likened to an experiment on life-history evolution (Rijnsdorp, 1993; Heino *et al.*, 2015). This selective fisheries exploitation constrains the demographics and genetic variability of fished populations leading to a greater sensitivity to climate variability, potentially leading to reduced resilience to climate change (Perry *et al.*, 2010; Planque *et al.*, 2010).

Marine ecosystems are critically important as they provide services of high socio-economic value, including the provision of food and habitat, recreational and commercial opportunities, and uptake and storage of atmospheric carbon dioxide, in turn regulating Earth‘s climate (Harley *et al.*, 2006; Allison *et al.*, 2009; Henson *et al.*, 2017). However, marine systems' structure, functioning, and adaptive capacity are increasingly affected by the main drivers of climate change: temperature, pH, and oxygen concentration (Henson *et al.*, 2017). Coastal marine systems, as the regions extending from the intertidal zone to the continental shelf, form a relatively small proportion of the ocean surface yet are particularly valuable in their provision of economic resources providing disproportionately greater biomass than other oceanic regions (Costanza *et al.*, 1997; Pauly *et al.*, 2002). However, the benefits obtained from coastal systems are under threat as these areas are likely to be the most negatively affected marine environments due to the greater magnitude and variability of climate change drivers when compared with the more stable open ocean environment (Lehodey *et al.*, 2006; Frieder *et al.*, 2012).

Literature regarding anthropogenically driven climate change of the oceans has primarily focussed on warming sea surface temperature (SST) (Collins *et al*., 2013). While warming is predominant, with approximately 72% of the world‘s coastlines increasing on average by 0.25°C per decade (Lima and Wethey, 2012), regional trends in cooling also exist. The thermal variability is exemplified along the South African coastline where the eastern subtropical region is undergoing a positive SST trend of up to 0.55°C per decade, while the western cool-temperate and southern warm-temperate regions are experiencing a negative trend (Rouault *et al*., 2010). The negative trend of the western and southern coasts is predominantly driven by increased upwelling favourable winds, which are intensified during

the La Niña–Southern Oscillation phase (Rouault *et al*., 2010; Duncan *et al*., 2019a). The warm-temperate south coast, in particular, is driven by extreme localized sea temperature variability on both daily and seasonal time scales, with changes of up to 10°C within a few hours being documented (Schumann *et al*., 1995, Schlegel *et al*., 2017). As atmospheric CO_2 increases, radiative cooling will be suppressed, leading to disproportionate heating rates over land relative to that of the ocean (Bakun *et al*., 2010). This excessive heating is anticipated to lead to increased upwelling favourable winds, which, in turn, will likely increase the frequency and intensity of upwelling as well as an associated increase in the rate of offshore advection (Bakun *et al*., 2015).

Climate change impacts the production and distribution of marine species as populations respond either directly or indirectly to alterations to their environment. Changing temperatures directly affect physiological, behavioural, and reproductive processes and distribution (Fry 1971, Rijnsdorp *et al*., 2009; Dahlke *et al*., 2020). Because climate change affects many environmental factors and processes, resolving the effects of these changes is complicated (Rijnsdorp *et al*., 2009). Indirect effects on fish populations are manifested through changes in productivity and ecosystem processes (Harley *et al.*, 2006). For example, increasing temperatures are associated with increased stratification and changing circulation patterns, which affect nutrient supply and plankton distributions, limiting food supply at greater trophic levels (Rykaczewski and Dunne, 2010; Popova *et al.*, 2016). Ultimately, understanding the responses of fish and their populations to these environmental changes is critical for planning and management of marine resources (Pörtner and Peck, 2010; Poloczanska *et al.*, 2016).

As ectothermic animals, fish are physiologically and physically regulated by temperature. The effect of temperature on physiological rates is governed by oxygen demand and the capacities of various systems, such as the cardiorespiratory system, to supply oxygen to tissues (Fry 1971, Pörtner *et al.*, 2017). Temperature also regulates reproductive processes from gamete development and maturation to spawning, hatching, and development of embryos and larvae (Pankhurst and Munday, 2011). Temperature, therefore, firmly controls the production and distribution of fish populations, with some species, owing to differing oxygen supply capacities, being more sensitive to thermal variability than others (Pörtner and Peck, 2010). Also, thermal sensitivity varies among individuals within a species and throughout the lifecycle, with spawning adults, embryonic and larval stages being most vulnerable to thermal changes (Dahlke *et al.*, 2020). Increasing temperatures will also reduce dissolved oxygen saturation, while stratification can lead to low oxygen levels of subsurface waters (Vaquer-Sunyer and Duarte, 2008). Reduced oxygen saturation could reduce activity rates by limiting metabolism, while a mismatch between oxygen supply and

demand may be the first mechanism driving distributional shifts (Pörtner and Knust, 2007; Potts *et al.*, 2015). Low dissolved oxygen levels can also occur in shallow coastal waters due to upwelling, which may be increasing in frequency and severity associated with climate change (Frieder *et al.*, 2012; Wang *et al.*, 2015; Duncan *et al.*, 2019a). While larger fishes, due to their greater anaerobic capacities, might be somewhat more tolerant to periodic low oxygen levels, tolerances are generally lifestyle and species-specific (Nilsson and Östlund-Nilsson, 2008). However, it is suggested that embryonic and larval stages may temporarily tolerate low oxygen levels by inducing metabolic depression, which reduces and may even stop growth and development (Rombough, 1988; Breitburg, 1994; Nilsson and Östlund-Nilsson, 2008). Because of the importance of maintaining high growth rates during larval stages, changes in growth during early development can have persistent effects (Ali *et al.*, 2003) and influence recruitment rates to natural populations (Houde, 1987). Vagner *et al.* (2019) suggest that hypoxia may be one of the most challenging drivers of climate change to overcome, as the effects of exposure on early developmental stages will lead to adverse effects on later stages.

Rising atmospheric CO_2 is tempered mainly by oceanic uptake, absorbing nearly a third of anthropogenic carbon added to the atmosphere (Sabine *et al*., 2004; Orr *et al*., 2005). While this uptake will help moderate future climate change, the associated process of hydrolysis of CO_2 in seawater increases the hydrogen ion concentration, decreasing pH and reducing calcium carbonate saturation, leading to ocean acidification (OA) (Orr *et al*., 2005). Average ocean surface water pH has already declined by more than 0.1 units since preindustrial times from 8.21 to less than 8.10 and is expected to decline by a further 0.4 – 0.5 pH units by 2100 (Orr *et al*., 2005; Raven *et al*., 2005; Doney *et al*., 2008). Critically, the high rate of decline is happening at a rate many times greater than has been experienced in the past millennia, and pH values expected by 2100 are many times lower than has been experienced over this period (Raven *et al*., 2005). Like temperature, changes to ocean pH will not be uniform across the ocean, with greater rates of change occurring near upwelling regions (Hofmann *et al.*, 2011). For example, acidification along the west and south coasts of South Africa is expected to occur at a greater rate than along the east coast (Hoegh-Guldberg and Bruno, 2010; Potts *et al*., 2015).

Much of the observations and projections for ocean acidification have been based upon open-ocean conditions measured quarterly, annually, or less often and where variability is exceptionally low, ranging approximately 0.02 units (Hofmann *et al*., 2011). Fine-scale in situ observations are uncommon, limiting our understanding of coastal carbonate chemistry variability and fluctuations which may be further influenced by freshwater inflow, upwelling, and biological activity (Frieder *et al*., 2012, Carstensen *et al*., 2019). For instance, daily

nearshore pH variation at the Californian La Jolla kelp forest ranged as much as 0.36 units. In comparison, variation from mean conditions could be as much as 0.1 units during upwelling events, declining to levels as low as 7.9 (Frieder *et al*., 2012). Additionally, high water column stratification led to considerable differences in pH between 7 and 17 m with mean values of 8.07 and 7.87, respectively (Frieder *et al*., 2012). Locally, mean coastal values of 8.04 have been recorded in Algoa Bay along the south coast of South Africa (Edworthy, 2020). Variability in recorded pH was high, up to 0.56 units, varying at both daily and longer time scales and likely driven by a combination of biological activity (photosynthesis and respiration) and upwelling (Edworthy, 2020). Additional drivers of variability in coastal pH, such as freshwater inputs and eutrophication, result in complex patterns of change for these dynamic environments, making predictions of future climate change difficult (Carstensen *et al*., 2019).

In contrast to the open ocean, values higher than 1000 µatm have already been observed in many coastal surface waters. This has been attributed to eutrophication and high rates of productivity and respiration associated with these environments (Frieder *et al.*, 2012; Melzner *et al.*, 2012). Elevated environmental pCO_2 will directly impact physiological mechanisms in fish by disrupting the diffusion of metabolically-derived CO_2 across cells and body fluids and into the environment (Vagner *et al.*, 2019). Elevating CO_2 within the body leads to acidosis, an acid-base disturbance, disrupting cellular processes, impacting molecular, cellular, organ, and whole animal performance (Brauner *et al.*, 2019). As such, acid-base balance is a tightly controlled process regulated by hydrogen and chloride ion exchange, a process primarily conducted at the gills (Fu *et al.*, 2010). Due to the absence of gills and/or high surface area: volume ratio, larval fish track environmental pCO_2 more closely than other life stages (Brauner *et al.*, 2019). For larval fish, reduced acid-base regulation capacity or increased metabolic costs associated with regulation may be responsible for findings of organ damage (Frommel *et al.*, 2012, 2014, 2016), reduced growth, and increased mortality rates (Franke and Clemmesen, 2011; Baumann *et al.*, 2012). Therefore, ocean acidification can severely affect population persistence by impacting recruitment dynamics (Stiasny *et al.*, 2016).

The effect of climate change on marine ecosystems is complex, and the drivers of change are diverse and spatially variable, making predictions difficult. Changing pH, temperature, oxygen concentration, and food availability have been identified as four principal climate drivers that will increasingly affect marine ecosystem structure, functioning, and adaptive capacity (Henson *et al*., 2017). However, changes at the base of the ecosystem, via changes to net primary productivity, are similarly affected by these drivers but additionally through changing distribution and composition of phytoplankton and habitat alterations

(Constable *et al*., 2014). Changing SST, upwelling intensity and frequency, current strength, and declining pH have been identified as some of the relevant changes likely to occur along the South African coastline (Potts *et al*., 2015). These climate stressors, if not directly, will have diverse indirect consequences on ecosystems and fish communities.

Fish populations facing climate change and/or variability will have to respond by changing their distribution, acclimating, or adapting (Munday *et al.*, 2017, 2019). Acclimation and adaptation are forms of environmental responsiveness that have a genetic basis. Broadly, acclimation relates to an individual‘s ability to produce more than one alternative phenotype in response to environmental conditions, while adaptation involves genetic selection over time (Munday *et al.*, 2019; Vagner *et al.*, 2019). The potential for acclimation and adaptation is, thus, directly related to phenotypic and genotypic diversity (Foo and Byrne, 2016). Individuals with phenotypic traits suited to the new conditions will begin to dominate the populations in response to environmental stress. This new phenotype will slowly undergo genetic assimilation and spread within the population (Lande, 2009). The ability to tolerate change, therefore, depends on the phenotypic and genotypic composition of a population and the variation within it, which differs amongst regions or environments relative to specific selective pressures, stressors, or external forms of selection (De Almeida-Val *et al.*, 2005; Hollins *et al.*, 2018; Petitjean *et al.*, 2019).

Fishing has been shown to constrain the phenotypic diversity of exploited populations, leading to increased sensitivity to environmental stress, which Morrongiello *et al.* (2019) described as fishing-induced homogenization of individual sensitivity. This process is best described as selecting bold, active individuals with higher metabolic phenotypes (Biro *et al.*, 2008; Diaz Pauli and Sih, 2017). For instance, thermal performance is thought to have strong links with behaviour and physiology, and thus, exploited populations may be more sensitive to the effects of temperature variability after prolonged, selective removal of relatively high-performance phenotypes (Morrongiello *et al.*, 2019; Duncan *et al.*, 2019b). The removal of high-performance phenotypes from the gene pool, in turn, affects the structure and function at higher levels of organization (Perry *et al.*, 2010). This process may occur rapidly, where self-recruitment or retention of larvae is high, as metabolism and other phenotypic traits are heritable (Long *et al.*, 2021), leaving exploited populations more sensitive to climate change and variability (Perry *et al.*, 2010; Hollins *et al.*, 2018). Because of the relatively more remarkable plasticity of larval stages (Gilbert, 2001) and their connective potential through planktonic dispersal, they are suggested to provide the best adaptive potential to climate change (Vagner *et al.*, 2019). However, excessive exploitation may decrease genetic variability and erode the potential for local adaptation leading to populations that are more sensitive to climatic extremes. This may be particularly pervasive

for populations where self-recruitment is common. Self-recruitment of reef fish populations is suggested to be far higher than previously expected (Borges *et al.*, 2007; Teske *et al.*, 2016), and larval retention may be a standard feature along coastal regions of the South African southern coast (Porri *et al.*, 2014). Indeed high residency and larval retention may not only accelerate this process but may have severe implications on the persistence of exploited populations under increasing climate variability and change.

1.2 Early life-history research: a perspective

Most fishes undergo indirect development, where the free-living organism transitions through a series of developmental stages from egg to larval and juvenile stages. To use conventional terminology, the embryonic stage encompasses fertilization through to hatching. At hatching, the fish becomes a larva and, while utilizing endogenous resources, is termed a yolk-sac larva until yolk reserves are absorbed. The period after the yolk-sac is absorbed and before the flexion of the notochord is called the preflexion stage. Once the flexion of the notochord begins, it is referred to as the flexion stage. Subsequently, the postflexion stage is characterized by the upturned notochord while ventral and caudal rays and supporting elements are not yet fully developed. This period extends until metamorphosis, where the fin rays and scales are formed, and the juvenile phase begins (Miller and Kendall, 2009). While development from a single cell to a complex organism occurs during the embryonic stage, development of the organs, muscles, and body parts necessary to survive the juvenile habitat often only develop during the larval stage (Miller and Kendall, 2009; Downie *et al.*, 2020). Variability in survival rates of the vulnerable larval stages strongly relates to fish stock dynamics. Early research by Hjort (1914) established the field of research into recruitment dynamics which later expanded into many subfields on early life-history biology, ecology, and oceanography (Houde, 2008; Llopiz *et al.*, 2014). Hjort (1914) proposed two main theories, the ~~c~~ritical period" and the ~~a~~berrant drift" hypotheses, to explain recruitment variability or year-class success. Briefly, the two theories propose that early larval stages can determine that year-class strength. Firstly, exogenously feeding larvae must ~~fi~~nd" suitable food in suitable quantities, and secondly, the dispersal patterns of planktonic stages by currents will determine recruitment success (Houde, 2008; Llopiz *et al.*, 2014). While significant advances in the field of recruitment variability have been made, Houde (2008) suggests that progress toward understanding recruitment in the context of variability within marine ecosystems and implications for management are worthy goals. As oceans currently undergo rates of change greater than any time over the past 300 million years (Hönisch *et al.*, 2012), research into the biology and ecology of the early life-history stages becomes more pressing than ever before.

Physiological research, which can be defined as the study of how an organism functions and adapts to changes in its external and internal environment (Finn and Kapoor, 2008), can be invaluable for gaining insight into how environmental change will affect fishes at varying levels of organization. Understanding the physiological responses of individuals to altered temperature, pH, and oxygen concentrations may prove vital in managing the preservation or recovery of fish populations (Burggren and Blank, 2009). Although embryonic and larval stages may lack cells, tissues, and organs present in later stages, they must still face their environment's same physical and chemical challenges (Finn and Kapoor, 2008). For instance, the gills are the predominant site for O_2 and CO_2 exchange, nitrogenous waste excretion, and acid-base regulation. Yet, gills only develop late into the larval stages for many marine fish species (Rombough, 1999, 2007). Embryonic and early larval stages must therefore exchange gases and ions through the epithelium, primarily performed by bulk diffusion (Pelster, 2008) and, therefore, reduced in its regulatory capacity. This implies that the internal environment will more closely match that of the external environment, and these stages may therefore be more vulnerable to environmental irregularities due to incomplete or inadequate regulation of ions or elevated metabolic rates associated with regulation (Brauner *et al.*, 2019; Munday *et al.*, 2019).

While larval fishes' small size and delicate nature may make experiments and physiological studies difficult, they can be effective models for understanding evolutionary and developmental processes. Studies of larval physiology can be invaluable for increasing the effectiveness of fisheries management (Cury and Roy, 1989; Kingsford *et al.*, 2002) and will also increasingly serve as important indicators for the impacts of global climate change (Burggren and Blank, 2009).

1.3 Aims of this thesis

The broad aims of this thesis are to determine the effect of ocean acidification, as an environmental stressor, on the physiology and development of the larvae from a commercially important marine fish species and to elucidate how exploitation and physiological traits may interact to shape the resilience of fish populations to climate change. To achieve this, this thesis is broken down into six chapters, which provide context to the study (Chapter 1 and 2), three research chapters (Chapters 3, 4, and 5), and a final synthesis chapter (Chapter 6).

More specifically, **Chapter 1** provides context and a perspective on the effects of global climate change and exploitation on fisheries populations and their environments. The value and importance of research on the early life history of fishes are briefly discussed, focusing on physiological research.

Chapter 2 provides a regional background to the South African coastal marine environment, emphasizing environmental change and variability occurring or predicted to occur due to increasing anthropogenic CO_2 release. This is followed by a description of the environmental conditions in the exploited and unexploited study sites. A brief description of the study species, *Chrysoblephus laticeps*, is presented, followed by a detailed explanation of the spawning and rearing processes and systems used to collect and maintain the eggs and larvae. This is concluded with a brief description of the larval stages.

A novel approach to determine minimum, routine and maximum oxygen consumption rates for fish larvae is presented in **Chapter 3**. By using rolling regression techniques on high-frequency respirometry data, this method produced reliable and repeatable results of oxygen consumption rates which may serve as relative proxies for Standard, Routine, and Maximum Metabolic Rates for larval fish.

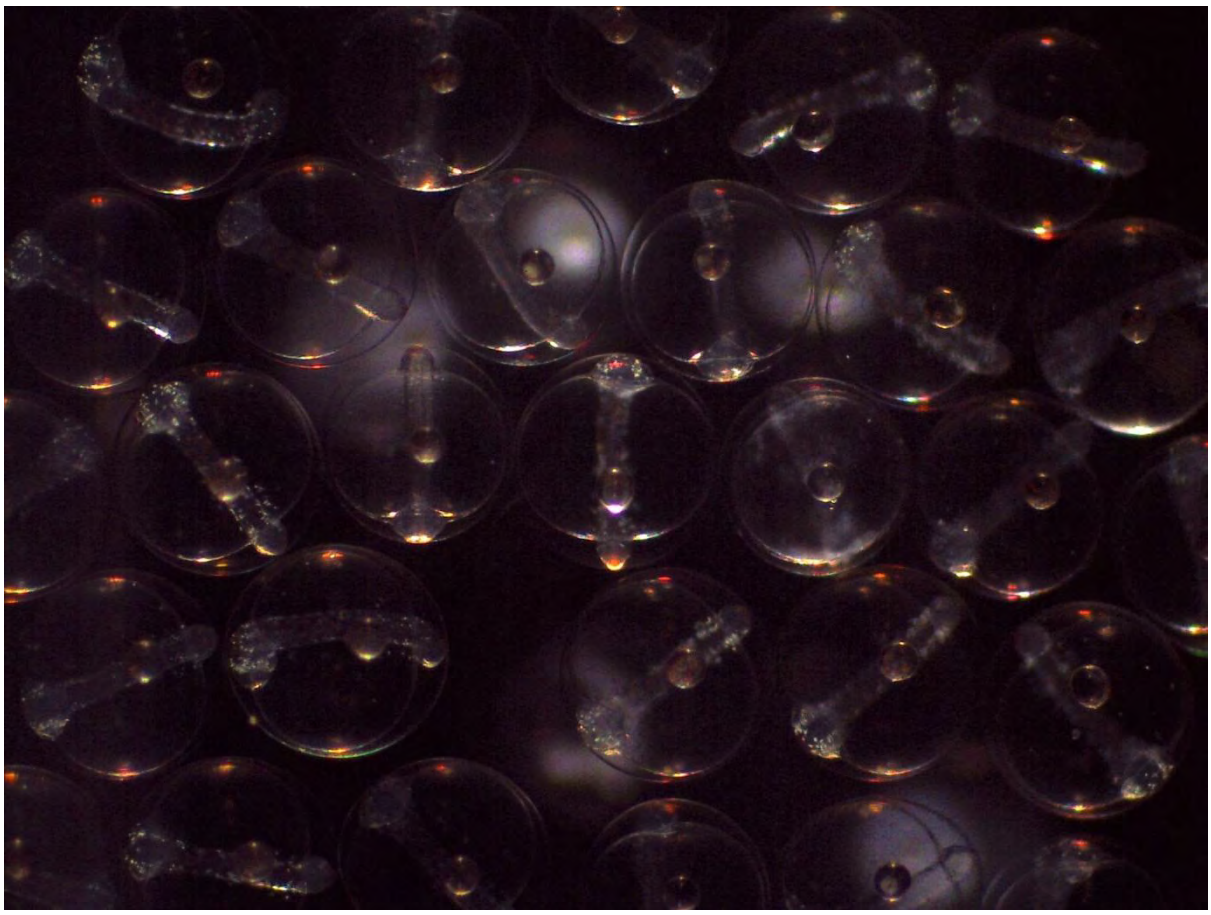
Chapter 4 assesses the impact of hypercapnic (high pCO_2) environmental conditions, expected by the year 2100, on the metabolic physiology and morphometrics of *C. laticeps,* by using the techniques investigated in the previous chapter. The chapter pays particular attention to the differences in responses between the early life stages, from shortly after hatching to flexion.

Chapter 5 compares the response of *C. laticeps* larvae spawned from adults in exploited and protected populations to hypercapnic conditions. The interaction between environmental stress and exploitation is compared to determine the potential effects of constrained physiological/genetic diversity, due to selective fishing, on the resilience of offspring to climate change.

The final chapter, **Chapter 6**, synthesizes the results of the data chapters, focusing on the potential of Marine Protected Areas for promoting climate change resilience in fishes. A further focus is placed on using an energy-limited concept for stress modified for early life stages as a means to provide a conceptual framework for understanding the effects and severity of environmental stressors on physiology, growth, and survival. This framework should be applicable for studies experimenting with single and multiple stressors and help gauge the severity of a stressor by comparing metabolic rates between treatment and controls. Limitations to the study, as well as recommendations for future research, are also discussed.

Chapter 2

Study area, species profile, spawning and rearing



Roman seabream (*Chrysoblephus laticeps*) approximately 26 hour‘s post-fertilization

2.1 South African coastline

The southern African coastline is one of the least convoluted worldwide and rugged in nature, dominated by strong ocean features on both the west and east coasts. The coastline extends 3650 km and predominantly comprises sandy beaches with mixed rocky shores (Bally *et al.*, 1984). The Agulhas Current is a warm western boundary current that flows close inshore along the eastern seaboard, sweeping warm equatorial water from the tropics down the east coast (Figure 2.1A). In the region of East London, the shelf widens and drives the current further offshore. Strong and persistent easterly winds along the south coast in the Austral summer and autumn drive surface waters offshore, causing seasonal upwelling of cold waters, while Agulhas meanders may occasionally drive warm water near the coast (Schumann *et al.*, 1995; Schlegel *et al.*, 2017). The Benguela Current drives cold water northwards on the western seaboard, while predominant south-easterly winds blow surface water offshore, causing cold, deep water to well up near the coast (Branch, 2017). The strong influence of these two currents separates the coastline into three distinct marine bioregions based on prevailing thermal regimes. These bioregions include the cool-temperate region of the west coast, warm-temperate south coast, and the subtropical east coast (Potts *et al.*, 2015; Whitfield and Pattrick, 2015) (Figure 2.1A).

The south coast, warm-temperate bioregion along South Africa‘s south coast is characterized by high thermal variability. This is particularly the case during summer and autumn when a strong thermocline is established, and protracted easterly winds can cause upwelling with changes in temperature of up to 10°C within a day (Schumann *et al*., 1988). These upwelling events originate at prominent headlands with the cold upwelled water then flowing alongshore in a westward direction before dispersing southwards onto the Agulhas bank (Figure 2.1 B-E) (Goschen and Schumann, 1995).

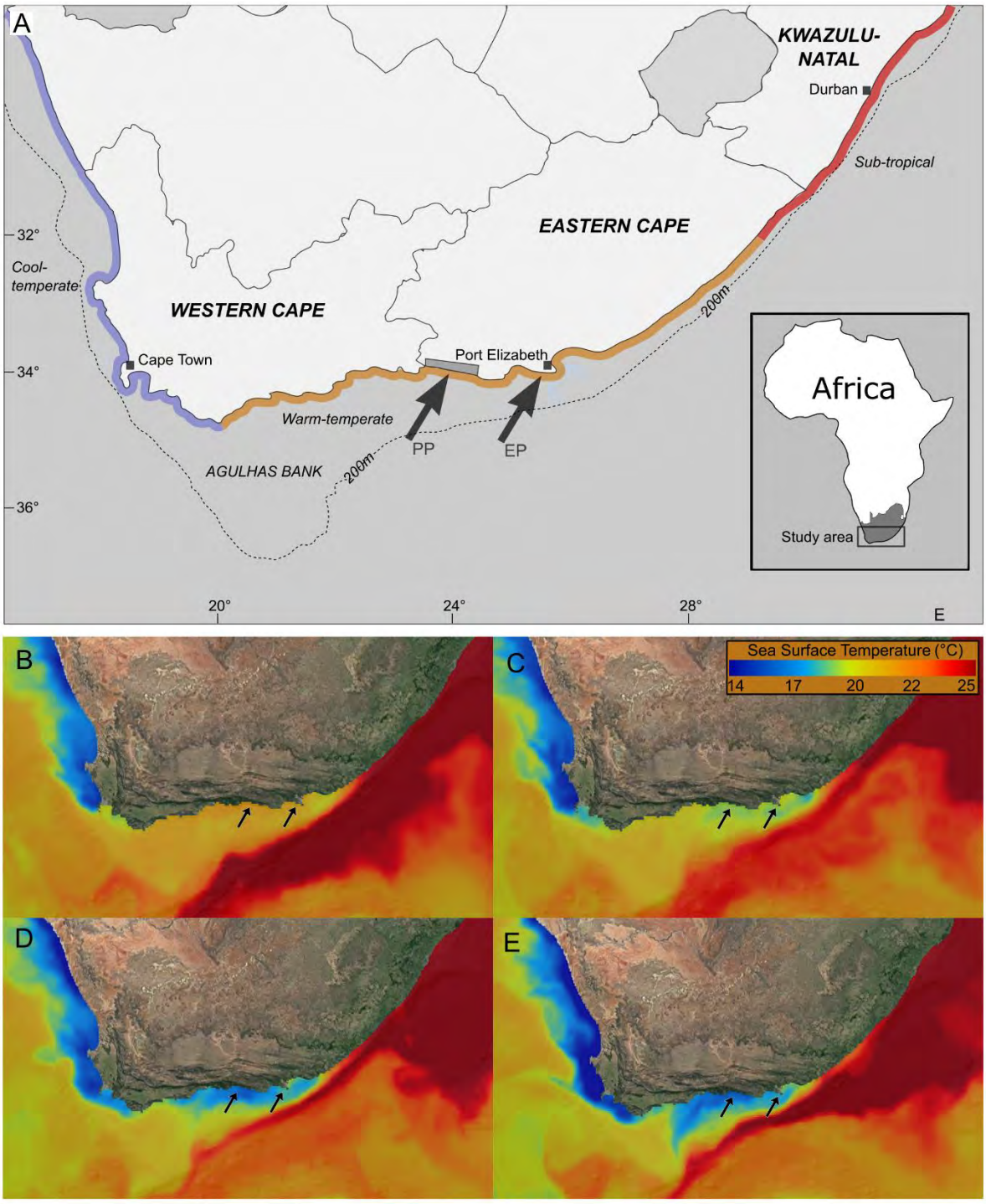


Figure 2. 1 Geographic map of South Africa (A) indicating divisions into three major biogeographic regions (top plate): cool-temperate (west coast), warm-temperate (south coast), and sub-tropical (east coast). Sampling locations indicated by black arrows: Tsitsikamma National Park (TNP or PP, left) as grey bordered region and Noordhoek, Port Elizabeth (NHK or EP, right). Bottom panels represent sea surface temperature (SST) map capturing pre-upwelling conditions (B, 16-03-2021); formation of upwelling cells (C, 28-03-2021); upwelling and alongshore advection (D, 03-04-2021); and offshore advection (E, 16-04-2021). Satellite images and SST provided by PacIOOS, U.S. Integrated Ocean Observing System (IOOS) and Voyager overlay (www.pacioos.org)

2.2 Sampling locations

In order to investigate the effects of exploitation on fish populations‘ resilience to changing environmental conditions, it was necessary to compare the responses of individuals from populations of contrasting levels of fishing pressure; while controlling for other factors as much as possible. Given a restricted spawning period and the protracted period required for larval rearing, the field spawning of fish in multiple exploited and protected sites was not possible and had to be restricted to one protected population (PP) and one exploited population (EP) (Figure 2.1 A).

2.2.1 Protected site

The coastline along the Tsitsikamma National Park (TNP) is the longest standing Marine Protected Area (MPA) in South Africa, established in 1964 and running for 60 km along the coast and stretching 5.6 km offshore (Lombard *et al.*, 2019) (Figure 2.1A). The coastline is primarily rocky and provides protected reef habitat for several overexploited linefish species, particularly for those which are long-lived and highly resident (Cowley *et al.*, 2010). The MPA also provides refuge to all life history stages for at least 17 commercially and recreationally exploited species (Wood *et al.*, 2000). Numerous studies have assessed the ichthyofaunal abundance, composition, and life-histories within the park, noting near-pristine populations and highlighting the importance of the MPA as critical for the protection of linefish species and maintenance of spawner biomass (Buxton, 1987, 1990a; Tilney and Buxton, 1994; Tilney *et al*., 1996; Wood *et al*., 2000; Cowley *et al*., 2010; Götz *et al.*, 2008; Lombard *et al.*, 2019).

2.2.2 Exploited site

For an exploited population comparison with the TNP, the coastal area directly south of Port Elizabeth, near the Noordhoek ski-boat club, was selected (Figure 2.1A). The area lies approximately 140 km east of the TNP, but given its proximity to a large city, the population has been subjected to decades of intense recreational and commercial fishing pressure (Smale and Buxton, 1985; Griffiths *et al.*, 2010). In addition to the location's exploitation history, Noordhoek (NHK) was selected due to its thermal and ecological similarity with the TNP, as noted in previous studies (Buxton and Smale, 1989; Buxton, 1993; Duncan *et al.*, 2019b) and below.

2.2.3 Thermal regimes of the two study sites

Among other headlands, both the TNP and NHK sampling locations appear to be focal regions for upwelling cells (Goschen and Schumann, 1995) (Figure 2.1 B-E). As the thermal

history of the populations, together with temperature-related ecological effects, may suggest varying tolerance or local adaptation, thermal regimes should be similar. To test the validity of the assumption that the thermal regimes were similar, the thermal history of the two locations was compared using temperature measurements captured from daily underwater temperature recorder (UTR) sea temperature data. UTR data was obtained from the Southern African Data Centre for Oceanography (SADCO) for the TNP (10 m depth) and Mangolds Pool, near Port Elizabeth (5 m depth) collected by the Department of Environmental Affairs (DEA) Oceans and Coasts. As the data had discontinuities in the collection, only concurrent data from both datasets were included in the analysis, resulting in continuous daily time series for both locations for periods January 1992 – April 1997 and April 2000 – November 2005.

The time series was decomposed into its trend, seasonal and random components using the ~~"d~~ecompose" function. The random component of each time series is an irregular variance that represents a departure from mean sea temperatures, such as by upwelling or downwelling events. The similarity in direction and magnitude of the random component between sampling areas was tested for synchrony using the ~~"m~~eancorr" function of the synchrony package (Gouhier and Guichard, 2014). Spatial and temporal autocorrelation are dealt with by instituting a naïve randomization procedure with tests for significant correlations performed using 999 Monte Carlo randomizations.

Visual inspection of the UTR time series showed similar yearly (Figure 2.2 A) and monthly (Figure 2.2 B) patterns, with high overlap for trends and ranges between the two locations. Temperature variability showed a seasonal pattern, with relatively more stable winter conditions and higher variability (greater standard deviation) during summer (Figure 2.2 C), which was typical for both locations. There was significant and high synchrony in thermal variability (Pearson's correlation: 0.681, p-value < 0.001), indicating a similar direction and magnitude of departure from mean sea temperature between locations.

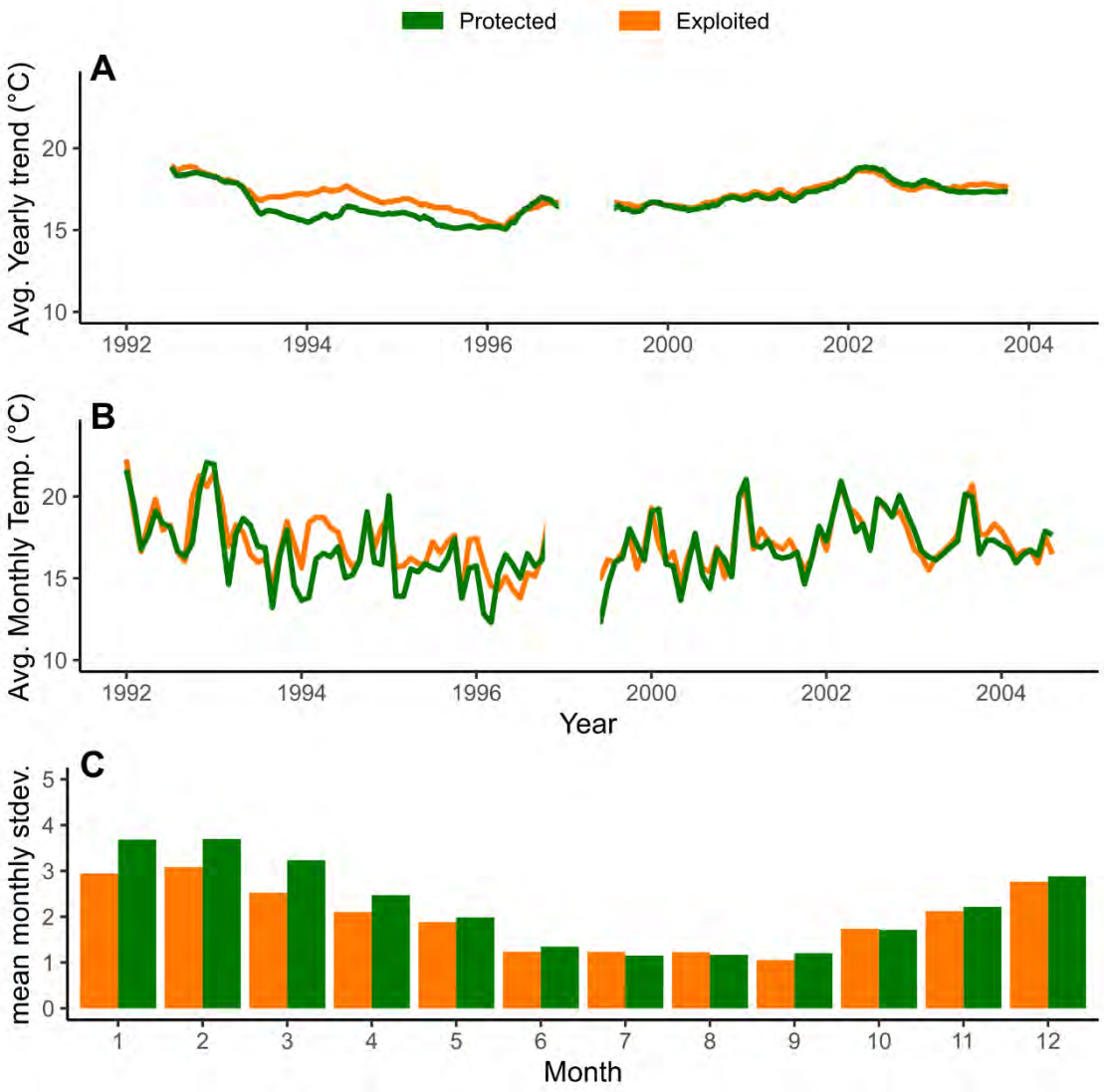


Figure 2. 2 Temperature profiles captured from in situ measurements showing: A) the average yearly trend, B) the mean monthly, and C) monthly averaged standard deviation from protected (Tsitsikamma National Park, green) and exploited (Port Elizabeth, yellow) sampling areas. Continuous measurements of both sites from periods January 1992 – April 1997 and April 2000 – November 2005

2.3 Study species

This thesis aimed to advance on previous work by Duncan (2019) and Duncan *et al.* (2019b), which examined metabolic phenotypes of adult fish from exploited and unexploited populations and found that exploitation removed high-performance individuals of the roman seabream *Chrysoblephus laticeps*. Duncan (2019) selected *C. laticeps* as a study species from the family Sparidae as it was the only species within the family that met key criteria to ensure that physiological data were representative of exploited and unexploited populations. These included a core distribution through the sampling area, commercial exploitation, high site residency, hardiness, shallow habitat depth, intra-specific competitiveness, and

panmixia (Duncan, 2019). The Sparidae family, commonly known as the sea breams, is a morphologically and ecologically diverse family that comprises approximately 100 species, which range throughout the world‘s oceans (Heemstra and Heemstra, 2004; Pavlidis and Mylonas, 2011). Southern Africa, however, appears to form the family‘s centre of distribution, where 41 species occur, 25 of which are endemic to the region. They occupy a range of habitats, from shallow estuaries to deeper offshore reefs (Van der Elst and Borchert, 1993). In southern Africa, they rank among the most important species for angling and as a food source (Van der Elst and Borchert, 1993; Mann, 2013). Many are hermaphroditic, either protandrous, changing sex from male to female, or protogynous, being female first and later becoming male (Buxton and Garratt, 1990; Pavlidis and Mylonas, 2011). Many larger species are slow-growing, reaching ages greater than ten years and becoming increasingly territorial with size (Heemstra and Heemstra, 2004). Reproduction typically takes place seasonally as many small pelagic eggs are produced, hatching into pelagic larvae which are small and poorly developed (Pavlidis and Mylonas, 2011; Pattrick *et al.*, 2013).

Chrysoblephus laticeps are endemic to the warm-temperate south coast, where genetic studies have suggested they are a single well-mixed population (Teske *et al.*, 2010). They are exploited throughout their distribution by the commercial boat-based linefishery and recreational ski-boat fishery (Smale and Buxton, 1985; Mann, 2013). Mean catch rates per boat have been steadily declining throughout their distribution over the past century (Griffiths, 2000). Monitoring data collected through the National Linefish Monitoring System indicates that the commercial linefishery catch of *C. laticeps* in the NHK area was well over 100 000 kg between 1985 to 2000 (Kerwath *et al.*, 2013; Duncan *et al.*, 2019b). *Chrysoblephus laticeps* (Figure 2.3) is a long-lived, hermaphroditic species, maturing from ~3 years, changing sex from female to male from ~7 years, and reaching at least 17 years (Buxton, 1993; Götz *et al.*, 2008). Spawning occurs throughout their distribution as the large, resident males increasingly defend their territory forming harems (Provost and Jensen, 2015). Gametes are released well above the substratum as pairs spawn, producing many small pelagic eggs (Buxton, 1990). Post-recruits are highly resident, residing in home ranges as little as 100 $m^{2,}$ and exhibit high territoriality, which increases with age (Kerwath *et al.*, 2007). As such, most dispersal and population connectivity are likely to be restricted to the planktonic embryo- and larval stages (Teske *et al.*, 2010), with settlement occurring from approximately 28 days after hatching, or approximately 30 days pelagic duration. Nonetheless, this species's slow growth, late maturation, and high residency make it particularly vulnerable to recruitment failure due to a reduction in the number of large males (Buxton, 1993). On the other hand, the small home range and high site fidelity after settlement lead to effective protection of *C. laticeps* in MPA‘s (Götz *et al.*, 2008). While *C.*

laticeps adults have been well studied, including their physiology (Duncan *et al*., 2019b; Skeeles *et al*., 2020), exploitation (Götz *et al*., 2008; Götz *et al*., 2009; Kerwath *et al*., 2013), biology (Buxton, 1990, 1993) and behaviour (Kerwath *et al*., 2007), considerably less work has been carried-out on their early-developmental stages. Indeed, ichthyoplankton studies along the southern coast rarely capture *C. laticeps* larvae in any appreciable numbers (Brownell, 1979; Wood, 1998; Pattrick, 2013).

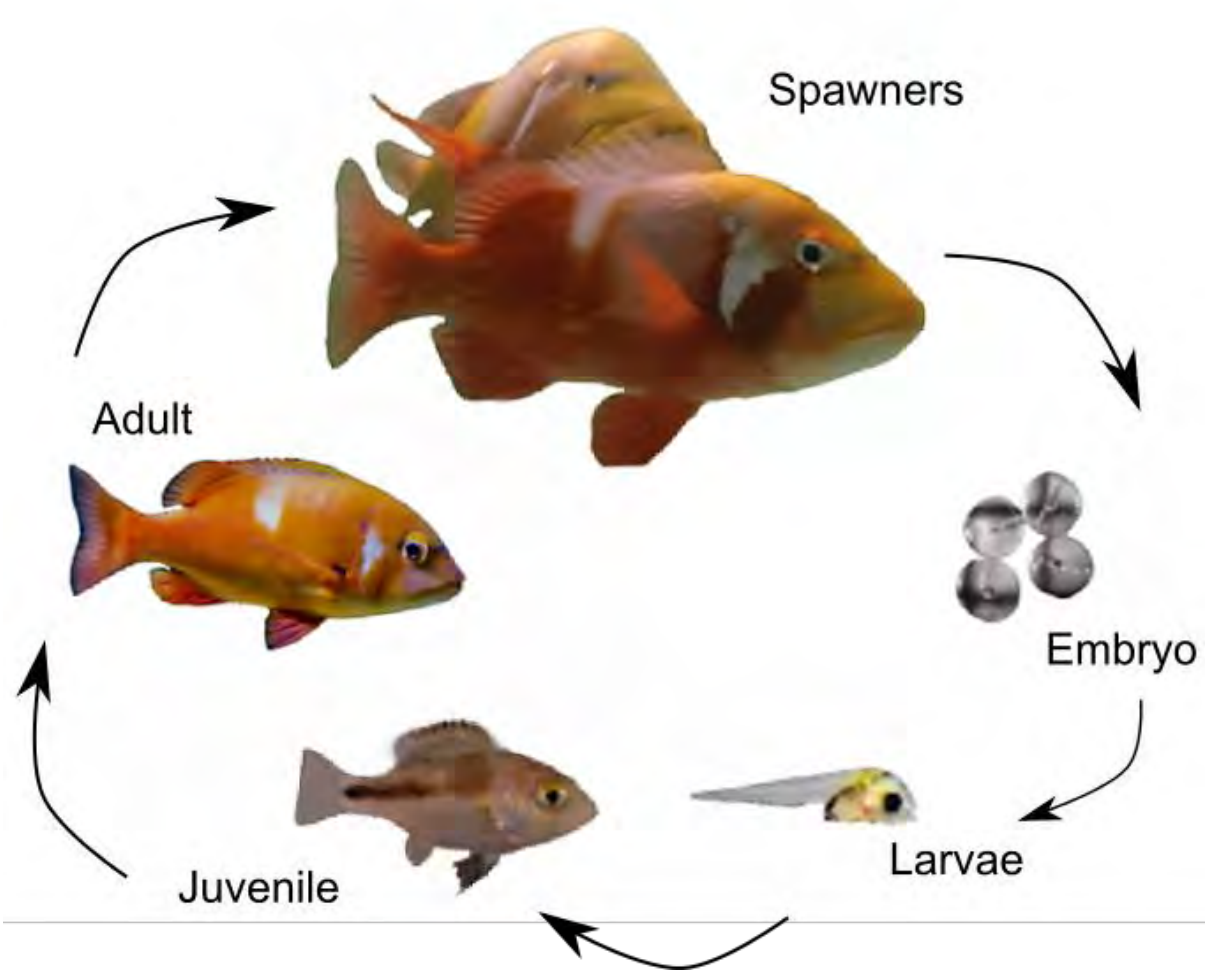


Figure 2. 3 Sequence of life stages of the roman seabream *Chrysoblephus laticeps* (Valenciennes 1830)

The combination of high site fidelity and exploitation makes this species a good model for investigations into fisheries-induced evolution. In addition, site fidelity, slow growth, hermaphroditism, and late maturation are shared features among many other sparids. This suggests that findings from this thesis will apply to C. laticeps and several other exploited species that share similar life histories and behaviours in South Africa and other temperate coastal waters.

2.4 Spawning and rearing

Rearing of larvae has been carried out before on only two occasions. The first of these was from eggs recovered from offshore ichthyoplankton trawls, with only two individuals being reared through metamorphosis (Brownell, 1979). The second occasion was by Davis (1996), who, for his MSc dissertation, investigated *C. laticeps* as a potential candidate for aquaculture. In this instance, the survival rates were low, ranging from 0.1 – 0.5 % at post-metamorphosis. To carry out the objectives of this thesis (specifically, the long-term exposure of larval *C. laticeps* to control and hypercapnic conditions from the embryonic stage up to flexion), it was necessary to have a supply of fertilized eggs and a suitable rearing environment. These were considerable challenges due to a number of variables; including the absence of rearing information or protocols for the species, the notoriously high mortality rates of larvae for this species and sparids in general, the lack of existing captive broodstock for breeding nor a naturally restricted spawning season, and little existing rearing facilities on-site in Makhanda, which is situated inland and 150 km from the nearest sampling site.

The capture of wild fish involves considerable stress, which has an inhibitory effect on reproductive performance for both males and females. Inhibitory effects can include suppressing ovarian and testicular development, inhibition of ovulation and spawning, and the production of smaller eggs and larvae (Pankhurst, 2016). Ovarian development and the reproductive cycle are regulated by environmental stimuli such as photoperiod, temperature, and nutritional condition. These signals are transduced into an endocrine signal as a result of the hypothalamus synthesizing and releasing gonadotropin-releasing hormones (GnRH). This, in turn, controls the activity of the pituitary gland (Pavlidis and Mylonas, 2011; Pankhurst, 2016). Even short episodes of stress disrupt this process, increasing plasma cortisol levels and depressing concentrations of gonadal steroids, such as 17ß-estradiol (E2), an estrogen steroid hormone, leading to the cessation of reproductive development and subsequent gonadal atresia (Pankhurst and Sharples, 1992).

In many cases, artificially generated stress by confinement and husbandry has shown a consistent inhibitory effect on reproduction, which appears to be most common for wild-caught fish with asynchronous ovarian development (Pankhurst, 2016), such as sparids. Therefore, successful reproduction of wild-caught broodstock may only occur if fish can recover from stress and adapt to captive conditions, which may take several years. However, some sparids may not spawn in captivity at all (Pavlidis and Mylonas, 2011).

Fortunately, aquaculture research has increased steadily with the increasing economic importance of farmed fish, as has our understanding of the hypothalamic-pituitary-gonadal

axis (Pankhurst, 2016). The development of synthetic GnRH substitutes, such as the commercially available AquaSpawn, is an efficient reproductive stimulator despite findings that teleosts exhibit up to eight GnRH variants (Zohar *et al.*, 2010). AquaSpawn has been used successfully for many wild-caught species to induce ovulation with improved success when fish are administered the dosage as soon after capture as possible (Pavlidis and Mylonas, 2011). Davis (1996) successfully utilized AquaSpawn to induce ovulation and spermiation of *C. laticeps* in the field during their peak spawning period. Given the constraints on time and space, this thesis adopted Davis' (1996) approach by capturing wild adults and stripping their gametes on-site with the application of AquaSpawn GnRH.

2.4.1 Spawning methodology

Sexually reproductive *C. laticeps* were targeted between November and January, coinciding with peak gonado-somatic index (Buxton, 1990), using regular hook and line fishing techniques from a ski boat. All fish were captured on shallow reefs (<25 m in depth) to reduce the effects of barotrauma, from the exploited NHK (ca. -34°04‘14”S 25°37‘59”E – -34°02‘22”S 25°41‘56”E) and protected TNP (ca. 34°01‘16”S 23°50‘45”E). Upon capture, fish were vented with an 18-gauge hypodermic needle inserted into the swim bladder and then kept in one of two 250 L water drums which were aerated and underwent frequent 50% water exchanges while on the boat. At least 18 adults were targeted for stripping with a minimum of four males, which were identified by size. Once either all 18 individuals had been captured or a maximum of 6 hours had elapsed, the fish was brought to respective NHK or TNP slipways where two 6000 L tanks had been positioned with full flow-through water supply. Shortly after returning to shore (within 2 hours), all fish were weighed to the nearest gram, injected with 0.5 ml. kg-1 AquaSpawn, and returned to flow-through tanks covered with shade cloth to keep low lighting and prevent temperature fluctuations. After 22 and 48 hours, all fish were stripped of gametes by applying light pressure to the abdomen. Ripe individuals had swollen abdomens that were pale in colour. Gametes from the first stripping were discarded, and only those from the second (48 hours after injection) were kept. It has been found that egg quality and fertilization rates are improved with subsequent stripping‘s of asynchronous spawners (Davis, 1996; Pavlidis and Mylonas, 2011).

Sperm was collected first, with the milt from all individuals being mixed and stored on ice in a glass beaker; eggs were collected into a 2 L plastic bowl with careful removal of excrement. Before stripping, the abdomen was wiped with paper towels to prevent water contamination, and all containers were sterilized with a 500 ppm hypochlorite solution before being thoroughly dried. Once all individuals had been stripped, small quantities of sperm (total of ~ 10 mL) were added to the eggs along with increments of fresh seawater, totalling 1 L. At the

same time, the solution was continuously mixed with a fine-bristled brush for 10 minutes. Following this, the solution was aerated for a further 15 minutes, after which 0.27 mL of formaldehyde was added to disinfect the eggs of potential pathogens, during which it was heavily aerated for a further 15 minutes (De Swaef *et al.*, 2016). Following this, the eggs were repeatedly strained with fresh seawater using a 250 µm mesh sieve while remaining submerged. All eggs were then equally distributed into Ziplock bags and stored in a polystyrene cooler while transported to the NRF-SAIAB Aquatic Ecophysiology Research Platform (AERP) Laboratory at Rhodes University, Makhanda.

On arrival at the laboratory, all eggs were mixed into a single 25 L glass tank with heavy aeration while acclimating to the control temperature (18 - 19°C). After approximately 30 minutes, the airline was removed, and the eggs were allowed to separate into sinking and floating, which were assumed to be unfertilized/dead and fertilized/developing, respectively. All floating eggs were collected in a beaker with light aeration to ensure complete mixing throughout the water volume. Eggs were then enumerated by drawing ten 2 mL samples and then randomly distributed into eight rearing tanks at 80 eggs L^{-1} or 6000 eggs per tank, with four tanks per treatment.

2.4.2 Rearing

Rearing tanks were fibreglass moulded and lined with white epoxy-based paint, square in shape with a squared conical bottom and 75 L in volume. A water supply inlet was located in the top corner of the tank, and the outlet was centrally placed at the surface to prevent a surface film while 250 µm mesh screens covered the outlet. A single airline fitted with a diffuser was placed approximately 5 cm above the conical bottom and maintained at a constant, slow stream of bubbles using a needle valve to keep shearing forces of water turbulence to a minimum. The temperature was controlled with standard 200-watt aquarium heaters in each tank.

All rearing tanks were connected to a single recirculating system with a total water volume of approximately 2000 L, with partial water replacement of approximately 3-4% per day to ensure water quality, pH and carbonate chemistry reflected natural coastal conditions. The system consisted of a central storage tank. Water was consistently recirculated through a bubble-bead filter and into a 500 L protein skimmer with ozone treatment via a flow-metered check valve to ensure adequate contact time. Water was partially supplied from the storage tank through carbon filters to a 1.8 m tall bio-filter with heavy aeration of bio-media for nitrification and degassing. Water supply to rearing tanks was gravity fed from the bottom of the bio-filter while effluent water from the rearing tanks drained into a drainage tank which was heavily aerated. When the drainage tank was filled, a float-level switch was closed and

pumped the contents of the drainage tank back into the storage tank. Make-up water was stored in a 5000 L tank with a closed-loop filtration system, sand filter, and UV steriliser. Make-up water flowed into the rearing laboratory through three cartridge filters of decreasing filter size (50, 25, and 4 µm), topping up the storage tank of the recirculation system.

Water flow was introduced to each tank, along with the eggs, at a rate of approximately 3 L per hour, or nearly one tank volume exchange per day. At the time of hatching, water flow was stopped, and there was no new water exchange up until first feeding or DAH 4 when water flow was introduced at night only (7 p.m. – 7 a.m.) with rates of 1 L per hour for each tank. From DAH 7 until the completion of the experiments, the water flow was increased to 4.5 L per hour at night only. The photoperiod was set to a 14-hour light: 10-hour dark cycle with low light conditions before first feeding, after which additional lighting was added for 10 of the 14-hour day cycle. Low lighting or morning/evening conditions were simulated using 3-foot, 8000 K, and high-light or daytime conditions with 5-foot 6000 K fluorescent tubes placed approximately 1.2 m directly above rearing tanks.

A green-water culturing technique was used to rear larvae with daily additions of algal paste (Nanno 3600, Reed Mariculture, FL, USA) at a concentration of approximately 400 000 cells per mL from the first feeding. Rotifers *Brachionus plicatilis* were added into rearing tanks from DAH 3 up to flexion and maintained at densities of at least ten mL^{-1} in all tanks by a daily sampling of five 1 mL samples each morning. Rotifers were added in three feedings at a ratio of 50:40:10 for the morning, afternoon, and evening. Nightly flow with an outlet screen of 250 µm mesh allowed the removal of rotifers from tanks so that newly enriched rotifers could be added daily. Rotifers were reared on a diet of *Nannochloropsis oculata* and *Saccharomyces cerevisiae* (baker‘s yeast) before being cultured on an enrichment diet of ORI-ONE (Skretting Stavangar, Norway) before feeding to larvae. Even at the flexion stage, the mouth gape-size of larvae was still too small to introduce artemia nauplii.

2.4.3 Descriptions of the early life history of Chrysoblephus laticeps

The pigmentation description is for unpreserved, live specimens which were photographed using a Leica EZ4 stereomicroscope. The description, therefore, includes the presence of chromatophores that would otherwise be absent in preserved specimens. An illustration of specimens accompanies their description in Figure 2.4. Photographs of larvae and indications of developmental elements are shown in Figure 2.5

Eggs were spherical and transparent with a homogenous yolk which had a light orange-yellow tint. Fertilized eggs were buoyant and rapidly accumulated at the surface of the water column. The chorion was smooth with little perivitelline space. Egg diameter was

approximately 0.75 ± 0.029 mm (n=16) with a single oil globule of approximately 0.14 ± 0.008 mm. Before hatching, the pigmentation consisted of a series of small melanophores scattered along the dorsal surface of the body. Yellow chromatophores (xanthophores) were observed along the flanks. These were particularly prominent at the fore and rear of the unpigmented eye, the mid-body, and the posterior. At the same time, the rest of the egg was unpigmented. Hatching time varied with temperature as eggs incubated at 20°C began at approximately 30 hours post fertilization (HPF), nearly 100% hatched by 45 HPF. Eggs incubated at 18°C began hatching at approximately 42 HPF with approximately 100% hatched at 52 HPF.

At hatch, larvae measured 2.07 ± 0.193 mm (notochord length), and the elongated yolk-sac was approximately 37% of body length (0.76 ± 0.08 mm), and the posteriorly positioned oil globule was ~7% of body length (0.15 ± 0.01 mm). Newly hatched larvae had no formed mouth, unpigmented eyes, and undeveloped pectoral fins. Newly hatched larvae had a dorsal row of melanophores that extended to the head. Paired xanthophores remained at the fore and rear of unpigmented eyes, all the while continuing to develop along the posterior of the oil globule and along a prominent ridge dorsally above the anus. Two chromatophore bands were present midway between the anus and the head and midway between the anus and notochord tip. By DAH 3, a series of small melanophores presented along the ventral midline from the anus to the notochord tip. Chromatophore pigments decreased around the eye, which was still not fully pigmented. By DAH 4, the eyes were fully pigmented, the mouth was developed, and the gut appeared to be formed with a single coil. The chromatophore bands had decreased, with pigments remaining only along the dorsal midline. By DAH 8, chromatophores were only present dorsally along the head, around the otic capsule, and peritoneal cavity. Melanophores remained dotted along the ventral-lateral ridge at most myomeres' base while increasing density at the base of the anus and dorsally of the swim bladder. Flexion stage larvae at DAH 21 had scattered chromatophores on the head and along the jawline. Melanophores joined along the dorsal surface of the hindgut, forming a continuous line from the colon to the anterior edge of the swim bladder.

Nares began to differentiate from the olfactory pit at flexion when teeth were first visible at the tips of both jaws. The total myomere count was 24, with pre-anal to post-anal counts changing from 7+17, 9+15, and 11+13 for early-preflexion, late-preflexion, and flexion stages.

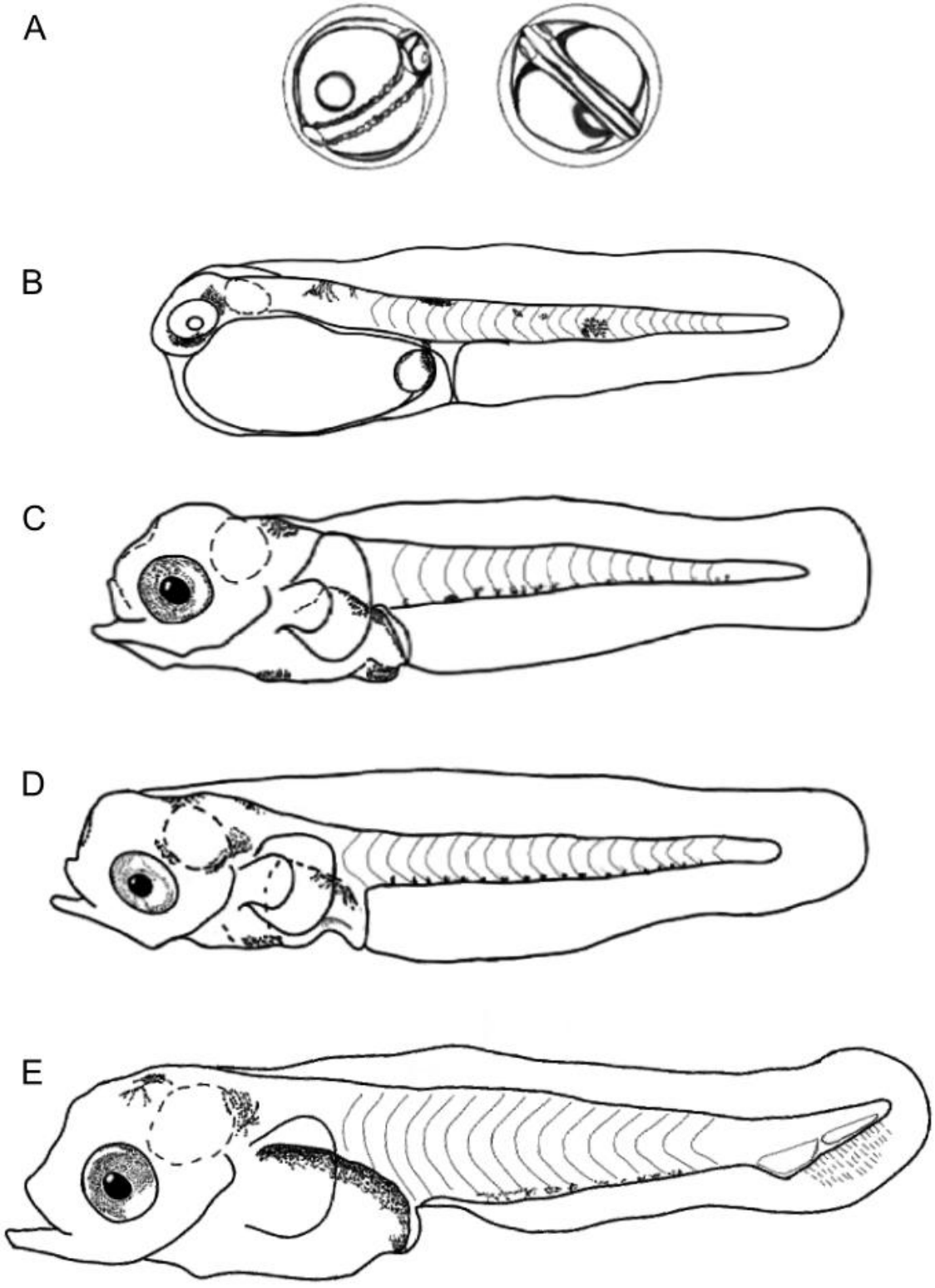


Figure 2. 4 Larval development of *Chrysoblephus laticeps* up to flexion stage. Late embryonic stage (A) diameter ~ 0.75mm; early yolk-sac (B) ~ 2.4 – 2.5 mm; early-preflexion (C) ~ 2.5 – 2.8 mm; late-preflexion (D) ~ 2.7 – 3.4 mm; and flexion (E) ~ 3.4 – 4.8 mm. Illustrations by author.

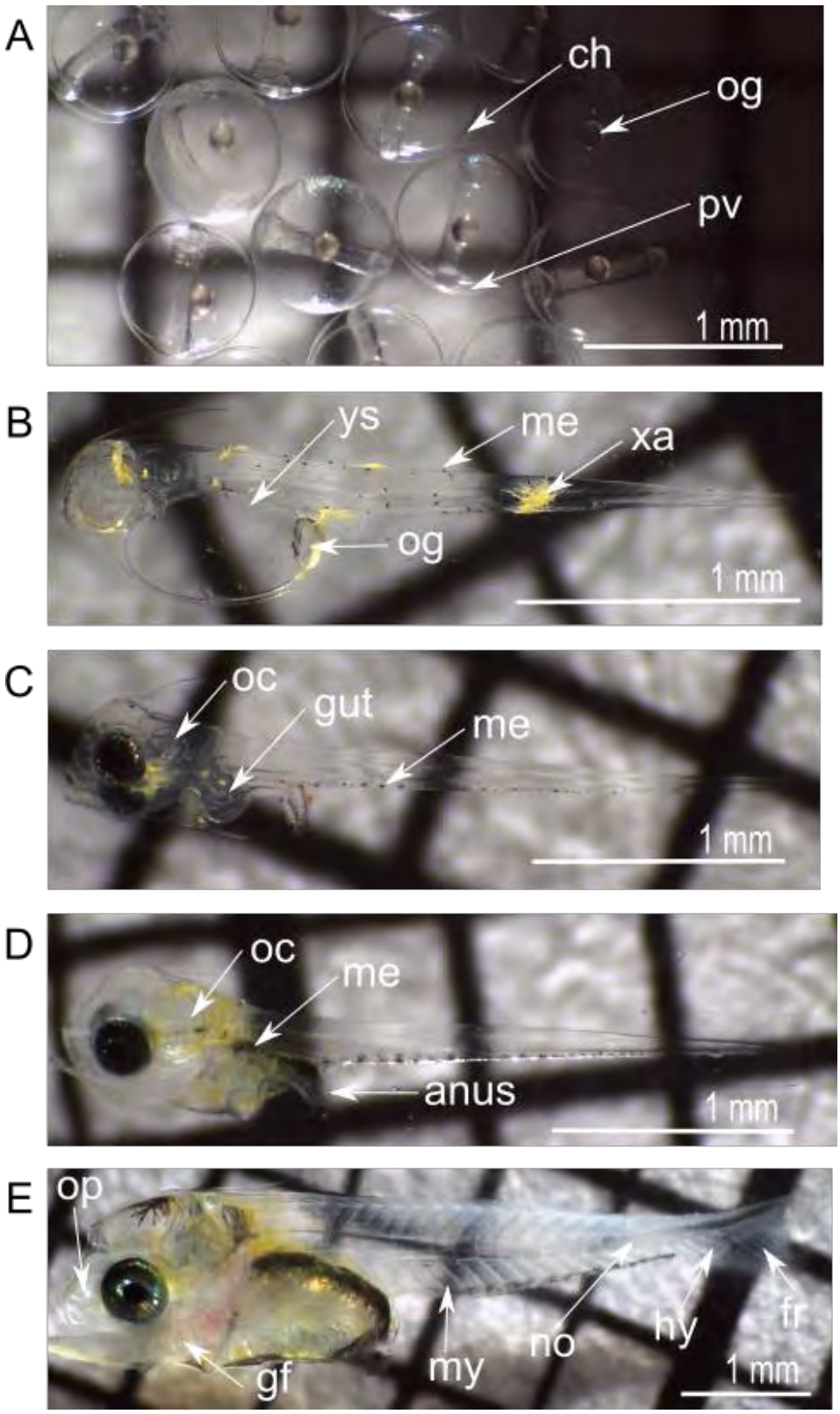
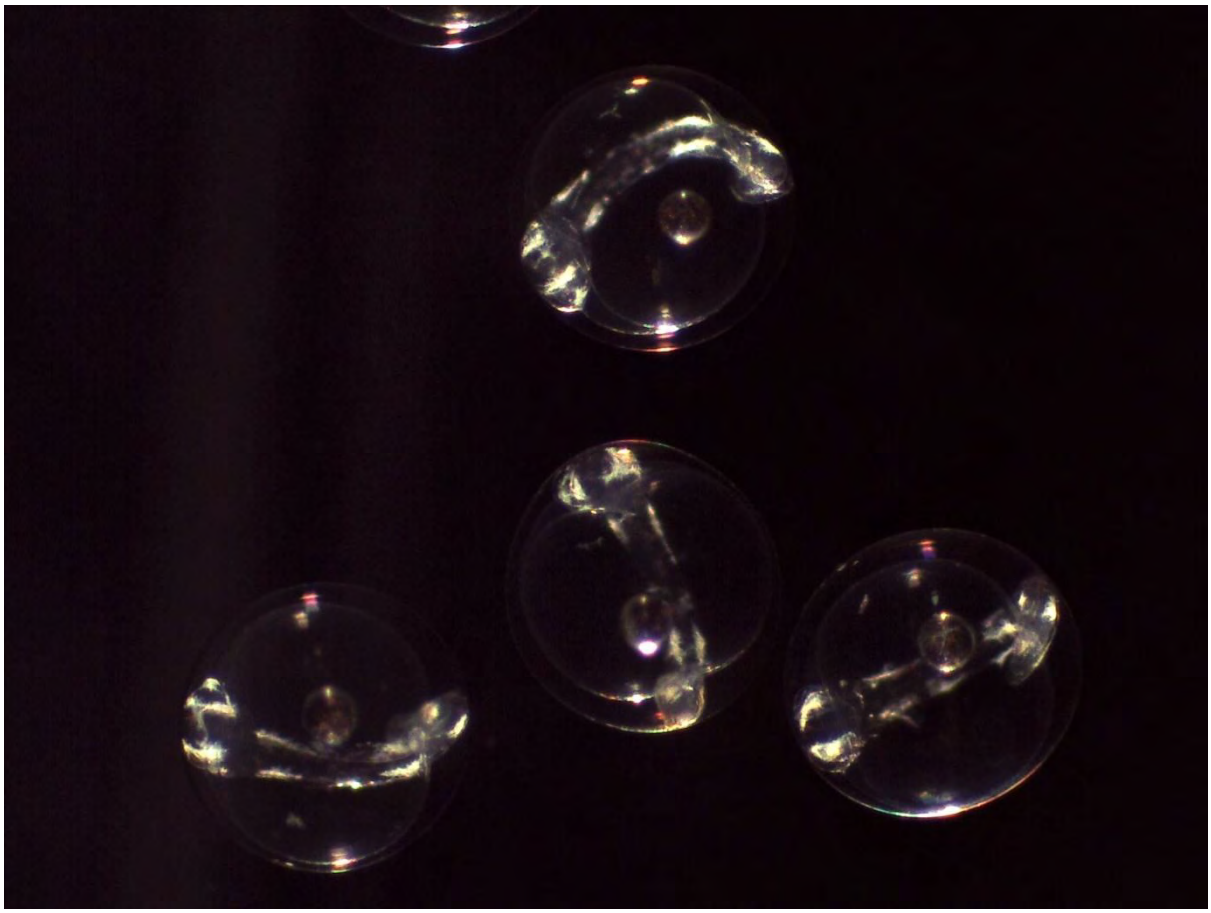


Figure 2. 5 Larval development of *Chrysoblephus laticeps* as per illustrations. Late embryonic stage (A); early yolk-sac (B); early-preflexion (C); late-preflexion (D); and flexion (E). ch = chorion, og = oil globule, pv = perivitteline space, ys = yolk sac, me = melanophore, xa = xanthophore, oc = otic capsule, op = olfactory pit, gf = gill filaments, my = myomere, no = notochord, hy = hypural plates, fr = fin rays

Chapter 3

An analytical method for obtaining consistent metabolic rate estimates of larval fishes from static respirometry



Roman seabream *Chrysoblephus laticeps* shortly before hatching, approximately 45 hour‘s post-fertilization

3.1 Introduction

Individuals form the basis upon which natural selection takes place by experiencing and responding to their environment, and it is through the individual that common traits emerge from population to ecosystem-levels (Ward *et al.*, 2016). The physiological and behavioural diversity within populations underlies their resilience to various pressures, such as exploitation, loss of habitat, and climate change-related warming and acidification (Ward *et al.*, 2016; Herbold *et al.*, 2018; Duncan *et al.*, 2019b). Maintaining phenotypic and genotypic diversity within populations may be critical to ensure the population's adaptability and persistence when exposed to anthropogenic-related stressors (Ricklefs and Wikelski, 2002). By understanding physiological and behavioural responses to stress by individuals and inter-individual variance in response, more effective interventions can be developed to ensure the persistence of species in the Anthropocene (Wikelski and Cooke, 2006; Cooke *et al.*, 2014).

Given that suites of traits are often correlated, in that particular life-history traits are often associated with physiological and behavioural characteristics, studies that focus on the individual level are particularly informative. Individual-based studies have been used to develop theories like the metabolic theory of ecology, pace-of-life syndrome, or shy-bold continuum, where the metabolic phenotype is related to risk-taking, dominance, and aggression (e.g., Brown *et al.*, 2004; Binder *et al.*, 2016; Metcalfe *et al.*, 2016; Arlinghaus *et al.*, 2017). Incidentally, individual variation in phenotypic traits, such as metabolic rate, should not be considered statistical noise but rather a reflection of important biological processes (Martins *et al.*, 2011).

Metabolic rate may be among the most widely measured of all physiological traits, given its value in assessing biological and anthropogenic effects, including growth (Pettersen *et al.*, 2018), exploitation (Killen *et al.*, 2015; Duncan *et al.*, 2019b), hypoxia (Guppy and Withers, 1999a; Stoffels, 2015; Claireaux and Chabot, 2016) and climate change (Lefevre, 2019; Munday *et al.*, 2019; Mcmahon *et al.*, 2020) on individual and population-level variability (Burton *et al.*, 2011). Measured rates typically include standard metabolic (SMR) and maximum metabolic rates (MMR), two fundamental physiological variables providing the floor and ceiling in aerobic energy metabolism (Rosewarne *et al.*, 2016). The total energy available between these two variables constitutes the aerobic scope (AS) or the potential amount of aerobic energy available, i.e., the energy available above maintenance demands to perform work (Norin and Malte, 2011; Clark *et al.*, 2013). Finally, routine metabolic rate (RMR) is a frequently reported estimate which includes SMR along with routine activity within a semi-confined chamber (Peck and Moyano, 2016)

Methods of measuring these distinct rates of oxygen consumption are varied and, as such, there are several studies focused on the refinement of techniques (Clark *et al.*, 2013; Chabot *et al.*, 2016; Norin and Clark, 2016; Rodgers *et al.*, 2016; Rosewarne *et al.*, 2016). Nevertheless, the approaches used to derive these fundamental metabolic rates show some consistency. Specifically, as SMR represents the basic cost of living and is defined as the minimal maintenance metabolic rate, measurements are made on unstressed, post-absorptive, non-breeding fish acclimated to experimental conditions (Chabot *et al.*, 2016; Rosewarne *et al.*, 2016). Conversely, MMR, as the upper boundary of aerobic metabolism (Norin and Clark, 2016), is measured at the point of exhaustion, which is attained by various methods such as maximum sustainable swimming speed, chasing, and/or air exposure (Clark *et al.*, 2013; Rosewarne *et al.*, 2016). However, the range of measurement techniques, approaches, and data analysis can lead to significantly different estimates of metabolic rates (Chabot *et al.*, 2016; Rodgers *et al.*, 2016).

Traditional methods for determining oxygen consumption rates from fishes were carried out by intermittently sampling water from a sealed respirometer and measuring the oxygen content of samples. As such, it was typically not possible to identify periods of spontaneous activity that may have influenced measurements (Clark *et al.*, 2013). In contrast, contemporary methods are of higher temporal resolution, often with multiple measurements per minute, and higher accuracy, with technological advancements such as optical sensors allowing precise measurements from small volumes of water. This high resolution allows regression techniques to be employed, providing a quality criterion or goodness of fit estimate and identifying variable linear regions that may be attributed to behavioural changes.

Gathered data is affected by respirator design and the materials used in respirometer construction, as some materials may store and release oxygen (reviewed in Stevens, 1992). Three respirometry designs are used, static (closed), intermittent-flow and flow-through systems, each with their own drawbacks (Rosewarne *et al.*, 2016; Svendsen *et al.*, 2016). Briefly, static respirometry involves placing an organism in a closed chamber and measuring the decline in oxygen concentration over time; flow-through measures the difference in oxygen concentration between the inlet and outlet flow, and intermittent-flow combines both techniques with flushing periods followed by closed measurement cycles (Svendsen *et al.*, 2016). While intermittent flow reduces some drawbacks from static (such as a build-up of metabolites and relatively short measurement phases) and flow-through (high chance of measurement error between inflow and outflow), it is still constrained by species and/or life-stage characteristics. For example, embryonic and early-life stages may not tolerate flush cycles and be damaged in the process. As such, static respirometry using small chamber

volumes and short measurement phases remains the most commonly used technique for small, sensitive organisms (Peck and Moyano, 2016).

Larval marine fish are among the smallest self-supporting vertebrates, often measuring only 3 – 5 mm at hatching. Due to exceedingly rapid growth and development, these early life stages exhibit a higher mass-specific metabolism than later stage conspecifics (Wieser, 1995). Despite technological advances in respirometry and measurement equipment, estimates of SMR and MMR are exceedingly rare in larval fish studies, with most studies only reporting RMR (reviewed in Peck and Moyano, 2016). This is due primarily to the difficulty in eliciting these extreme states, as static respirometry methods are carried out over relatively short durations compared to studies using juveniles or adults with intermittent-flow designs. Additionally, the SMR of pre-metamorphic larval fish may never be truly attained via measurements of oxygen consumption due to inherent growth costs and feeding on endogenous resources (Rombough, 1988). Similarly, poor swimming abilities and general unresponsiveness to chase or fright stimulus mean that measuring MMR is problematic. However, it is important to note that even measurements of RMR are not without issue, as measurements from individuals can vary substantially in response to a variety of factors and spontaneous bursts of activity (Wieser, 1985).

Of the pre-metamorphic larval stages, the measurement of MMR is particularly challenging for the pre-flexion stage because they lack the differentiated muscles and caudal fin required to respond to the traditional methods for eliciting MMR. Low mitochondrial densities in the swimming muscles (Wieser, 1995) also likely keep swimming activity restricted to short bursts. However, pre-flexion larvae must exert considerable force for movement in their viscous environment (indicated by a low Reynold‘s number (Downie *et al.*, 2020)). They routinely exhibit stages of sporadic high energy movement, followed by periods of quiescence. As such, respirometry carried out over periods long enough to capture this range of activity should incorporate periods of high energetic activity followed by periods of recovery. Thus, rates of oxygen consumption (VO_2) from high-resolution respirometry should reflect distinct periods of maximum and minimum O_2 decline associated with behavioural activity changes. As these rates may not similarly measure SMR or MMR captured for juveniles or adults, for reasons mentioned above, it may be more appropriate to refer to rates of VO_2 as minimum (VO_2min), routine (VO_2rout), or maximum (VO_2max) (Rombough, 1988; Wieser *et al.*, 1988).

Regardless of the terminology, estimation of these rates has occasionally been determined using various approaches (Table 3.1). For example, VO_2 has been measured whilst larvae were anaesthetized or in darkness to estimate SMR/VO_2min during low activity (Houlihan *et*

al., 1995; Flynn and Todgham, 2018; Moyano *et al.*, 2018). MMR/VO_2max has been estimated during periods of observed activity under high lighting (Ruzicka and Gallager, 2006; Geist *et al.*, 2013) or, for more developed larvae, using swim tunnels (Nilsson *et al.*, 2006; Killen *et al.*, 2007). When many individuals have been sampled, a quantile approach has also been used to calculate the mean of the 5, 50, and 95% quantiles, defined as the SMR, RMR, and MMR, respectively (Geist *et al.*, 2013; Edworthy *et al.*, 2018). Although the determination of these rates for juveniles and adults is becoming increasingly standardized, it is not the case for the early-life stages, which Rombough (1988) noted and is still lacking at present (Peck and Moyano, 2016). With the increasing interest in the metabolic physiology of larvae, particularly in response to climate change and anthropogenically introduced stressors along with resilience or adaptation, there is a need to explore methods to obtain more refined estimates of VO_2min and VO_2max for larval fishes. Due to high intraspecific variation of metabolic phenotypes (Maciak and Konarzewski, 2010; Boldsen et al., 2013), these methods should not ignore interindividual variability as done by the quantile technique. Given the high resolution and accuracy of modern O_2 measurement equipment and the sporadic behaviour of larvae, an analytical approach may provide a means to solve this problem.

Rolling regression has been shown to produce consistent estimates of MMR for adult fish when compared to alternative sequential and segmented regression methods (Prinzing *et al.*, 2021). This method uses overlapping windows, where Ordinary Least Squares regressions step forward one data point at a time, providing extremely high resolution and reducing the chance of missing the window of greatest or lowest O_2 decline (Harianto *et al.*, 2019). While this method has not yet been used on respirometry data of premetamorphic fish, it has successfully been used for juveniles and adults to determine distinct metabolic rates (Zhang *et al.*, 2019; Durtsche *et al.*, 2021).

Using rolling regression methods on individual VO_2 data from larval roman seabream *Chrysoblephus laticeps*, this chapter aimed to demonstrate that distinct metabolic rate metrics, namely VO_2min and VO_2max, could be easily and consistently determined for individuals with little subjectivity. Repeated sequential measurements were carried out over the same day on the same individuals to validate the method. The repeatability (test-retest reliability) of metabolic rates was estimated. Further validity was provided by comparing the derivatives of metabolic rates (absolute aerobic scope, factorial aerobic scope, and routine factorial scope) with estimates for larvae in published works (Table 3.1). The results from this chapter would then be used to determine the suitability of this approach both for comparing metabolic responses to hypercapnic environmental conditions (Chapter 4), and for differing population responses to hypercapnic conditions (Chapter 5).

Table 3. 1 Studies which have measured minimum and/or maximum metabolic rates of marine fish larvae, the method used to elicit behavioural states, and the method of estimation used to approximate metabolic scope (either Routine Factorial Scope, Factorial Aerobic Scope, or Absolute Aerobic Scope)

Family	Species		Stage	MR	Method	Estimation[#]	Reference
Bathydraconidae	Dragonfish	*Gymnodraco acuticeps*	Larvae	SMR	Darkness	Lin. reg	(Flynn and Todgham, 2018)
Clupeidae	Herring	*Clupea harengus*	Larvae	SMR*	Anaesthetized	Lin. reg	(Kiorboe *et al.*, 1987)
			Larvae	SMR*	Anaesthetized	Difference	(de Silva and Tytler, 1973)
			Early-larvae	SMR*	Anaesthetized	Lin. reg	(Houlihan *et al.*, 1995)
			Early-larvae	SMR	Anaesthetized	Lin. reg	(Moyano *et al.*, 2018)
	European sardine	*Sardina pilchardus*	Larvae	SMR*	Anaesthetized	Lin. reg	(Moyano *et al.*, 2014)
Gadidae	Atlantic cod	*Gadus morhua*	Larvae	SMR*, AMR*	Darkness, activity	Difference	(Ruzicka and Gallager, 2006)
	Walleye Pollock	*Theragra chlcogramma*	Larvae	SMR*, AMR*	Darkness, activity	Difference	(Yamashita and Bailey, 1989)
Carangidae	Cape horse mackerel	*Trachurus capensis*	Early-larvae[1]	SMR*, AMR*	Darkness, light	Lin. reg Quantile	(Geist *et al.*, 2013)
Pomacentridae	Ambon damsel	*Pomacentrus amboinensis*	Late-larvae[2]	AMR*	Swimming		
	Black-axil chromis	*Chromis atripectoralis*	Late-larvae[2]	AMR*	Swimming	Lin. reg	(Nilsson *et al.*, 2006)
	Spiny chromis	*Acanthochromis polycanthus*	Late-larvae[2]	AMR*	Swimming		
Sciaenidae	Dusky kob	*Argyrosomus japonicus*	Larvae	SMR, AMR	na	Lin. reg, Quantile	(Edworthy *et al.*, 2018)
Sparidae	Guilthead seabream	*Sparus aurata*	Embryo + Larvae	SMR*	Darkness	Difference	(Rønnestad *et al.*, 1994)
Pleuronectidae	Plaice	*Pleuronectes platessa*	Larvae	SMR*	Anaesthetized	Difference	(de Silva and Tytler, 1973)
Cottidae	Shorthorn sculpin	*Myoxocephalus scorpius*	Larvae	SMR*, AMR*	Darkness, swimming	Lin. reg	(Killen *et al.*, 2007)
Cyclopteridae	Lumpfish	*Cyclopterus lumpus*	Larvae	SMR*, AMR*	Darkness, swimming	Lin. reg	

*used to determine Figure 3.3 B and/or C; [#]Linear regression when multiple points, difference when measured at start and end; [1] pre-flexion; [2] settlement stage

3.2 Materials and methods

3.2.1 Field Spawning, Egg Incubation and Larval Rearing

Mature *Chrysoblephus laticeps* (n = 18) were collected from the exploited population (EP) at Noordhoek, Port Elizabeth (NHK) on the 14th and 15th of November 2019. Fish were captured by hook and line from a ski boat at depths between 10 to 25 m. The surface temperature during the capture event was 20 °C. Details of the spawning and rearing methodology are provided in Chapter 2.

3.2.2 Respirometry procedure

Respirometry was performed at successive developmental stages from hatching through to flexion using static-respirometry methods, with O_2 measurements taken every 15 seconds using a 24-channel optical fluorescence, oxygen-sensing system (Loligo Systems, Copenhagen, Denmark). Twenty-four well microplates were used throughout the experiment, with well volume adjusted for size and activity of specimens, with a minimum of four wells used as blanks to account for background respiration. At DAH 7, 13, 21 and 22, corresponding to early-, late-preflexion and the onset of flexion, respectively, oxygen consumption measurements were performed in duplicate or triplicate on the same individuals. Blank wells used to attain background respiration were rotated with subsequent treatments and treated as all other wells, i.e. replacement water came from fish holding containers. Larvae were captured by siphoning from throughout the water column of the rearing tanks into glass beakers and subsequently siphoning again by visually targeting individuals into a beaker with 30 µm filtered tank water to separate the rotifers from the larvae. Beakers were then placed in a water bath at the control temperature for a three-hour fasting period to ensure the testing of post-absorptive larvae and avoid confounding the measurements of metabolic rates with specific dynamic action (Andrade *et al.*, 2011; Moyano *et al.*, 2018). After the fasting period, specimens were placed individually into wells, which had been filled with oxygen saturated, filtered (1 µm) and sterilised seawater at the same temperature as acclimation (20°C).

The microplate surface was covered with parafilm, a silicone membrane and PVC weighted cover to ensure an oxygen impermeable seal on each individual well. The assembled microplate was then placed into a temperature-controlled flow-through water bath positioned above the Sensor Dish Reader for optical O_2 measurement. The water bath was covered approximately ten minutes after measurements had begun and uncovered shortly before the cessation of measurements to aid the capture of both active and inactive behaviour under light and dark conditions. Following the first trial, the microplate was removed from the water

bath and opened by removing the PVC weight, silicone membrane and parafilm. A micro-pipette was used to drain and recharge individual wells with fresh, fully oxygen saturated seawater, reassembled with new parafilm and placed back into the water bath with measurements resuming within five minutes. This process was repeated a third time to acquire a total of three oxygen consumption measurements per individual, with measurement periods lasting approximately 60 minutes per trial. At least four blank or empty wells were used to record background respiration rates and were treated as all other wells. As the microplates were not fitted with mixing devices, they were selected based on the size and activity of the larvae to ensure adequate mixing from movement while preventing confinement stress. 80 µL wells were used for early-preflexion, 200 µL for late-preflexion, and 1700 µL for flexion stage larvae.

3.2.3 Statistical analysis

Respirometry data were filtered by removing the first five minutes of measurements and those below 70% oxygen saturation (5.0 mg $O_2.l^{-1}$). A quality threshold with an $R^2 > 0.80$ was implemented to filter the linear decline in oxygen. Oxygen consumption measurements were analysed for each individual using the R package ~~"~~RespR" (Harianto *et al.*, 2019). Three metabolic measurements (VO_2min, VO_2rout and VO_2max) were obtained for each specimen during each trial. Oxygen consumption rates of blank cells were averaged and subtracted from the individual measurements before determining metabolic rates to account for background respiration. The average volume of specimens was calculated using the formula for an oblate ellipsoid:

$$v = \pi r^2 \frac{h}{3}$$

where r represents the radius at the head, and h is the length from the tail to the eye. This value was subtracted from the well volume to obtain actual chamber fluid volume to calculate oxygen consumption independent of mass over time. Metabolic rates were estimated by fitting regressions to different windows of time across the VO_2 data and searching for the steepest and most gradual slope to determine VO_2max and VO_2min, respectively. While shorter regression windows yielded higher rates of VO_2max, they would potentially reflect more system noise than actual signal. Based on observations of numerous VO_2 traces and comparisons of outputs from differing time intervals, regression windows were standardized as follows: VO_2min was estimated from the smallest rate of linear decline over a 10-minute period, VO_2rout as the most linear rate of decline observed using 75% of the data, and VO_2max as the greatest rate of decline over a 5-minute period.

The absolute aerobic scope (AAS), factorial aerobic scope (FAS) and routine factorial scope (RFS) were estimated as follows:

$$AAS = VO_2 - VO_2min$$

$$FAS = VO_2 / VO_2min$$

$$RFS = VO_2 / VO_2min$$

To test for effects of developmental stage and trial on metabolic rate estimates, linear mixed-effects models (LMM) were built using the ~~l~~merTest" package (Kuznetsova *et al.*, 2017). Metabolic rates (VO_2min, VO_2rout, VO_2max and AAS) were included as the response variable, developmental stage (Late Preflexion and Flexion) and trial (Respirometry trial 1, 2 and 3), and their interaction was included as fixed effects, while individual ID was included as a random effect. Effect results were determined from the Anova function of the ~~c~~ar" package (Fox and Weisberg, 2019) using type 3, Kenward-Roger test with Satterthwaite degrees freedom. Fixed and random effect responses were estimated using the ~~s~~umm" function of the ~~j~~tools" package (Long, 2019), where p-values were similarly estimated with Satterthwaite degrees freedom. Raw metabolic rate data violated the assumptions of normality and homoscedasticity based on visual assessment of respective Q-Q and residual fitted plots and were therefore ln-transformed to meet the criteria for parametric testing.

Metabolic metrics of AAS, FAS and RFS, derived from VO_2min, VO_2rout and VO_2max, were compared amongst life stages using a Kruskal-Wallis rank-sum test to determine if differences occurred between life stages for any of these metrics. Subsequently, to compare the results obtained here with other methods of metabolic rate measurements, AAS, FAS and RFS were analysed descriptively and compared with previously published results described in Peck and Moyano (2016) and presented in Table 3.1.

The repeatability of metabolic rates was estimated using Bayesian generalised linear mixed models (GLMM) with the R package ~~M~~MCMCglmm" (Hadfield, 2010). The GLMM was built using the MCMCglmm( ) function and the posterior.mode( ) and HPDinterval( ) functions were used to estimate repeatability and the corresponding 95% confidence intervals (CI) (code adapted from Clements *et al.* 2020). Essentially, estimates and CI were estimated as the variance explained by individual identity divided by the total variance in metabolic rate derived from posterior distributions. Agreement repeatability (R_{agr}) was estimated using a model with metabolic rate as the response variable, trial as the fixed effect and individual ID as a random variable. A subsequent model of adjusted repeatability (R_{adj}) was created to account for variance associated with the development stage. The model was built with

metabolic rate as the response variable, trial and developmental stage (preflexion & flexion) as fixed effects, their interaction (trial x developmental stage), with individual ID as a random variable. Weakly informative inverse Wishart priors were used (V = 1, nu = 1.002) and the settings for the model fitting were: nitt = 390 000, burnin = 9000 and thin = 100.

3.3 Results

Observations of individual VO_2 traces typically showed linear declines in O_2 with greater rates of decline always followed or preceded by more gradual periods (Figure 3.1). There were few irregularities or abnormal fluctuations in O_2 measurements, which would have indicated poor mixing within the chamber, suggesting that well volumes were adequately sized for the larvae. Behavioural observations of larvae during respirometry trials showed sporadic bursts of activity followed by periods of inactivity. Activity did appear to be reduced when the lights within the room were turned off, but behavioural observations were not carried out when the chamber was covered.

Developmental stage significantly influenced all metabolic rate estimates, with VO_2 (min, rout, max) and AAS rates significantly greater for the later developmental stage (VO_2min: $F(1,14) = 11.73$, $P = 0.004$; VO_2rout: $F(1,14) = 25.27$, $P = <0.001$; VO_2max: $F(1,14) = 68.99$, $P = <0.001$; AAS: $F(1,14) = 70.80$, $P = <0.001$) (Figure 3.2, Appendix Table A.1). There was no significant difference among trials for any of the metabolic rate estimates; VO_2min ($F(2,28) = 0.35$, $P = 0.273$), VO_2rout ($F(2,28) = 0.93$, $P = 0.407$), VO_2max ($F(2,28) = 1.22$, $P = 0.311$) or AAS ($F(2,28) = 0.872$, $P = 0.429$) (Figure 3.2, Appendix Table A.1). The interaction between development and trial was also not significant for any VO_2 estimates or AAS (Appendix Table A.1).

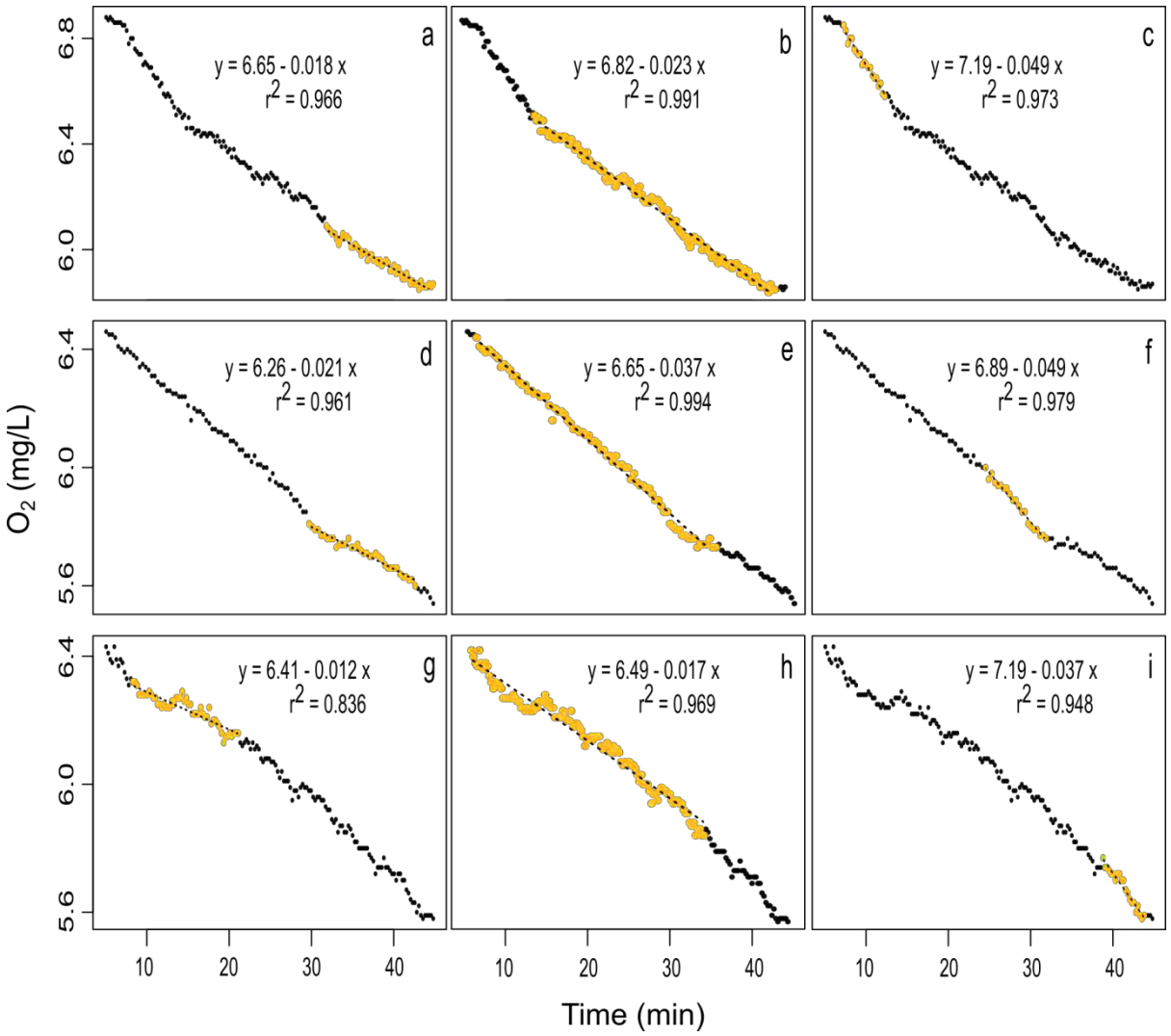


Figure 3. 1 Oxygen consumption data of the same 13 DAH individual over three trials (trial 1 a-c; trial 2 d-f; trial 3 g-i). Slope regions shown in yellow indicate estimates derived for VO_2min (a, d, g), VO_2rout (b, e, h) and VO_2max (c, f, i) along with linear regression trend line, regression equation and goodness of fit estimate (as per Harianto *et al*., 2019)

To compare differences between life stages and prior published results (listed in Table 3.1), additional aerobic metabolic metrics were estimated for all individuals where repeated duplicate and triplicate measurements were obtained. Mean absolute aerobic scope (AAS) increased from 5.75 ± 2.93 nmol O_2. individual^{-1}. hr^{-1} for early-preflexion to 11.28 ± 4.09 nmol O_2. individual^{-1}. hr^{-1} for late-preflexion and 48.32 ± 27.21 nmol O_2. individual^{-1}. hr^{-1} (Figure 3.3A, Table A.2)). There was a significant difference in FAS among developmental stages (X_2 = 7.29, *df* = 2, *P* = 0.026), with highest average rates for early-preflexion larvae (5.14 ± 3.34), followed by flexion (4.17 ± 2.92) and late-preflexion (3.02 ± 1.65). FAS showed a moderate degree of variation among individuals, ranging from a minimum of 1.72 ± 0.10 to a maximum of 9.08 ± 6.36, with an overall mean of 3.80 ± 2.58. All mean values for

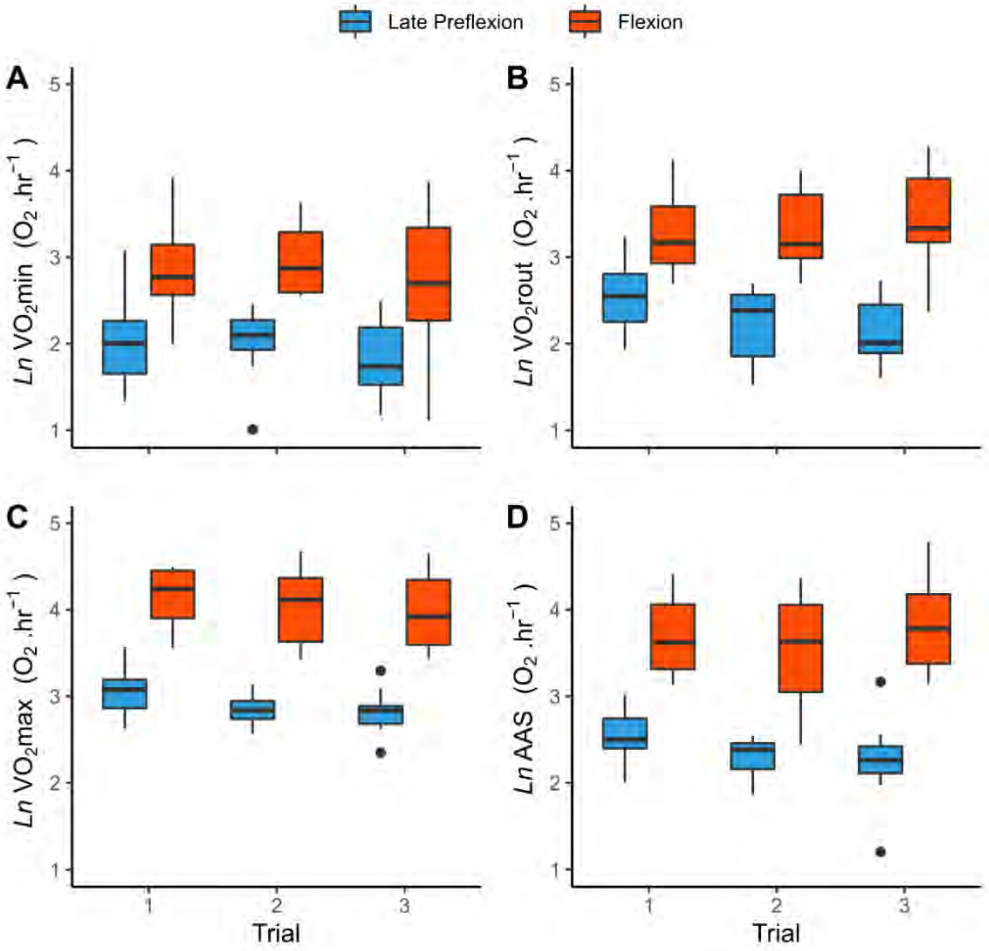


Figure 3. 2 Metabolic rate estimates for *Chrysoblephus laticeps* VO_2 measures of (a) minimum, (b) routine, (c) maximum, and (d) absolute aerobic scope for developmental stage by trial. VO_2 measures are ln-transformed from nanomole O_2. individual^{-1}. hour^{-1}. Box and whisker plots show medians (centre line), upper and lower quartiles (box) and total range (whiskers) excluding outliers (points). Plotted are triplicate observations only (*n*=16)

the early life stages and the overall mean fell within previously published results for early-stage FAS (1.36 – 6.72 (red rectangle), Figure 3.3B). RFS showed little variation among individuals and no significant difference between developmental stages (X^2 = 1.52, *df* = 2, *P* = 0.468). The overall mean of 1.66 ± 0.70 fell within the range of previously published results for early-stage RFS (1.4 – 3.37 (blue rectangle), Figure 3.3C).

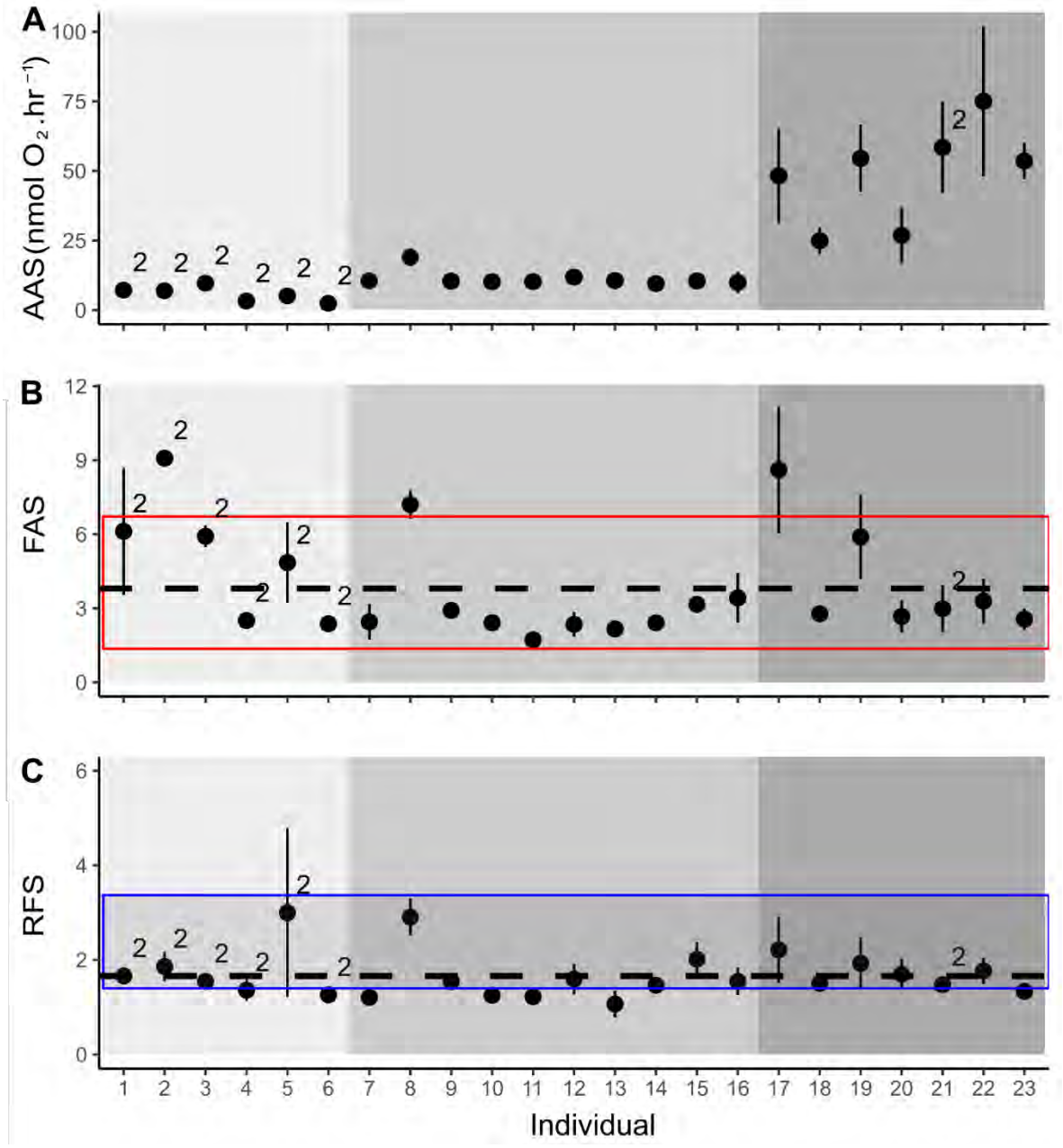


Figure 3. 3 Measures of aerobic scope for early-stage *Chrysoblephus laticeps* including Absolute Aerobic Scope (AAS) (a), Factorial Aerobic scope (FAS) (b) and Routine Factorial Scope (RFS) (c) of individuals (point indicates mean, bars indicate standard error, value above point indicates when only 2 repeat measures were made and not 3, dashed black line indicates grouped mean for b and c). Red and blue boxes indicate the range of respective measurements from prior reported studies on larval fish metabolism (see Table 3.1). Shaded area indicates stage of development with specimens 1 – 6, early-preflexion; 7 – 16, late-preflexion; and 17 – 23 at the onset of flexion

Out of a possible 23 individuals, repeat measurements in triplicate were successfully obtained for 16 individuals due to mortality during successive respirometry trials. This mortality was typically associated with the handling and transferring of the sensitive larvae and the opening and reassembly of the respirometry chambers. Repeatability analysis was

only performed on those individuals that had metabolic rate estimates in triplicate. Estimates of repeatability indicated moderate to high levels of test-retest reliability and measurement accuracy. Measures of agreement repeatability (R_{agr}) were high, with values of 0.83 (95% CI, range 0.64 – 0.93) for VO_2max, 0.82 (range 0.62 – 0.92) for VO_2min, and 0.73 (range 0.49 – 0.88) for VO_2rout (Figure 3.4). Adjusted values (R_{adj}), which accounted for the effect of development (with separate slopes and intercepts per developmental stage. i.e. reduced variance among groups), were marginally lower than R_{agr} but nevertheless indicated a substantial degree of individual repeatability for larval *C. laticeps*. R_{adj} was greatest for VO_2min (0.73, range 0.49 – 0.88), followed by VO_2max (0.64, range 0.38 – 0.82) and VO_2rout (0.55, range 0.30 – 0.78).

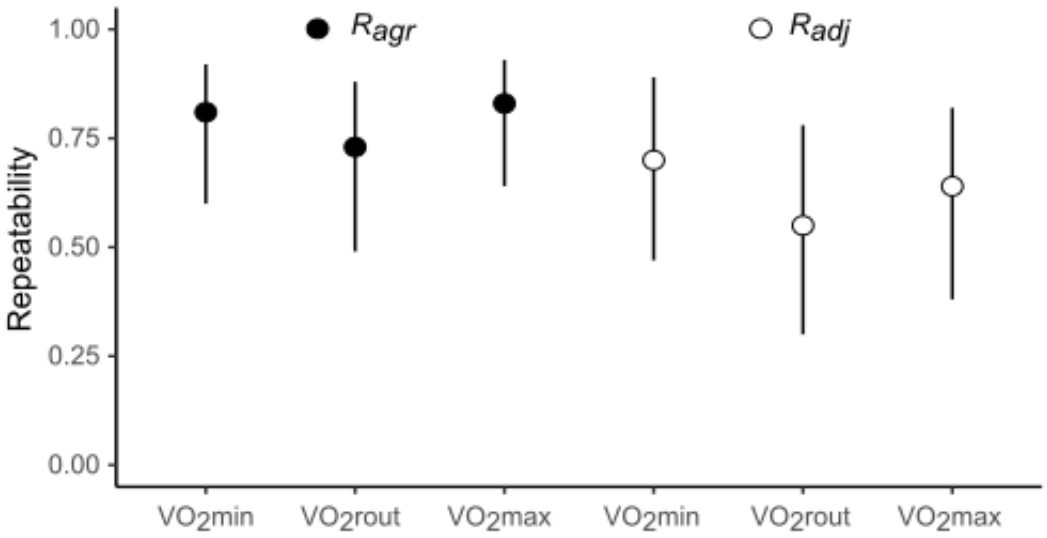


Figure 3. 4 Agreement (R_{agr}) and adjusted (R_{adj}) repeatability of VO_2min, VO_2rout and VO_2max for larval *Chrysoblephus laticeps* from triplicate within-day measurements. Point and whisker shows respective estimate along with 95% confidence intervals

3.4 Discussion

This study demonstrated that reliable and consistent individual estimates of metabolic rates could be obtained from early developmental stages of *Chrysoblephus laticeps* using static respirometry. Subsetting VO_2 datasets into behaviourally relevant time periods and determining maximum and minimum rates of decline via rolling regression provided consistent results that did not differ significantly between repeated trials. The method used here to determine these rates were easily applied to the data and could be consistently used across individual O_2 measurement traces reducing subjectivity and operator bias. Rates of FAS and RFS fell within the range of values reported from previous studies (Figure 3.3). The studies in question used more exhaustive protocols to determine these estimates, such as

by anaesthesia or chasing (see Table 3.1), providing further evidence that VO_2 measurements obtained using the current methodology are highly comparable. Although there was high inter-individual variation in metabolic rates, even among individuals at the same stage of development, rates of repeatability were moderate to high, indicating that these differences were consistent among individuals. Test-retest reliability measures (R_{agr}) for both VO_2min and VO_2max were high, indicating that the method captured consistent rates for these estimates and that the methodology, static respirometry over 60 minutes, captured the necessary range of behaviour for preflexion larvae with mixed active and inactive states.

Measurement reliability is generally influenced by two components: within-individual differences in the value of an attribute, and differences due to measurement error (Matheson, 2019). A measure of reliability that maintains the performance ranking of individuals through repeated measurements is an important measure to assess the accuracy of measurements and subsequently the quality of data (Nakagawa and Schielzeth, 2010). Just as high within-individual variation of a measured trait can lead to low estimates of repeatability, so does low between-individual variation (Nakagawa and Schielzeth, 2010). This was evident in the comparable measures of the two *R* estimates. Here, the *Ragr* values were greater than *Radj* due to the significant life-stage differences in VO_2. However, values for *Radj* may have been greater given a greater sample size, which, together with a greater number of trials, would increase both the accuracy and power in determining these estimates (Dingemanse and Dochtermann, 2013). Although the measurement and comparison of phenotypes is a central practice in the biological sciences and, as such, the accuracy of measurements should be of priority to researchers (Garamszegi *et al.*, 2009; Nakagawa and Schielzeth, 2010), no analogous studies on the consistency of larval metabolic measurements are available for comparison with the current results.

The lack of comparative research is not necessarily owing to the uniqueness of this study but rather the difficulty or, indeed, the dissuasion for estimating the metabolic rates, particularly minimum and maximum rates, of larval fishes (Chabot *et al.*, 2016; Peck and Moyano, 2016). Certainly, measurements of larval VO_2min may include growth (Peck and Moyano, 2016) and limited activity costs (Chabot *et al.*, 2016) and therefore never truly estimate standard metabolic rates (SMR) while MMR may be underestimated due to a larvae‘s poor response to forced swimming. However, during even prolonged measurements, juveniles are prone to bursts of spontaneous activity. On the other hand, adults may be undergoing gonad maturation, and both may be experiencing stress-related costs or conversely undergoing starvation-related metabolic suppression after prolonged fasting (Chabot *et al.*, 2016). Alternatively, maximum metabolic rate (MMR) may be

underestimated when the exhaustive technique is not suited to the species or potentially modified under varying salinities and daily or seasonal cycles (Norin and Clark, 2016). As such, all measurements have the potential to be underestimated or overestimated. In the particular case of larvae, factors like growth costs, organogenesis, skeletal ossification, osmoregulation, ion regulation, and sporadic activity may be relatively high compared with juveniles, and a confounding factor in determining upper and lower metabolic factors rates. This is not to say that given these difficulties, ~~it~~ is preferable to report routine metabolic rates" (Chabot *et al.*, 2016). Instead, one should determine the context/environment-specific minimum and maximum rates, which are shown here to be more consistent than rates of VO_2rout, particularly since Rombough noted a lack of absolute or factorial measurements for fish larvae in 1988 and were still lacking by 2016 (Peck and Moyano, 2016).

Studies have shown that metabolic rates of juveniles and adults are not only repeatable over time but that there is high intraspecific variation (Maciak and Konarzewski, 2010; Boldsen *et al.*, 2013; Svendsen *et al.*, 2014; Reemeyer and Rees, 2020). Consistent individual differences in metabolic rate measurements have been linked to differences in individual behaviour and growth (Biro and Stamps, 2010; Metcalfe *et al.*, 2016). As the mortality rates of planktonic larvae decline with increasing size (Peterson and Wroblewski, 1984; Sogard, 1997), it follows that growth and metabolic rates would be high for individual larvae (Osse *et al.*, 1997). Indeed, to bolster this assertion, pre-metamorphic metabolism does scale at a greater rate than post-metamorphic fish (Giguère *et al.*, 1988; Killen *et al.*, 2007). Counteracting this, Bochdansky *et al.* (2005) provided evidence that high metabolic phenotypes are selected against under food limitation. While the production of offspring with a wide range of physiological phenotypes may have evolved as a bet-hedging strategy against unpredictable resource supplies (Sasaki and Ellner, 1995), external forms of selection, such as by intense fishing pressure, may inadvertently target high metabolic phenotypes. This process would therefore select a more timid, slower growing and later maturing population (Biro *et al.*, 2008), as metabolism is a heritable trait (Metcalfe *et al.*, 2016; Long *et al.*, 2021). This highlights the importance of measuring individual metabolic physiology to assess potential population fitness as natural (and artificial) selection acts on individuals and not populations (Bochdansky *et al*., 2005; Burton *et al*., 2011).

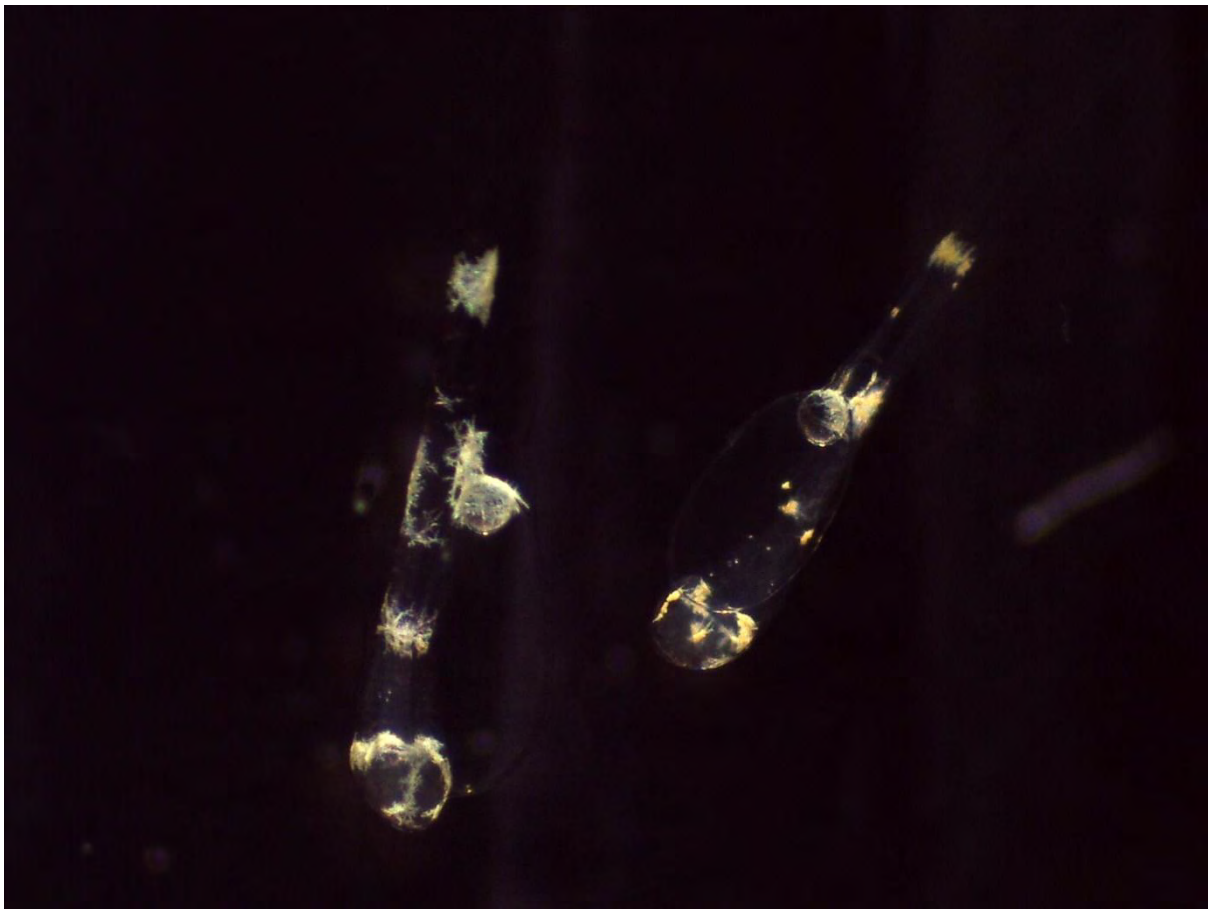
The assessment of intraspecific variability in the metabolic rates of fishes is a critical research area for predicting the response of fish populations to the combined impacts of exploitation and climate change, particularly given the relationship with physiology and behaviour (Metcalfe *et al.*, 2016; Pettersen *et al.*, 2018). While studies investigating this variability have focused on juvenile and adult stages, it is clear that larval fish similarly exhibit great variability in their metabolic phenotypes, with potential consequences of

physiology-related recruitment dynamics (Leis, 2007; Pimentel *et al.*, 2014). To explore these relations further, individual rates need to be determined, which cannot be derived using cumulative frequency or quantile approaches (described by Chabot *et al.*, 2016). They ignore inter-individual variability and preclude statistical comparisons amongst treatments. The method proposed in this chapter shows promise. It provided consistent results of metabolic rates derived from individual oxygen consumption data within the limited scope provided by static respirometry methods.

In summary, this study provided evidence that distinct rates of minimum, routine and maximum oxygen consumption could be accurately captured for individuals. These estimates were repeatable, demonstrating the accuracy of the measurements and high inter-individual consistency of metabolic phenotypes. Furthermore, factorial estimates (FAS and RFS) were comparable with and fell within the range of previously published results. The capture of these distinct metabolic rates is facilitated by the common behaviour of small fish larvae to exhibit abrupt active swimming followed by periods of inactivity. At the same time, the rolling regression technique allowed for easy and consistent application with little bias for estimating metabolic rates. This method is particularly suited for preflexion stages, where individuals are small, lack a caudal fin, and the musculature is undifferentiated. In this case, the relatively high viscosity of the medium makes swimming energetically expensive and requires substantial physical exertion followed by a period of recovery. The determination of distinct metabolic rates following flexion may deteriorate, but given the current findings, determination of and comparison amongst individual or grouped VO_2min and VO_2max for preflexion larvae of *C. laticeps* is indeed possible. By determining VO_2 rates of individuals, this method can allow for comparisons between sites or populations and provide the means for statistical comparisons among groups. This is valuable information that has otherwise only been used for juvenile and adult fishes and may allow insight into the resilience or sensitivity of early stages to climate variability or change. In subsequent chapters, metabolic rates derived from this method will be used to compare the effects of environmental hypercapnia, first within the exploited population (Chapter 4) and then between the exploited and protected populations (Chapter 5) in order to evaluate the potential for combined effects of exploitation and environmental stress on the resilience of different populations to climate change.

Chapter 4

Effects of experimental ocean acidification on the larval morphology and metabolism of a temperate sparid, *Chrysoblephus laticeps*



Roman seabream *Chrysoblephus laticeps* hatchlings, approximately 52 hour‘s post-fertilisation

4.1 Introduction

Atmospheric carbon dioxide (CO_2) concentrations have been rising at an increasing rate since the industrial revolution. As a major sink, the oceans are expected to take up much of this CO_2, leading to a decrease in ocean pH, carbonate ion concentration (CO_3^{-2}) and calcium carbonate ($CaCO^3$) saturation state, collectively known as ocean acidification (OA) (Doney *et al.*, 2008). At the current rate of change, ocean pH may, over the next 300 years, decline to levels not encountered over the past 300 million years (Caldeira and Wickett, 2003). Average ocean surface waters have already dropped by 0.1 units since preindustrial times. They are expected to decline by a further 0.4 units by the end of the century, with coastal areas likely to experience an even greater rate of change (Doney *et al.*, 2008; Frieder *et al.*, 2012; McNeil and Matsumoto, 2019). While marine organisms will be adversely affected by OA, the severity of these effects appears to be highly variable among taxa and even within taxa among life-history stages (Pörtner *et al.*, 2004; Munday *et al.*, 2019). Much of the earlier research focussed on marine calcifying invertebrates, primarily due to their increased susceptibility to OA but also due to the early assumption that fish would be widely tolerant to rising CO_2 levels owing to their well-developed regulatory mechanisms (Ishimatsu *et al.*, 2008; Heuer and Grosell, 2014). Using extreme CO_2 levels (greater than 10 000 µatm), earlier studies revealed an efficient acid-base regulatory system capable of compensating for hypercapnic disturbances (Cameron, 1978; Toews *et al.*, 1983).

Recent studies using CO_2 levels relevant to near future and longer-term climate change over the 21st century (values typically below 2000 µatm) have revealed adverse effects on growth, metabolism, development, behaviour and survival of some fishes (Cattano *et al.*, 2018; Brauner *et al.*, 2019; Lefevre, 2019; Munday *et al.*, 2019). These relatively low rates of CO_2 increase have resulted in respiratory acidosis, causing a decline in cellular pH due to increasing CO_2 diffusion gradients (Esbaugh *et al.*, 2012). This, in turn, drives an acid-base compensation response, which includes an increase in plasma bicarbonate (HCO_3^-), resulting in downstream physiological consequences (Heuer and Grosell, 2014, 2016). For instance, acid-base compensation to environmental hypercapnia has been proposed to change the reversal potential of the GABA receptor in the brain, altering behavioural responses (Heuer *et al.*, 2016; Brauner *et al.*, 2019). Results, however, vary widely and may be species, life-stage or even population-specific, with several studies revealing no physiological consequences. Nevertheless, some generalisations have been made with species-specific responses being linked to their habitat affinity. For example, pelagic species appear more sensitive to environmental hypercapnia than benthic and bentho-pelagic species, which are naturally exposed to greater environmental variability (reviewed in Cattano *et al.*, (2018)).

External conditions influence the partial pressure of CO_2 in the arterial blood ($PaCO_2$). Therefore, environmental hypercapnia will lead to acid-base disturbances or acidosis, which cannot be brought to levels below the environment (Brauner, 2008; Esbaugh *et al.*, 2016). Therefore, fish must regulate acid-base disturbances through differential regulation of H+ and HCO3- or other acid-base equivalents (Brauner *et al.*, 2019). Apart from gas exchange and ammonia excretion, the gills are the predominant site for ionoregulation, an active process that carries an energetic cost (Claiborne *et al.*, 2002; Esbaugh *et al.*, 2012; Heuer and Grosell, 2016). While ionoregulation costs are generally only between 6 and 15% of standard metabolic rate (SMR), any increases may require energy reallocations from other processes (Ishimatsu *et al.*, 2008; Heuer and Grosell, 2016).

The early life stages, embryos and larvae specifically, are often more sensitive to increased CO_2 than are the juvenile and adult stages (Heuer and Grosell, 2014; Brauner *et al.*, 2019; Munday *et al.*, 2019). The greater sensitivity of early life stages to hypercapnia is likely due to two main factors, including the incomplete development of regulatory mechanisms, which leads to inadequate ionoregulation, and a greater surface area to volume ratio, which results in $PaCO_2$ levels that more closely match that of the environment (Brauner *et al.*, 2019; Dahlke *et al.*, 2020). Indeed, most explanations for the adverse effects of hypercapnia are centred on increases in internal pCO_2 and elevations in HCO_3^- (Heuer and Grosell, 2014).

The larvae of most teleosts represent a transitional stage between single-celled eggs and immature juveniles and, despite being exposed to chemical challenges imposed by an aquatic environment, typically lack the cells, tissues and organs present in later stages (Brauner, 2008). Early development of the gills appears to be more crucial for ionoregulation than gas exchange as cutaneous exchange becomes limited as the cuticle thickens and the ratio of surface area to body volume decreases across ontogeny (Fu *et al.*, 2010; Zimmer *et al.*, 2014). Consequently, larvae from broadcast spawners of temperate species appear to be more negatively affected by environmental hypercapnia. Here, they are typically smaller, slower growing, and potentially have less developed acid-base regulatory mechanisms than their tropical counterparts (Munday *et al.*, 2019). The reduced capacity for acid-base regulation during hypercapnia forms much of the current explanatory basis for the negative effects on fish larvae. These effects include neurosensory, behavioural and metabolic changes (Heuer and Grosell, 2014).

Environmental hypercapnia is expected to act as a loading stress, thereby increasing SMR and reducing available energy for activity, given by aerobic scope (AS), essentially reducing the energy available for growth (Fry, 1971; Heuer and Grosell, 2014). While the metabolic costs of ionoregulation may be relatively small for juvenile or adult fish, they may be

considerably larger for larvae undergoing rapid and energetically costly development. Furthermore, the necessary extracellular pH adjustments, facilitated by increased blood plasma HCO_3^-, can be far greater for larvae than for juveniles and adults given their relatively lower $PaCO_2$ (Brauner *et al.*, 2019). Unlike adults, fish larvae lack substantial energy reserves and are required to rapidly resume feeding after any interruption. For instance, once endogenous feeding ends, through consumption of yolk and oil reserves, exogenous feeding must commence within a brief period, often only hours before reaching a ~~-~~point-of-no-return", after which starvation becomes irreversible (Blaxter and Hempel, 1963; Garcia *et al.*, 2020). Therefore, even small additions to the energy budget may significantly affect individuals and, ultimately, population recruitment (Stiasny *et al.*, 2016).

This study investigated how ocean acidification conditions (ΔpH = -0.4, expected by 2100) might affect the metabolism, development, and morphometry of early-stage roman seabream *Chrysoblephus laticeps,* who were exposed from shortly after fertilisation up to flexion (25 days exposure). Increased pCO_2 and an associated decrease in pH were expected to result in elevated metabolic rates and reduced growth at age, leading to delayed development compared with control specimens due to increased energetic costs associated with acid-base regulation.

4.2 Materials and Methods

4.2.1 Field spawning, egg incubation and larval rearing

A full description of the capture, field spawning, egg incubation and rearing procedure are described previously in Chapter 2. Briefly, mature *C. laticeps* (n = 18) were collected from the exploited population (EP) at Noordhoek (NHK), immediately to the south of Port Elizabeth, Eastern Cape, South Africa, on the 14th and 15th of November 2019. Fish were captured using hook and line and returned to flow-through tanks at the Noordhoek ski-boat club slipway. Ovulation was ensured by injecting the recommended dosage of Aquaspawn (see Chapter 2) on the day of capture. Gametes were stripped from females (n = 12, 400 – 830 g) and males (n = 3, 1300 – 1460 g) after 48 hours by applying light pressure to the abdomen (Pavlidis and Mylonas, 2011). Eggs collected from all 12 females were mixed with 10 ml of sperm from the three males. The mixture was continuously mixed with a fine bristled brush for 10 minutes while adding small amounts of seawater to reach a total volume of 1 L. Eggs were left to stand for 15 minutes, after which they were aerated and disinfected with 0.27 ml of formaldehyde for a further 15 minutes (De Swaef *et al.*, 2016). All eggs were repeatedly strained with fresh seawater following disinfection. Then, they were equally distributed into large Ziplock bags and stored in a polystyrene cooler whilst being transported to the NRF-SAIAB Aquatic Ecophysiology Research Platform (AERP) Laboratory at Rhodes

University in Grahamstown, South Africa. Fertilised eggs were distributed into rearing tanks at 80 eggs per litre or 6000 eggs per tank.

Larvae were fed from three days after hatching (DAH) up to flexion with rotifers *Brachionus plicatilis*, cultured using an enrichment diet (ORI-ONE, Skretting Stavanger, Norway). Rotifers were maintained at levels of 10 mL^{-1} in rearing tanks and were added in three feedings at a ratio of 50:40:10 for morning, afternoon and evening. Nightly flow with an outlet screen of 250 µm mesh allowed removal of rotifers from tanks so that newly enriched rotifers could be added daily; rotifer densities were enumerated each morning by taking five 1 mL samples from each tank. A green-water culturing technique was used, involving daily additions of algal paste (Nanno 3600, Reed mariculture, Florida USA) at a concentration of approximately 400 000 cells per mL (Pavlidis and Mylonas, 2011).

4.2.2 Water chemistry

Tanks were randomly assigned to one of two pCO_2 treatments: (1) present-day control with pH = 8.03, $pCO_2 \approx 420$ µatm; and (2) hypercapnic or high pCO_2 treatment pH = 7.63, $pCO_2 \approx$ 1400 µatm, for coastal conditions expected for 2100 based on Δ-0.4 pH units (Caldeira and Wickett, 2003; Orr *et al.*, 2005). Present-day control conditions were determined from discrete monthly surface water samples collected inshore in Algoa Bay, Port Elizabeth, between June 2018 and January 2020 (mean pH = 8.03 ±0.07 SD; mean pCO_2 = 423.93 µatm ±84.6 SD) (Edworthy, 2020). The temperature was controlled using aquarium heaters in all tanks and set to 19°C. The pH was slowly brought to experimental conditions over 24 hours following the introduction of the eggs and was controlled with pH/CO_2 controllers (Tunze 7070.200, Aquarientechnik GmbH, Austria) fitted with a non-return valve and pH electrode (Tunze 7070.110) in each tank. Electrodes were calibrated regularly using buffer solution (Tunze 7040.130). The CO_2 was slowly bled into individual tanks with diffusers and needle valves to ensure a slow release. Temperature and pH (measured on the NBS scale) were measured in each tank twice daily, and salinity was measured daily with a Hanna HI 98194 multi-parameter meter. Oxygen levels were maintained above 90% saturation with air diffusers in each tank. Total alkalinity (TA) was measured twice weekly from supply water by using a total alkalinity mini-titrator (Hanna Hi 84531). Ammonium, nitrite and nitrate were measured twice weekly using a colourimeter (Hach DR900) and maintained at values near zero. Carbonate chemistry parameters (ΩCa and ΩAr) were calculated using TA, pH, temperature and salinity as inputs using the CO2SYS program with the constants K1 and K2

Table 4. 1 Mean (±SD) seawater parameters and carbonate chemistry maintained throughout the study. Temperature and pH_{NBS} were measured twice daily, salinity and dissolved oxygen (DO) daily in each rearing tank, total alkalinity (TA) was measured twice weekly. Carbonate chemistry and pCO_2 (ΩCa and ΩAr) were estimated from these parameters in CO2SYS

	Control	High pCO_2
Temperature (°C)	19.51 (±0.59)	18.91 (±0.81)
pH_{NBS}	8.01 (±0.05)	7.62 (±0.05)
Salinity	36.78 (±0.79)	36.76 (±0.79)
DO (mg/L)	7.71 (±0.47)	7.80 (±0.47)
TA µmol kg^{-1} SW	2007.38 (±166.28)	2007.38 (±166.28)
pCO_2 (µatm)	490.16 (±37.05)	1436.18 (±139.79)
ΩCa	3.81 (±0.38)	1.60 (±0.17)
ΩAr	2.48 (±0.25)	1.04 (±0.12)

(Mehrbach *et al.*, 1973) refit by Dickson and Millero (Dickson and Millero, 1987) and using the NBS pH scale. Water conditions and carbonate chemistry parameters maintained throughout the study period are summarised in Table 4.1.

4.2.3 Developmental stages and oxygen consumption measurements

Larvae were grouped into developmental stages based on microscopic examination of developmental milestones (see Chapter 2). Yolk-sac larvae included DAH 0, 1 and 2; early-preflexion included DAH 7 and 8; late-preflexion DAH 13; and flexion DAH 21. Oxygen consumption rates for all stages were performed using static-respirometry methods with O_2 measurements taken every 15 seconds using 24 well microplates placed in a water bath and a 24-channel optical fluorescence, oxygen-sensing system (Loligo Systems, Copenhagen, Denmark). Larvae of all stages were captured by siphoning from throughout the water column of rearing tanks into glass beakers and subsequently siphoned again by targeting into a beaker with 30 µm filtered tank water to separate rotifers from larvae. Following this, the separate beakers were placed into a water bath at control temperature for a three-hour fasting period to ensure post-absorptive larvae testing and avoid confounding digestion measurements on metabolic rates (Andrade *et al.*, 2011; Moyano *et al.*, 2018). Respirometry trials were performed twice per day for hatchling and early-preflexion stages; few remaining larvae resulted in a single trial per day for late-preflexion and flexion stages. The first or single trials started at 10:00, while the second began at 15:00. A minimum of four wells were designated as blanks to record background oxygen consumption rates and were treated as all other wells. As microplates were not fitted with mixing devices, they were selected based on the size and activity of larvae to ensure adequate mixing from movement while preventing confinement-related stress. 80 µL wells were used for yolk-sac larvae and early-preflexion,

200 µL for late-preflexion, and 1700 µL for flexion. Respirometry trials were carried out until most wells reached 80% oxygen saturation (5.6 mg O_2 L^{-1}), resulting in trials lasting up to two hours. Microplates were cleaned between trials by flushing with 70 % ethanol, while beakers and other equipment were rinsed with a 500 ppm hypochlorite solution.

4.2.4 Morphometrics and respirometry

Following respirometry trials, individual larvae were recovered from the wells and were then mounted on a millimetric slide for image capture. Only living individuals were captured due to the rapid change in morphology of dead larvae; it was not necessary to anaesthetise larvae for image capture. No physical damage of larvae was evident at this time as handling was kept to a minimum by siphoning and pipetting into and out of wells. Post respirometry mortality rates were on average less than 40 %. Micrographs were captured with a Leica EZ4 stereomicroscope and fitted camera and analysed with ImageJ software. Morphometric measurements (MM), including total length (TL), depth at vent (DV) and eye diameter (ED), were measured for all individuals.

In contrast, yolk length (YL), yolk depth (YD) and oil globule diameter (OD) (Figure 4.1) were measured for yolk-sac larvae using the measurement landmarks recommended by Chambers *et al.* (2014). Following respirometry or image capture, all specimens were preserved in 99 % ethanol. No specimens were returned to rearing tanks.

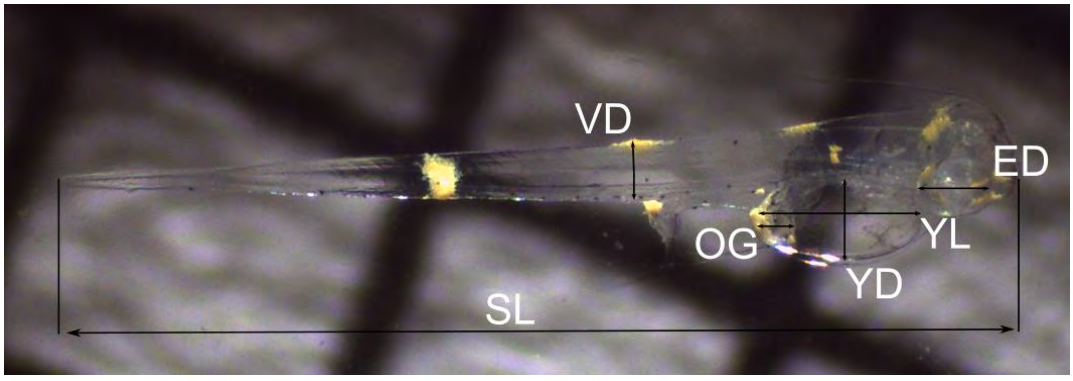


Figure 4. 1 Micrograph of DAH 1 *Chrysoblephus laticeps* showing morphometric traits measured. TL = total length; VD = muscle depth at vent; OG = oil globule diameter; YD = yolk depth; YL = yolk length; ED = eye diameter

4.2.5 Data preparation

Respirometry data were filtered by removing measurements below 80% oxygen saturation which, given the experimental temperature and air pressure, was 5.6 mg O_2 L^{-1}. A quality threshold, $R^2 > 0.85$, was implemented to filter the linear decline in oxygen. Individual

respirometry measurements were analysed using the R package ‗RespR" (Harianto *et al.*, 2019) as described in Chapter 3 to derive measurements of VO_2min, VO_2rout and VO_2max. Blank wells' oxygen consumption rates were averaged and subtracted from the individual measurements to account for background respiration. The average volume of specimens was calculated using the formula for an oblate ellipsoid:

$$V = \pi r^2 \frac{h}{3}$$

Where *r* represents the radius of the head, and *h* is the length from tail to the eye. This specimen volume was then subtracted from the measurement chamber volume (Harianto *et al.*, 2019). Absolute Aerobic Scope (AAS) was calculated using the equation:

$$Absolute\ aerobic\ scope = VO_2max - VO_2min$$

The volume of the yolk sac was approximated by the formula for a prolate spheroid:

$$V = \frac{\pi}{6} lh^2$$

where *l* is the yolk sac length and *h* the height (Blaxter and Hempel, 1963; Bagarinao, 1986)

4.2.5 Statistical analysis

The effects of environmental hypercapnia on metabolic rates (MR) (VO_2min, VO_2rout, VO_2max and AAS) and morphometric measurements (MM) across early development were examined using linear mixed-effects models (LMM) with the ‗lme4' package (Bates *et al.*, 2015). Treatment (control and high pCO_2), development (DAH), and their interaction were included as fixed factors. To avoid pseudoreplication, ‗tank' was added as a random effect, while ‗DAH' was included as a nested random effect to account for the hierarchy of measurements in each LMM. Differences in metabolic rates between treatments were tested by modelling a second-order polynomial relationship between metabolic rate and development (DAH). Orthogonal polynomials were used to reduce the effect of collinearity and because Y values did not approximate the fit curve as the relationship was curvilinear. With the above conditions, a model was produced of the form:

MR/MM = Treatment + DAH + DAH^2 + Treatment:DAH + Treatment:DAH^2 + (tank(DAH)) + error

Akaike information criterion (AIC) was used for model selection and inference (Zuur *et al.*, 2009). Residual diagnostics were carried out for hierarchical regression models using the ‗DHARMa' package (Hartig, 2018). All statistical analysis was conducted using R v3.6.3 (R

Core Team, 2018) with the Rstudio interface. The α level was set to $P < 0.05$ for all analyses. Metabolic rate estimates were $\log_{(X + 1)}$ transformed to meet parametric assumptions while the depth at vent required a box cox transformation.

Respirometry data are reported in nanomole O_2 per individual per hour (nmol O_2 $indiv^{-1}$ h^{-1}). Attempts were made to determine the mass of individuals to report oxygen consumption per gram, however weighing multiple individuals, even at later developmental stages, proved unsuccessful due to their small size even when using a micro-balance.

4.3 Results

4.3.1 Effect of pCO_2 on metabolic rates of developing larvae

Metabolic rates (VO_2min, VO_2rout, VO_2max, and FAS) all showed a positive increase from hatching through to flexion, a period of 24 days. From hatching through to late-preflexion (up to DAH 13), increasing rates were relatively uniform for both control and high pCO_2 larvae. At late-preflexion, a separation between treatments became evident for VO_2min and VO_2rout in particular (Figure 4.2A and B) with a far greater rate of increase for high pCO_2 treatment larvae, while metabolic rates for control larvae increased at a comparatively lower rate. The rate of increase in VO_2min was initially greater for control larvae, with the average VO_2min of yolk-sac larvae increasing by over 500% until late-preflexion (DAH 13) compared with high pCO_2 larvae, which showed a 340% increase over the same time period (Table 4.2). Rates of increase in VO_2min from late-preflexion to flexion (DAH 13 – 21) were vastly dissimilar, increasing by 137% and 900% for control and high pCO_2 larvae, respectively (Figure 4.2A).

Developmental rates of increase in VO_2max between treatments followed the same pattern, although variance between treatments was not as pronounced (Figure 4.2C). Average rates of VO_2max increased from hatching to late-preflexion by 378% and 298% for control and high pCO_2 larvae, respectively. In contrast, the average rate of increase from late-preflexion up to flexion was 181% and 393% for control and high pCO_2, respectively.

Development showed a significant positive relationship with all metabolic rates (VO_2min, VO_2rout, VO_2max and AAS) with increasing oxygen consumption rates over larval development (Table 4.3, Figure 4.2). Although treatment (control/high pCO_2) was not a significant factor for any rates, the interaction between treatment and development (treatment: DAH^2) was significant for VO_2min, VO_2rout and VO_2max. The significance of the polynomial interaction term suggests that the rate of change differed between the control and treatment and that the relationship is a non-linear one. There was no significant effect of treatment or its interaction with development on AAS (Figure 4.2D, Table 4.3).

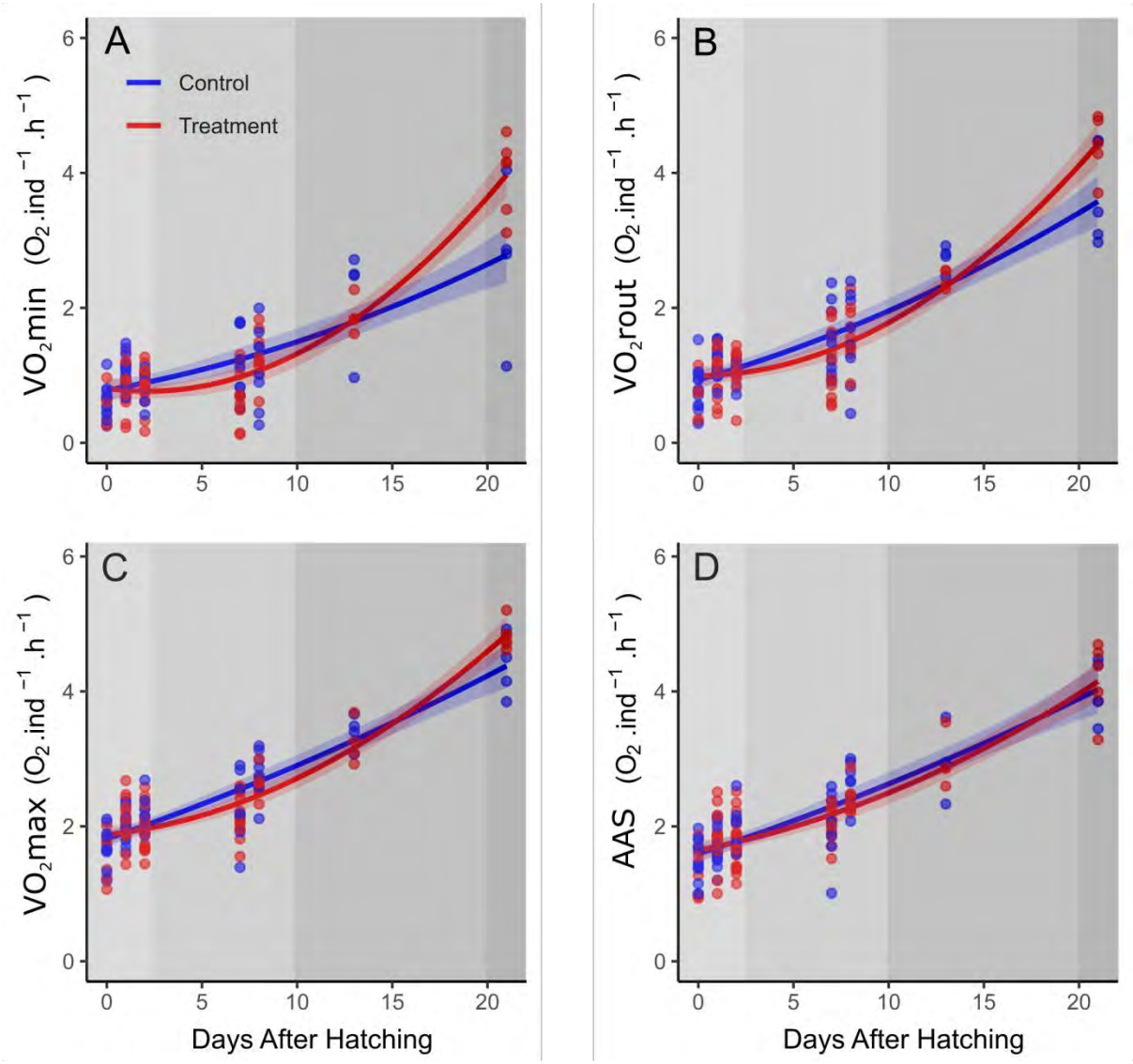


Figure 4. 2 $Log_{(x+1)}$ transformed metabolic rates for *Chrysoblephus laticeps* larvae from control and treatment (blue, current day; and red, high pCO_2) for VO_2min (a), VO_2rout (b), VO_2max (c), and AAS (d) measured across development (DAH). Points indicate individual data, and the modelled second-order polynomial relationship with development is the solid line with shaded 95% confidence intervals. Shaded grey regions indicate approximate developmental stages from lightest to darkest: yolk-sac, early preflexion, late preflexion and flexion.

Table 4. 2 Summary of mean (± SD) metabolic rates (nmol O_2 ind^{-1}. h^{-1}) of early *Chrysoblephus laticeps* by developmental stage with corresponding days after hatch (DAH), treatment (control, pCO_2 ≈ 420 µatm; treatment, pCO_2 ≈ 1600 µatm) and number of individuals sampled (n)

Lifestage	pCO_2	n	VO_2min	VO_2rout	VO_2max	AAS
Yolk-sac (0-2)	Control	39	1.44 ± 0.70	1.91 ± 0.82	6.23 ± 2.26	4.79 ± 2.05
	High	44	1.32 ± 0.65	1.95 ± 0.81	6.44 ± 2.56	5.12 ± 2.41
Early preflexion (7-8)	Control	17	2.61 ± 1.85	4.45 ± 3.03	12.69 ± 5.79	10.08 ± 4.84
	High	21	1.88 ± 1.34	3.39 ± 2.18	10.15 ± 3.74	8.27 ± 2.85
Late preflexion (13)	Control	4	9.47 ± 5.42	14.68 ± 2.82	29.76 ± 7.21	20.29 ± 11.38
	High	4	5.81 ± 2.00	10.49 ± 1.59	25.62 ± 9.40	19.81 ± 9.43
Flexion (21)	Control	4	22.51 ± 23.26	39.10 ± 32.51	83.56 ± 39.6	61.05 ± 27.29
	High	6	58.25 ± 28.44	87.10 ± 31.22	126.32 ± 28.5	68.07 ± 31.65

4.3.2 Effect of pCO_2 on larval morphometrics

Total length and depth at vent measurements mirrored metabolic rates with lower average values for larvae in the high pCO_2 treatment up until flexion, where values of both measurements were greater than control larvae (Figure 4.3A and B). Average values of total length and depth at the vent for flexion stage larvae were approximately 12 and 61% greater, respectively, for high pCO_2 larvae compared to control larvae. While treatment was not a significant factor, the interaction between treatment and development was significant for the quadratic term (Table 4.4).

Despite the exposure of embryos to high pCO_2 conditions, total length at DAH 1 and 2 showed no significant difference between treatment nor in the interaction between treatment and DAH (LME, $P > 0.05$) (Figure 3C). Oil globule diameter at 2 DAH was 2% larger for high pCO_2 larvae, but yolk volume at 1 DAH was approximately 15% less for high pCO_2 larvae compared to control larvae (Figure 4D and E). Oil globule diameter and yolk volume did not show any significant difference between treatments (LME, $P > 0.05$). By 2 DAH, yolk reserves were spent for most individuals, regardless of treatment, and could not be compared.

Table 4. 3 Results of the linear mixed-effects models (LMM) analyses of metabolic rates of *Chrysoblephus laticeps* larvae in response to high pCO_2 treatments across early development presented as a linear (DAH) and quadratic (DAH^2) function, with treatment as an interaction term. Pseudo-R^2 reported significant values in bold

Metabolic rate	Effect	Estimate	Std. Error	*t-value*	*P*
VO_2min Pseudo-R^2 0.713	**Intercept**	**1.139**	**0.072**	**15.831**	**0.000**
	Treatment	-0.021	0.101	-0.206	0.839
	DAH	**6.004**	**0.772**	**7.781**	**0.000**
	DAH^2	0.675	0.793	0.852	0.400
	Treatment : DAH	**2.737**	**1.117**	**2.451**	**0.020**
	Treatment : DAH^2	**2.840**	**1.126**	**2.522**	**0.017**
VO_2rout Pseudo-R^2 0.811	**Intercept**	**1.457**	**0.056**	**25.801**	**0.000**
	Treatment	-0.017	0.078	-0.224	0.830
	DAH	**8.201**	**0.628**	**13.066**	**0.000**
	DAH^2	0.903	0.631	1.431	0.159
	Treatment : DAH	1.696	0.874	1.939	0.060
	Treatment : DAH^2	**2.404**	**0.877**	**2.742**	**0.009**
VO_2max Pseudo-R^2 0.827	**Intercept**	**2.376**	**0.054**	**44.096**	**0.000**
	Treatment	-0.046	0.076	-0.612	0.545
	DAH	**8.134**	**0.584**	**13.936**	**0.000**
	DAH^2	0.364	0.597	0.610	0.545
	Treatment : DAH	0.705	0.840	0.840	0.406
	Treatment : DAH^2	**1.763**	**0.846**	**2.084**	**0.043**
AAS Pseudo-R^2 0.758	**Intercept**	**2.129**	**0.056**	**38.294**	**0.000**
	Treatment	-0.033	0.077	-0.430	0.670
	DAH	**7.744**	**0.620**	**12.495**	**0.000**
	DAH^2	0.414	0.628	0.660	0.512
	Treatment : DAH	-0.005	0.876	-0.005	0.996
	Treatment : DAH^2	0.949	0.881	1.078	0.287

Table 4. 4 Results of the linear mixed-effects models (LMM) analyses of total length and depth at the vent of *Chrysoblephus laticeps* larvae in response to control and high pCO_2 treatments across early development presented as a linear (DAH) and quadratic (DAH^2) function, with treatment as an interaction term. Pseudo-R^2 reported significant values in bold

Metric	Effect	Estimate	Std. Error	*t-value*	*p*
Total length Pseudo-R^2 0.830	**Intercept**	**2.720**	**0.039**	**69.581**	**0.000**
	Treatment	-0.006	0.053	-0.108	0.915
	DAH	**3.942**	**0.343**	**11.484**	**0.000**
	DAH^2	**-0.932**	**0.347**	**-2.689**	**0.010**
	Treatment : DAH	0.386	0.470	0.821	0.419
	Treatment : DAH^2	**1.317**	**0.462**	**2.850**	**0.008**
Depth at vent* Pseudo-R^2 0.765	Intercept	-0.025	0.121	-0.205	0.844
	Treatment	0.005	0.169	0.028	0.979
	DAH	**7.260**	**0.745**	**9.742**	**0.000**
	DAH^2	-1.239	0.773	-1.603	0.117
	Treatment : DAH	1.107	1.028	1.077	0.291
	Treatment : DAH^2	**3.365**	**1.021**	**3.296**	**0.002**

*Box Cox transformed response variable

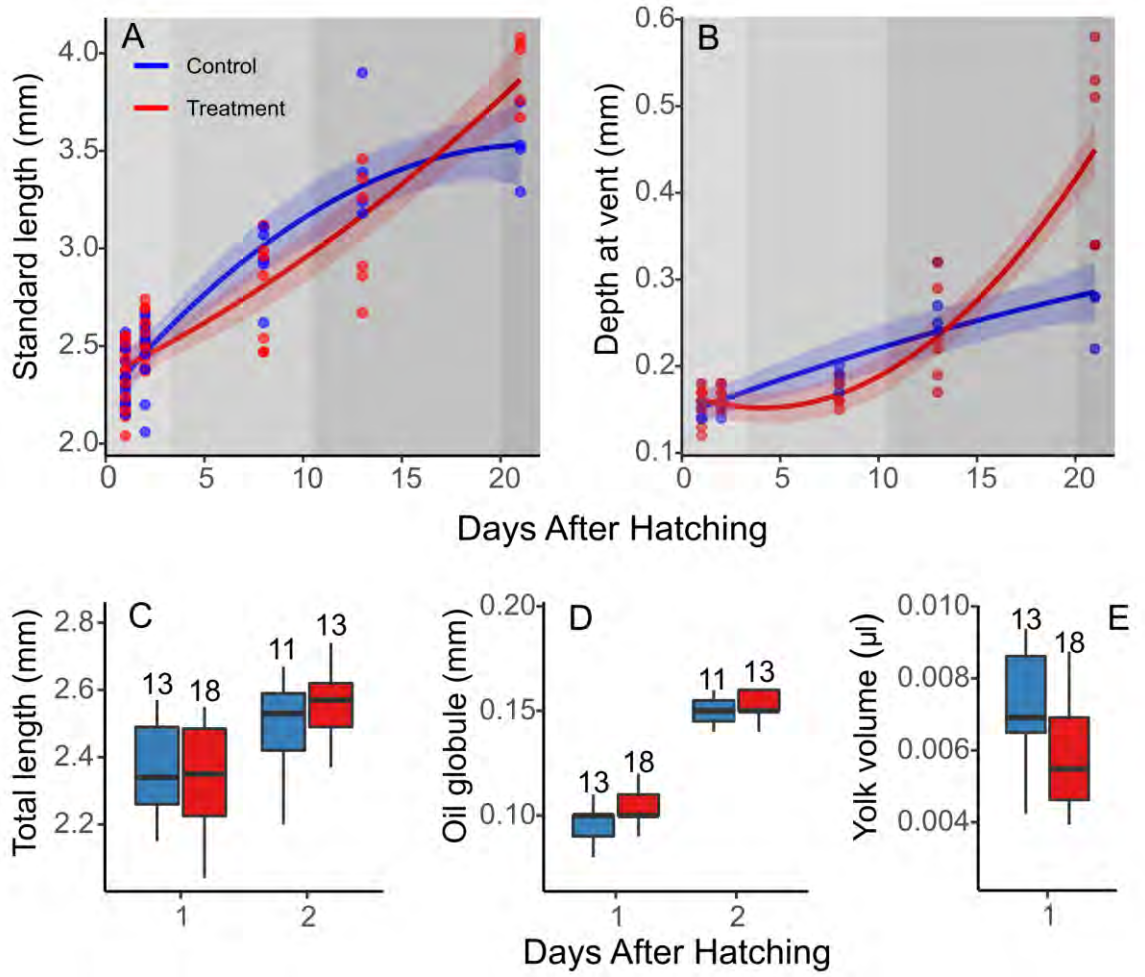


Figure 4. 3 Effect of elevated pCO_2 on morphometrics of early *Chrysoblephus laticeps*. Total length (mm) (a) and depth at vent (mm) (b) throughout early development, total length by day for 1 and 2 DAH (c), oil globule diameter (mm) (d), and yolk volume (µl) for 1 DAH (e). Box and whisker plots where whiskers denote 10th and 90th percentiles, the box denotes 25th and 75th percentiles, the median value indicated by a horizontal bar, values above whisker indicate n per treatment.

4.4 Discussion

This study revealed that experimental ocean acidification produced stage-specific opposing results during the early development of *Chrysoblephus laticeps*. Yolk-sac larvae appeared to exhibit some resilience to high pCO_2 experimental conditions, relevant to future predicted Ocean Acidification by 2100. Yolk-sac larvae displayed little to no difference in metabolic or growth metrics between control and treatment conditions apart from a small, non-significant decrease in yolk volume. Early-preflexion larvae in the high pCO_2 treatment began to exhibit reduced metabolism and growth, which became more evident by the late-preflexion stage. However, although sample sizes were reduced and should be interpreted with caution, it appeared that this pattern in metabolic and growth metrics was reversed by the onset of

flexion with greater metabolic and growth rates in larvae exposed to high pCO_2 conditions by 21 DAH.

Although embryos from high pCO_2 treatment conditions had been exposed to hypercapnic conditions for two days, yolk-sac larvae displayed no significant differences in metabolism, growth or endogenous resource supplies compared with control larvae. Although yolk volume was 15% smaller at 1 DAH in larvae from the high pCO_2 treatment, there was little difference in oil globule diameter between treatments. Reduced energy reserves, in the form of smaller yolk and/or oil globule, has been observed in other marine larvae exposed to elevated pCO_2 treatments (up to 4714 µatm, 7.5 pH), including summer flounder *Paralichthys dentatus*, yellowtail kingfish *Seriola lalandi*, Senegalese sole *Solea senegalensis* and blacktail seabream *Diplodus sargus* (Chambers *et al.*, 2014; Munday *et al.*, 2015; Faria *et al.*, 2017). The smaller yolk volume in the larvae exposed to high pCO_2 levels may suggest that they utilised a larger proportion of their endogenous energy reserve to attain the same size and may indicate a trade-off between growth, necessary for first feeding and the window to begin exogenous feeding (Chambers *et al.*, 2014). This critical period begins at yolk exhaustion and can be as little as 12 hours (Garcia *et al.*, 2020). Therefore, even minor reductions in yolk size may have large ecological implications as it will reduce the length of the window between depletion of endogenous reserves and feeding. This is of particular concern in a natural setting, where food availability may be sporadic (Voss *et al.*, 2006).

During preflexion (7, 8 and 13 DAH), *C. laticeps* from the high pCO_2 treatment were smaller and exhibited lower average metabolic rates (Table 4.2, Figure 4.2 and 4.3). The smaller size of treatment larvae at this stage may be related to the smaller yolk reserve of yolk-sac larvae but may also be due to metabolic depression induced by acidosis. Metabolic depression is an important strategy that allows organisms to enhance their tolerance and survival time when exposed to stressful environmental conditions (Hand and Hardewig, 1996; Richards, 2010). By down-regulating energy utilising processes, such as protein synthesis, in favour of maintenance mechanisms, such as ionoregulation, organisms may reduce energetic demands while further reducing energetic costs associated with ionoregulation as a consequence of declines in intracellular pH (Guppy and Withers, 1999a). Indeed, a characteristic decline in intracellular pH is associated with but may even cause metabolic depression (Guppy *et al.*, 1994; Guppy and Withers, 1999a). Given the increasing environmental pCO_2 and a concomitant decline in pH, modifications to the diffusion gradient will increase organismal pCO_2 and lead to extracellular acidosis, which may result in metabolic depression and reductions in growth if there is no active compensation (Esbaugh *et al.*, 2012; Nilsson *et al.*, 2012).

Additionally, a greater overall decline in active or maximum metabolic rate is suspected to lead to reductions in aerobic scope (Pörtner and Peck, 2010) which, although not significant, was observed in the absolute aerobic scope of preflexion larvae in this study (Table 4.2). Reductions in early growth in response to elevated CO_2 have also been observed in other species, including the gilt-head bream *Sparus aurata,* the inland silverside *Menidia beryllina,* and the yellowfin tuna *Thunnus albacares* (Baumann *et al.*, 2012; Frommel *et al.*, 2016; Pimentel *et al.*, 2016). This may be one of the more common findings in general (Cattano *et al.*, 2018; Munday *et al.*, 2019). However, as many of these studies did not simultaneously measure metabolism, it is unclear whether growth rate reductions were related to metabolic depression. Given the current findings, it seems reasonable to suggest that there was metabolic depression among treatment fish and the observed reductions in somatic growth may be attributed to the down-regulation of protein synthesis. This is especially likely given that the gills, the primary site for ionoregulation and acid-base regulation of preflexion *C. laticeps,* appeared to be in a primitive state of development.

By 20 DAH, the majority of *C. laticeps* appeared to be undergoing notochord flexion. For pelagic-spawned eggs and larvae, this period generally coincides with muscle differentiation, increasing density of ionocytes at the gill surface and further gill development of secondary lamellae to cope with increasing oxygen demands (Rombough, 1988, 2007). While there was a considerable difference between lengths and depth at vent at 21 DAH, with high pCO_2 larvae larger on average than control larvae, the latter was particularly apparent (Figure 4.3B). The deepening of the trunk occurs along with muscle differentiation and formation of the caudal fin to aid in a transition to increased active swimming (Downie *et al.*, 2020). Therefore, the greater depth at the vent for high pCO_2 larvae indicates a more rapid rate of development, having reached flexion before control larvae. While this may introduce a confounding factor when considering metabolism, routine metabolic rates at 21 DAH for high pCO_2 treatment were more than double that of the control. Other studies have similarly found increased length at age during the flexion period. Dolphinfish *Coryphaena hippurus* from high pCO_2 treatments (1460 µatm) were larger than those from ambient (350 to 490 µatm) conditions at 8 DAH while also displaying a greater proportion at the flexion stage. However, by 9 DAH, once all larvae had reached flexion, the differences in length between treatments had disappeared (Bignami *et al.*, 2014). Similarly, at 28 DAH, larval *P. dentatus* were larger and had developed faster in elevated CO_2 treatments (4714 µatm pCO_2) compared with low and intermediate treatments (775 and 1808 µatm) (Chambers *et al.*, 2014). Furthermore, Frommel *et al.* (2012) found that the growth of larval Atlantic cod *Gadus morhua* was positively affected by high pCO_2 (4200 µatm) between 25 and 39 DAH, coinciding again with flexion (Herbing *et al.*, 1996). However, by 46 DAH, there was no

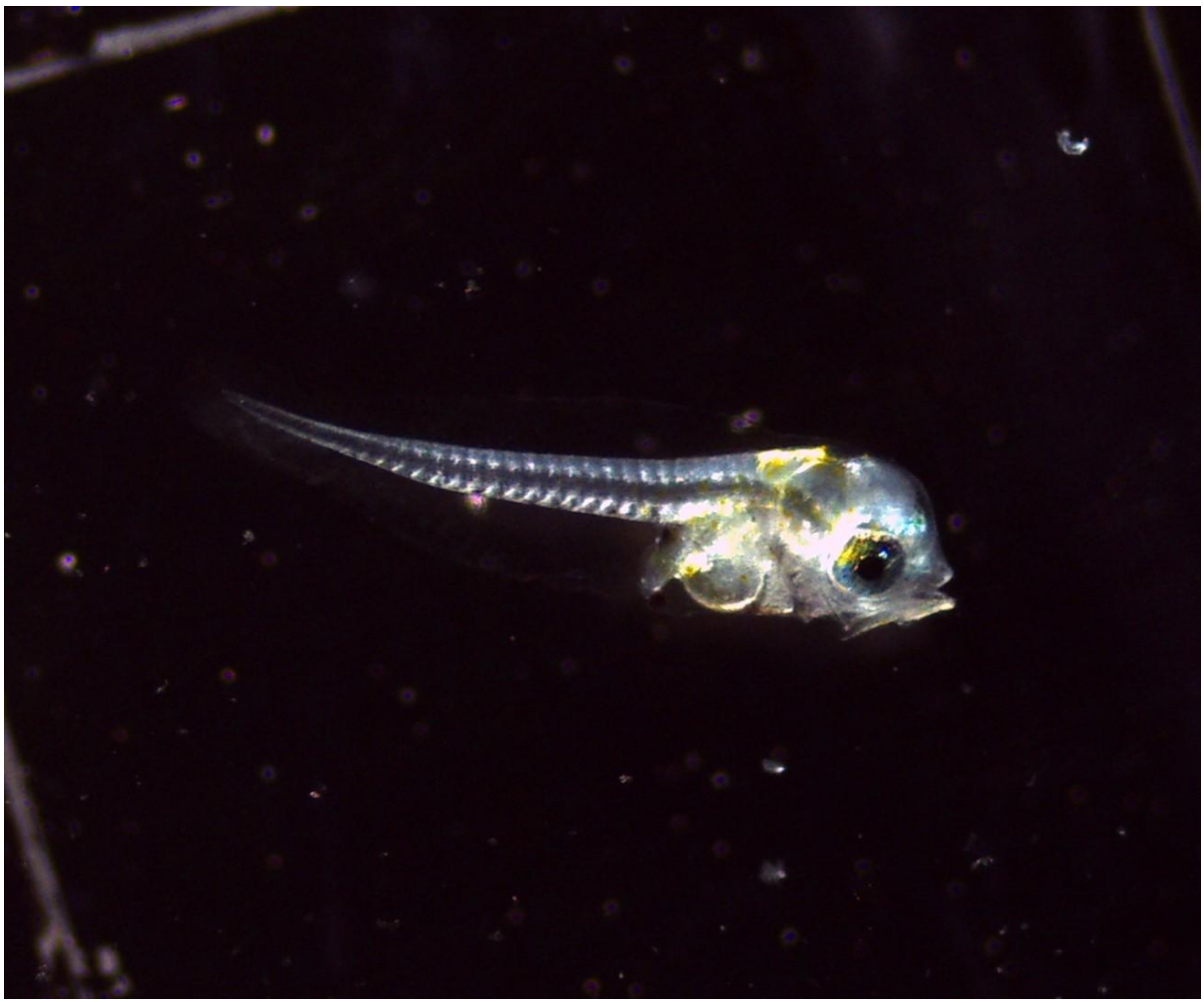
difference between treatments. The increased growth rate during a critical developmental stage for *G. morhua* came at a high cost, though, as severe to lethal tissue damage of internal organs was associated with elevated CO_2 concentrations (Frommel *et al.*, 2012). Pimentel *et al.* (2016) likewise observed a greater incidence of body malformations in 15 DAH (preflexion) *S. aurata* exposed to elevated CO_2 (1400 µatm).

The present study provides some perspective on the potential impacts of OA on metabolism and growth during the early life stages of a large, commercially important fishery species. Few studies have examined the impacts of OA throughout early ontogeny but instead focussed on key developmental periods or arbitrary stages post-hatching. The literature is interspersed with negative, neutral and positive findings on development and growth (reviewed in Munday *et al.*, 2019). Indeed, if only specific life stages were investigated in the current study, results could support either of these findings. This highlights the importance of conducting studies of this nature throughout the various larval developmental stages. Nevertheless, it would appear in the current instance that elevated CO_2 does present an environmentally relevant stressor to developing *C. laticeps*.

Yolk-sac larvae appear otherwise tolerant apart from potentially greater energy expenditure evidenced through reduced yolk reserves. At preflexion, an inability to respond to and adequately compensate for acid-base disturbance leads to metabolic depression, potentially through a direct environmental effect on extra- and intracellular pH (Guppy and Withers, 1999a). Consequently, the response to elevated CO_2 resulted in reduced growth-at-age which may be due to related behavioural and physiological adjustments in line with the theory of metabolic depression, such as reduced activity and protein synthesis, respectively (Richards, 2010). Toward the end of preflexion and along with an increase in gill surface area and mitochondria-rich ionocyte densities, it appears that acid-base regulation may begin to compensate for imbalances. This is most likely facilitated by an increase in metabolic rate, necessary to maintain acid-base balance and is fuelled by an increase in energy intake, which promotes compensatory growth (Munday *et al.*, 2009; Bignami *et al.*, 2014). However, this compensatory growth may be associated with developmental abnormalities or tissue damage (Frommel *et al.*, 2012, 2014; Pimentel *et al.*, 2016). Finally, potentially negative effects of elevated CO_2 encountered here, and possibly even cryptic impacts, are likely mediated by a limitless supply of food, potentially leading to an underestimate of negative outcomes (Munday *et al.*, 2019; Stiasny *et al.*, 2019). Future studies would benefit by including varying prey availability scenarios. In contrast, investigations into the development of gills and regulatory potential under varying pCO_2 conditions through ontogeny would provide valuable insights for our understanding of pH regulation in the early life stages of fishes.

Chapter 5

Climate change and larval red roman seabream *Chrysoblephus laticeps,* does exploitation influence resilience to an environmental stressor?



Preflexion roman seabream *Chrysoblephus laticeps*, approximately 13 days after hatching

5.1 Introduction

All forms of fishing are selective, the most obvious being for size, where the largest individuals are routinely targeted (Heino *et al.*, 2015). This leads to population evolution of smaller average-sized individuals, with exploited populations commonly displaying demographic shifts toward smaller and/or younger individuals (e.g. Conover and Munch, 2002; Stergiou, 2002; Jakobsdóttir *et al.*, 2011). Additionally, this unnatural selection may affect size- and age-at-maturity, the timing of reproduction, dispersal and even behaviour (Allendorf and Hard, 2009). Even seemingly non-selective forms of fishing, such as purse-seining, still exhibit selection at the population level due to the non-random distribution of stocks, which is typically determined by interactions between the environment and physiology of an organism (Planque *et al.*, 2011). While selection operates on individuals, it is the gene pools of populations where selective events ‗memory' is stored (Conover and Schultz, 1997).

As many of the traits impacted by exploitation are heritable, this unnatural selection process may reduce the frequency of desirable phenotypes within a population (Allendorf and Hard, 2009). This process, referred to as fisheries-induced evolution (FIE), has been well studied and reviewed (e.g. Law, 2000; Biro *et al.*, 2008; Hutchings and Fraser, 2008; Enberg *et al.*, 2012; Heino *et al.*, 2015). Field and experimental studies have revealed aspects of FIE related to reduced growth and fecundity (Conover and Munch, 2002), age or size at reproduction (Jorgensen, 1990; Kendall *et al.*, 2014), metabolism (Cooke *et al.*, 2007; Duncan *et al.*, 2019b) and behaviour (Biro *et al.*, 2008; Prokkola *et al.*, 2021). Despite convincing evidence for FIE, conventional fisheries management does not appear to have considered the influence of fisheries on the evolutionary trajectory of a stock and its consequences, nor has it considered the impact of co-occurring environmental change (Stergiou, 2002; Heino *et al.*, 2015).

Climatic variability is known to affect fish biomass (e.g. Lehodey *et al.*, 2006). For example, short-lived species, such as sardine exhibit enormous biomass fluctuations over decadal to centennial scales coinciding with climate oscillations (Mcfarlane, 2002). Although these changes may be less dramatic for large, long-lived species, biomass and distributional shifts related to large-scale changes in temperature and ocean circulation have been observed (Nye *et al.*, 2009). Generally, changes to population biomass or distribution are considered to be related to the variation in larval survival and recruitment success (Houde, 1997; Lehodey *et al.*, 2006; Nye *et al.*, 2009).

The eggs and larval stages of many marine spawned species are exceedingly small, have little endogenous reserves in the form of egg yolk, are limited in their movement capabilities

and exhibit narrow environmental tolerances (Duarte and Alearaz, 1989; Dahlke *et al.*, 2020; Downie *et al.*, 2020). With anthropogenic-driven climate change anticipated to lead to greater ocean-climate variability, including changes in temperature, current strength and acidification, the survival and recruitment of marine fish larvae will likely be negatively affected (Potts *et al.*, 2015; Dahlke *et al.*, 2020). Through the process of FIE, exploitation may further aggravate these adverse effects by reducing the resilience of these early-life stages to climate variability (Hollins *et al.*, 2018). Size-selective processes may reduce resilience to perturbations; processes like age truncation toward smaller, earlier spawning adults which produce fewer, smaller, and lower quality eggs (Conover and Munch, 2002; Planque *et al.*, 2010). Additional processes include the removal of bold, aggressive phenotypes with genetic corollaries that ultimately change the genetic composition of the population (timidity syndrome Arlinghaus *et al.*, 2017). Physiological covariation between bold and shy behaviours has been found to exist (Redpath *et al.*, 2010; Killen *et al.*, 2011). Furthermore, metabolic phenotypes show a degree of heritability (Munday *et al.*, 2017; Long *et al.*, 2021). For this reason, selective fishing by passive gear may result in the selection of more timid and low-performance metabolic phenotypes that may be less resilient to environmental stressors.

The energy budget of all organisms is limited and must be partitioned into a range of functions, such as activity, growth, and maintenance (Sokolova *et al.*, 2012; Sokolova, 2013). During physiological stress, more energy must be redirected towards maintenance or regulatory processes, which reduces the energetic capacity for growth and activity (Sokolova, 2013). Physiological stress may further affect energy budgets by reducing energy acquisition and increasing expenditure (Maltby *et al.*, 2001). As such, organisms with higher residual energy levels (or greater aerobic scope) may be comparatively better equipped to endure periods of stress (Sokolova *et al.*, 2012).

Fish larvae present a good model to determine bioenergetic compromises because their residual energy is highly constrained. They must partition a limited energy supply among many competing life processes (Rombough, 1994). High growth rates are essential as mortality declines with increasing size, and as such, high growth rates are typical for larval fish (Peterson and Wroblewski, 1984; Pedersen, 1997). Nevertheless, growth is an energetically expensive process, likely demanding a significant portion of the available energy budget (Wieser *et al.*, 1988). Additionally, the development of structures and organs must, to some degree, reflect priorities for feeding, locomotion and respiration, and these tend to characterise important developmental milestones (Fuiman and Higgs, 1997; Osse *et al.*, 1997). These specific ontogenetic stages during early development similarly represent

critical energetic bottlenecks where physiological stress may compromise survival (Edworthy *et al.*, 2018; Joly *et al.*, 2021).

Findings from Chapter 4 suggest that *Chrysoblephus laticeps* larvae from the exploited population (EP) reared in hypercapnic conditions relative to control conditions experienced a critical period during preflexion where metabolic rates, aerobic scope and growth were depressed. Additionally, the significantly higher metabolic rates of flexion larvae reared in hypercapnic conditions and growth over-compensation suggested exposure to physiological stress. Given these findings, it is likely that survival would have been compromised under sporadic or limited feeding conditions. Duncan *et al*. (2019b) found that adult *C. laticeps* from the TNP protected population (PP) were physiologically more resilient to increasing temperatures than adults from the NHK exploited population. As such, the larvae from protected populations may also have an inherent resilience to environmental stressors, such as ocean acidification. This chapter aimed to determine if increased resilience to climate change, specifically ocean acidification, may be found in the progeny from the protected population compared to those from the exploited population. To do this, larvae from adults from the PP were assessed in contemporary (control, pCO_2 = ~400 µatm, pH = 8.03) and predicted future ocean acidification conditions (high pCO_2 = ~1600 µatm, pH = 7.63). They were assessed according to larvae's aerobic performance (VO_2min, VO_2rout, VO_2max and Aerobic Scope) and development (total length, depth at vent, head depth, and eye diameter). These results were then compared with those from larvae from the EP (Chapter 4) throughout early development up to flexion.

5.2 Materials and methods

5.2.1 Field spawning, egg incubation and rearing

The rearing of larvae in this study should ideally have been performed alongside those of Chapter 4, with capture and spawning taking place in a similar time interval. However, several limitations (such as equipment availability, sampling teams, rearing space and weather conditions) made this approach logistically unfeasible. In light of this, a decision was made to replicate the study one year later during the species‘ peak spawning season. To ensure comparability, the methods used were, as far as possible, identical for both populations. As average *in situ* temperatures at the time of collection were lower at TNP, the set-point for rearing tanks was reduced from 19°C to 18°C, to improve survival rates and ensure sufficient larvae for experiments.

The process of capture, egg collection and rearing has been previously described in Chapter2. Briefly, mature *C. laticeps* (n=19) were collected from the Protected Population

(PP) of the Tsitsikamma National Park (see Chapter 2) on a shallow-water reef (34°01‘13.2”S 23°50‘29.7”E) of less than 15 m in depth on the 14 December 2020. Fish were injected with Aquaspawn to induce ovulation and maintained in flow-through tanks (2 x 5000 L) near the capture site. Gametes were stripped from males (n = 4, mass ranging from 1500 – 2180 g) and females (n=15, mass 360 – 1060 g) 48 hours after injection by applying light pressure to the abdomen. Once all eggs had been collected, they were mixed and added to a 2 L bowl to which a small volume (approximately 10 mL) of sperm was added. Small volumes of seawater were added over 10 minutes whilst mixing with a fine-bristled brush to reach a final volume of 1 L. The mixture was then heavily aerated for a further 15 minutes, after which 0.27 mL of formaldehyde was added to disinfect the eggs of potential pathogens. After 15-minutes, the mixture was repeatedly strained and rinsed with fresh seawater, equally distributed into sealable bags and stored in a cooler for transport back to the NRF-SAIAB Aquatic Ecophysiology Research Platform (AERP) at Rhodes University, Makhanda.

On arrival, all eggs were mixed into a 20 L glass tank with heavy aeration while acclimating to control temperatures. Once control temperatures were reached (18°C), aeration was removed, and eggs were allowed to separate into floating (fertilised) and sinking (non-fertilised) fractions. Floating eggs were skimmed from the surface and held in a 1 L beaker with light aeration to ensure thorough mixing of eggs throughout the water column. Eggs were enumerated by drawing ten 2 mL samples and then randomly distributed into each of eight tanks at 60 eggs L^{-1} or 6000 eggs per tank.

5.2.2 Water chemistry

Tanks were randomly assigned to one of two pCO_2 treatments: (control) present-day conditions with pH = 8.03, pCO_2 ≈ 420 µatm; and high-pCO_2/hypercapnic treatment with pH = 7.63, pCO_2 ≈ 1400 µatm, for coastal conditions expected to occur by the end of the century with a ∆-0.4 pH units (Caldeira and Wickett, 2003). Present-day control conditions were determined from discrete monthly surface water samples collected inshore in Algoa Bay, Port Elizabeth, between June 2018 and January 2020 (mean pH = 8.03 ±0.07 SD; mean pCO_2 = 423.93 µatm ±84.6 SD) (Edworthy, 2020). The pH was slowly brought to experimental conditions over 24 hours following the introduction of the eggs. The temperature was set to 18°C for all tanks and controlled using aquarium heaters.

Temperature, pH (NBS scale) and dissolved oxygen were measured in each tank twice daily using a Hanna HI 98194. Salinity was measured daily with a refractometer, and total alkalinity was measured weekly by using a total-alkalinity mini-titrator (Hanna Hi 84531). Ammonium, nitrate and nitrite were measured twice weekly using a Hach DR900 colorimeter and kept at levels near zero with frequent water changes. Dissolved oxygen was maintained

at levels above 90% saturation with air diffusers. Carbonate chemistry parameters were calculated using TA, pH, temperature and salinity as inputs, using the CO2SYS program with the constants K1 and K2 refitted by Dickson and Millero (1987) and using the pH_{NBS} scale. Rearing conditions for the current study and Chapter 4 are summarised in Table 5.1.

5.2.3 Respirometry and morphometrics

Larvae from the EP (Chapter 4) and PP were subject to respirometry based on a microscopic examination of larval developmental stages or milestones, as described in Chapter 2 (Figure 2.4). This was done as a means to ensure comparability in the oxygen consumption and morphometric measurements between larvae from the EP and PP. Completion of the yolk-sac stage was identified by the complete absorption of endogenous resources (both yolk-sac and oil-globule). The full pigmentation of the eyes noted Early-preflexion, mouth opened, development of gut, and the presence of prey in the gut of the majority of individuals indicating successful feeding and mortality of individuals who had not survived first feeding. Late-preflexion was noted by the increase in body length and gut pigmentation with further development. Flexion was noted as the time when most individuals (>50%) were undergoing notochord flexion along with the presence of the lower hypural plate and caudal fin rays. The yolk-sac stage continued up to DAH 3, preflexion from DAH 4 – ca.20, and flexion from DAH ca.20 – 22. Respirometry trials and data filtering were carried out as per Chapter 4 with all trials being carried out at 19°C.

Table 5. 1 Mean (±SD) seawater parameters and carbonate chemistry maintained throughout the study for EP (Chapter 4) and PP (current study). Temperature and pH_{NBS} were measured twice daily, salinity was measured daily, and total alkalinity (TA) was measured twice weekly. pCO_2 was estimated from these parameters in CO2SYS

	Control		High pCO_2	
	EP (Chapter 4)	PP	EP (Chapter 4)	PP
Temperature (°C)	19.51 (0.59)	17.93 (0.26)	18.91 (0.81)	17.98 (0.22)
pH_{NBS}	8.01 (0.05)	8.01 (0.05)	7.62 (0.05)	7.62 (0.06)
Salinity	36.78 (0.79)	34.75 (0.43)	36.76 (0.79)	34.75 (0.43)
TA µmol kg^{-1}. SW	2007.38 (166.28)	2213.32 (77.07)	2007.38 (166.28)	2079.21 (100.87)
pCO_2 (µatm)	490.16 (37.05)	535.87 (33.28)	1436.18 (139.79)	1497.13 (37.67)

5.2.4 Statistical analysis

Data from the current study (PP) was included with that from Chapter 4 (EP) to compare the response of larvae from the EP and PP to the effects of high pCO_2/hypercapnia by life stage. As Chapter 4 showed significant differences among early life stages, analysis for the current chapter separated these periods into discrete stages for comparison. Linear mixed-effects

models (LMM) were used to test the effects of hypercapnia on the metabolic rates (VO_2min, VO_2rout, VO_2max and AAS) or morphometric measurements (Total length, depth at vent, head depth and eye diameter), respectively. This was done to evaluate the stage-specific effects of high pCO_2 exposure. Metabolic rates (MR) or morphometric measurements (MM) per life stage were included as response variables with population, treatment, and their interaction as fixed effects. To avoid pseudoreplication, ‗tank' was included as a random effect in each model. A model was produced for each life stage (yolk-sac, preflexion and flexion) and MR/MM of the form:

MR/MM = Population + Treatment + Population:Treatment + (tank) + error

All statistical analyses were conducted using R-Studio v1.2.5033 (R Core Team, 2018). LMMs were run using the 'lme4' (Bates *et al.*, 2015) package using maximum likelihood estimates for fixed effects, while pairwise estimates were carried out using the ‗emmeans' package with Satterthwaite's method to determine degrees freedom and significance levels. Residuals were assessed visually and statistically using the ‗DHARMa' package (Hartig, 2018), while the best fit model was determined from Akaike Information Criterion (AIC).

5.3 Results

5.3.1 Yolk-sac stage

Mean values of VO_2min, VO_2rout and VO_2max were higher for yolk-sac stage larvae from the protected population (PP) than the exploited population (EP) in both control and high pCO_2 conditions (Figure 5.1A, Table 5.2). However, population was only found to be a significant factor for VO_2rout (P = 0.002, Table 5.3, Figure 5.1A). Larvae from both populations had higher mean VO_2min estimates in control conditions and higher mean VO_2rout and VO_2max estimates in hypercapnic conditions (Figure 5.1A, Table 5.2). However, treatment was not a significant factor for any of these metrics (Table 5.3, Figure 5.1A).

Mean morphometric values of total length (P < 0.001), depth at vent (P < 0.001), head depth (P < 0.001) and eye diameter (P < 0.001) were all significantly greater in PP larvae (Figure 5.1B and C, Table 5.4). Yolk-sac larvae of both populations reared in control conditions were on average smaller in total length than high pCO_2 larvae (Table 5.2). However, there was no significant effect of treatment on any of the morphometric measurements.

Table 5. 2 Summary data of metabolic rates and morphometric measurements for *Chrysoblephus laticeps* larvae by developmental stage and exploitation status. Metabolic rates include VO_2min, VO_2rout, VO_2max and AAS and are measured in nanomoles. individual^{-1}. hour^{-1} (± SD). Morphometric measurements include total length, depth at vent, head depth and eye diameter and are measured in mm (±SD)

Stage	Population	pCO_2	N	VO_2min	VO_2rout	VO_2max	AS
Yolk sac (0 – 3)	Exploited	Control	39	1.44 (0.70)	1.91 (0.82)	4.35 (1.90)	2.91 (1.61)
		High	44	1.32 (0.65)	1.95 (0.81)	4.82 (2.22)	3.50 (1.94)
	Protected	Control	40	1.65 (0.74)	2.50 (0.80)	5.07 (2.15)	3.42 (1.86)
		High	46	1.52 (0.67)	2.52 (0.87)	5.14 (1.94)	3.63 (1.72)
Preflexion (7 – 15)	Exploited	Control	21	3.92 (3.84)	6.40 (5.05)	16.71 (12.29)	12.79 (11.04)
		High	25	2.51 (2.04)	4.53 (3.36)	14.21 (14.91)	11.70 (13.30)
	Protected	Control	37	4.65 (2.40)	7.95 (6.17)	16.11 (8.29)	11.46 (6.89)
		High	40	5.29 (2.79)	7.84 (5.04)	14.84 (9.65)	9.55 (7.66)
Flexion (21 – 22)	Exploited	Control	6	17.93 (4.35)	24.98 (5.90)	59.32 (19.97)	41.39 (20.53)
		High	7	54.66 (28.68)	81.21 (34.82)	109.79 (42.19)	55.13 (42.12)
	Protected	Control	16	24.78 (14.34)	33.31 (15.09)	71.44 (25.95)	46.66 (19.13)
		High	12	45.11 (24.32)	62.03 (24.73)	105.08 (26.90)	59.97 (19.20)
				Total length	Depth at vent	Head depth	Eye diameter
Yolk sac (0 – 3)	Exploited	Control	24	2.41 (0.17)	0.16 (0.01)	0.31 (0.02)	0.20 (0.02)
		High	31	2.43 (0.18)	0.16 (0.01)	0.31 (0.03)	0.20 (0.03)
	Protected	Control	44	2.68 (0.14)	0.18 (0.01)	0.40 (0.05)	0.23 (0.03)
		High	55	2.74 (0.14)	0.18 (0.01)	0.42 (0.08)	0.24 (0.02)
Preflexion (7 – 15)	Exploited	Control	9	3.15 (0.36)	0.22 (0.05)	0.57 (0.09)	0.27 (0.03)
		High	15	2.93 (0.31)	0.20 (0.05)	0.54 (0.11)	0.25 (0.03)
	Protected	Control	28	2.71 (0.33)	0.17 (0.04)	0.53 (0.13)	0.26 (0.04)
		High	33	2.75 (0.38)	0.18 (0.01)	0.50 (0.10)	0.25 (0.03)
Flexion (21 – 22)	Exploited	Control	4	3.58 (0.19)	0.28 (0.05)	0.73 (0.17)	0.34 (0.02)
		High	5	3.92 (0.19)	0.46 (0.11)	1.00 (0.13)	0.42 (0.05)
	Protected	Control	16	4.64 (0.71)	0.46 (0.13)	1.07 (0.20)	0.43 (0.05)
		High	15	4.63 (0.52)	0.48 (0.11)	1.08 (0.14)	0.43 (0.05)

5.3.2 Preflexion

Mean values of VO_2min and VO_2rout were higher for PP preflexion larvae in both control and high pCO_2 conditions, while rates for VO_2max were similar between populations (Table 5.2, Figure 5.1D). Population was a significant term for VO_2min (P = 0.033, Table 5.3), with PP larvae exhibiting greater rates than EP larvae. Rates of VO_2max for preflexion larvae reared in high pCO_2 conditions were lower than that of control larvae. EP larvae responded to high pCO_2 with lower average rates for VO_2min and VO_2rout when compared with controls (Figure 5.1D). In contrast, PP larvae did not display differences between control and treatment conditions for VO_2min and VO_2rout.

Mean values for total length and depth at vent were lower for PP preflexion larvae for both the control and treatment conditions (Table 5.2, Figure 5.1E and F), while average values for head depth and eye diameter were similar across populations and treatments (Figure 5.1F). Although PP preflexion larvae exhibited little size-related difference between treatments, EP preflexion larvae from hypercapnic conditions were smaller than control counterparts (Figure 5.1E). However, despite observed differences in mean values (Table 5.2), population and treatment did not significantly affect any morphometric measurements for preflexion larvae (Table 5.4).

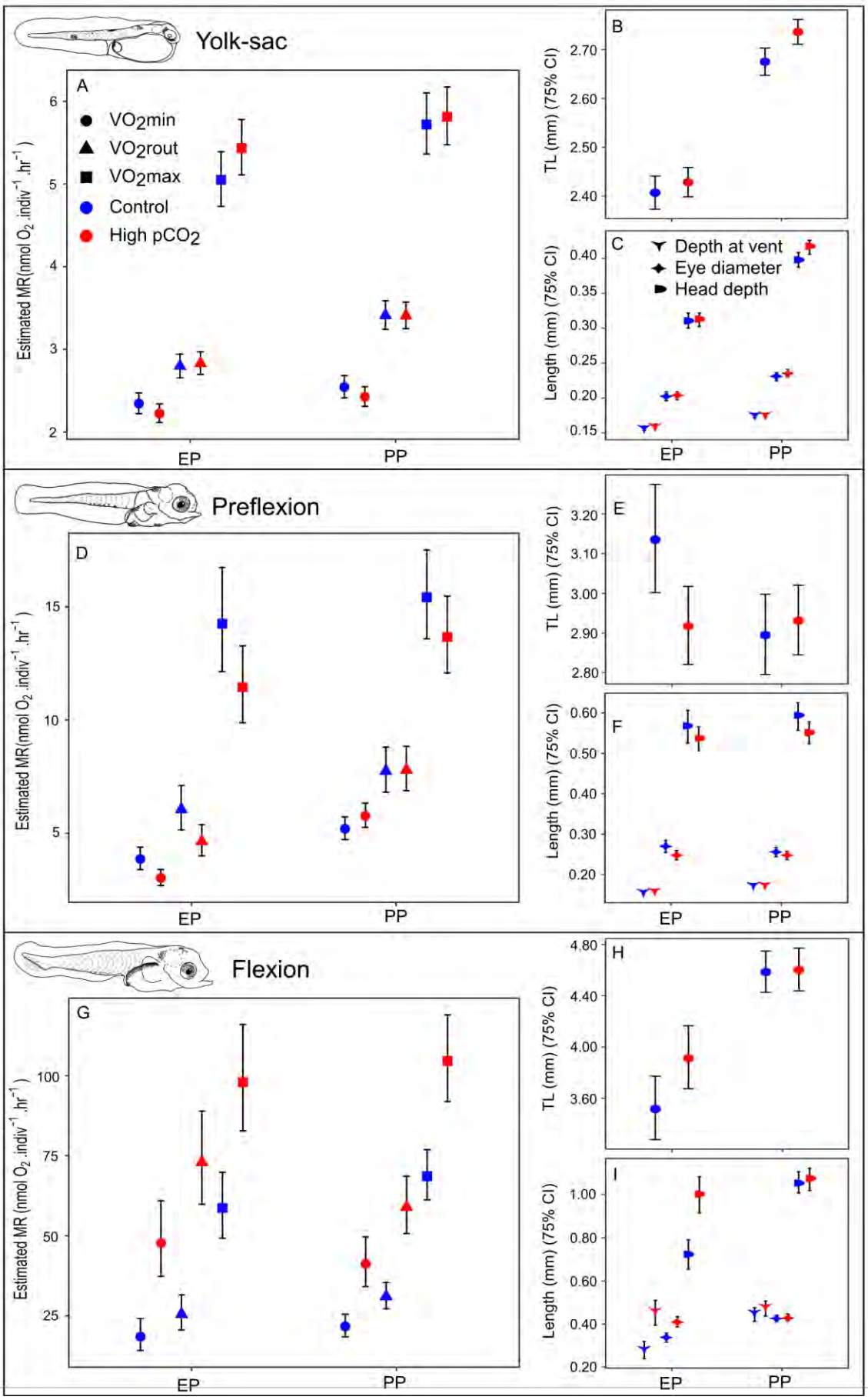

Yolk-sac
A
VO2min
VO2rout
VO2max
Control
High pCO2
Estimated MR(nmol O2 .indiv-1 .hr-1)
EP
PP
B
TL (mm) (75% CI)
C
Depth at vent
Eye diameter
Head depth
Length (mm) (75% CI)
Preflexion
D
E
F
Flexion
G
H
I

Figure 5. 1 Predicted metabolic rate and length measurements across two populations (EP – Exploited and PP – Protected) and two pCO_2 treatments (low pCO_2, control and high pCO_2, treatment) by developmental stage. Metabolic rates (VO_2min, VO_2rout, VO_2max), total length (TL) and length measurements (depth at vent, eye diameter and head depth) for yolk-sac (a, b, c), preflexion (d, e, f) and flexion (g, h, i) stages, respectively. Predicted estimates and 75% confidence intervals were obtained from LMM and back-transformed from logarithmic scale to obtain metabolic rates in nanomoles O_2. individual^{-1}. hour^{-1} and length measurements in millimetres

5.3.3 Flexion

The onset of flexion occurred at the same period after hatch for both populations and treatments, with approximately 50% of larvae undergoing flexion by 21 DAH. Larvae from both populations exhibited the same response to being reared in hypercapnic conditions, with elevated metabolic rates compared to their respective control larvae (Table 5.2). Treatment was a significant factor for VO_2min (P = 0.005), VO_2rout (P = 0.001) and VO_2max (P = 0.030) (Figure 5.1G, Table 5.3). However, the scale of the difference between control and high pCO_2 was not the same for both populations. Exploited population larvae from high pCO_2 conditions had average rates of VO_2min and VO_2max, which were 200% and 85% greater than controls. Protected population larvae exhibited a more modest difference, as high pCO_2 rates were 82% and 47% greater than control larvae for VO_2min and VO_2max, respectively. Despite this, there was no significant effect of population on the metabolic rates of flexion larvae (Figure 5.1G, Table 5.3).

Morphometric measurements of flexion larvae differed between populations and treatments. For total length, only population was a significant factor (P < 0.001), with PP larvae significantly larger than the EP population (Figure 5.1H, Table 5.2, Table 5.4). For depth at vent, head depth, and eye diameter treatment, population and the interaction between the two were significant factors (Table 5.4). Similar to total length, PP flexion larvae were significantly larger in-depth at vent (P = 0.002), head depth (P < 0.001) and eye diameter (P < 0.001) (Figure 5.1I, Table 5.2, Table 5.4). For the EP, high pCO_2 treatment larvae were significantly larger in total length, depth at vent and head, and eye diameter than their respective controls (Figure 5.1I, Table 5.2, Table 5.4). In comparison, the morphometric measurements of PP larvae in the control and treatment were not significantly different (Figure 5.1I, Table 5.2, and Table 5.4).

5.3.4 Absolute Aerobic scope (AAS)

Mean values for AAS increased by approximately 15-fold from yolk-sac through to flexion stages (Table 5.2) for larvae from both populations. Mean values for AAS of larvae from hypercapnic conditions were higher than control larvae during the yolk-sac and flexion stages but lower during preflexion stages for larvae from both populations (Figure 5.2).

However, there was no significant difference between populations or treatments at any developmental stages (Table 5.3).

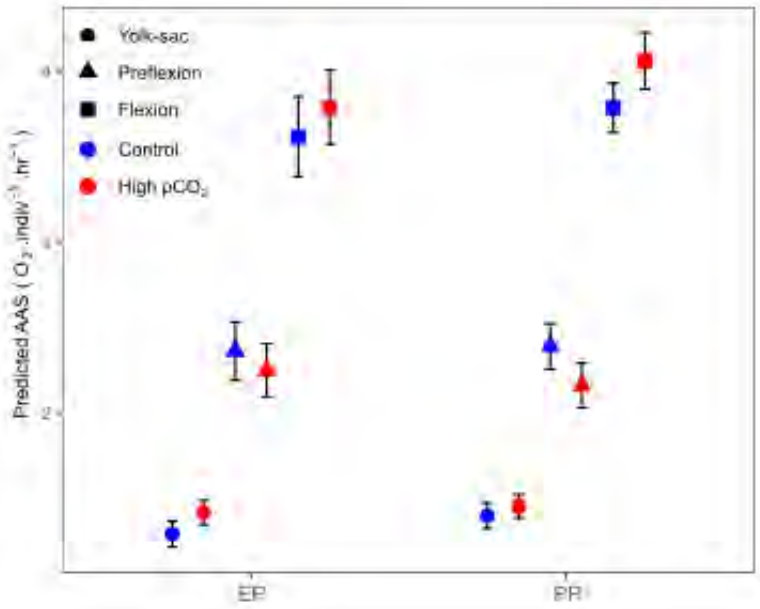


Figure 5. 2 Predicted log-transformed absolute aerobic scope (AAS) for *Chrysoblephus laticeps* larvae across two populations (EP – Exploited and PP – Protected) and two pCO_2 treatments (low pCO_2, control and high pCO_2, treatment) by developmental stage. Estimates and 75% confidence intervals back-transformed from LMM

Table 5. 3 Linear mixed-effects model (LMM) results from early developmental stages of *Chrysoblephus laticeps* from hatching, preflexion and at the onset of flexion. Zero level contrasts are Population: EP and Treatment: control (low pCO_2)

Stage	Metabolic rate	Effect	Estimate	Std. Error	t-Value	*P*
Yolk-sac	VO_2min	**Intercept**	**0.852**	**0.047**	**18.276**	**0.000**
		Population	0.082	0.065	1.253	0.212
		Treatment	-0.052	0.064	-0.812	0.418
		Population:Treatment	0.004	0.090	0.050	0.960
	VO_2rout	**Intercept**	**1.028**	**0.045**	**23.074**	**0.000**
		Population	**0.198**	**0.063**	**3.169**	**0.002**
		Treatment	0.012	0.061	0.196	0.845
		Population:Treatment	-0.013	0.086	-0.153	0.878
	VO_2max	**Intercept**	**1.619**	**0.057**	**28.502**	**0.000**
		Population	0.125	0.080	1.562	0.120
		Treatment	0.073	0.078	0.941	0.348
		Population:Treatment	-0.057	0.109	-0.524	0.601
	AAS	**Intercept**	**1.292**	**0.066**	**19.697**	**0.000**
		Population	0.107	0.092	1.166	0.245
		Treatment	0.126	0.090	1.396	0.165
		Population:Treatment	-0.070	0.126	-0.555	0.580
Preflexion	VO_2min	**Intercept**	**1.350**	**0.111**	**12.129**	**0.000**
		Population	**0.298**	**0.138**	**2.162**	**0.033**
		Treatment	-0.244	0.151	-1.617	0.121
		Population:Treatment	0.349	0.189	1.850	0.067
	VO_2rout	**Intercept**	**1.800**	**0.140**	**12.848**	**0.000**
		Population	0.246	0.153	1.606	0.111
		Treatment	-0.267	0.191	-1.400	0.182
		Population:Treatment	0.274	0.210	1.303	0.195
	VO_2max	**Intercept**	**2.657**	**0.140**	**19.009**	**0.000**
		Population	0.079	0.156	0.506	0.614
		Treatment	-0.219	0.190	-1.154	0.263
		Population:Treatment	0.098	0.214	0.460	0.646
	AAS	**Intercept**	**2.362**	**0.148**	**15.918**	**0.000**
		Population	0.027	0.168	0.162	0.872
		Treatment	-0.113	0.202	-0.559	0.583
		Population:Treatment	-0.115	0.230	-0.497	0.620
Flexion	VO_2min	**Intercept**	**2.920**	**0.230**	**12.714**	**0.000**
		Population	0.158	0.269	0.587	0.560
		Treatment	**0.945**	**0.313**	**3.021**	**0.005**
		Population:Treatment	-0.305	0.380	-0.803	0.427
	VO_2rout	**Intercept**	**3.239**	**0.185**	**17.513**	**0.000**
		Population	0.197	0.215	0.915	0.366
		Treatment	**1.050**	**0.253**	**4.154**	**0.001**
		Population:Treatment	-0.409	0.305	-1.344	0.189
	VO_2max	**Intercept**	**4.072**	**0.151**	**26.879**	**0.000**
		Population	0.156	0.168	0.927	0.360
		Treatment	0.512	0.211	2.431	0.030
		Population:Treatment	-0.091	0.243	-0.374	0.711
	AAS	**Intercept**	**3.615**	**0.204**	**17.734**	**0.000**
		Population	0.170	0.239	0.711	0.481
		Treatment	0.173	0.278	0.623	0.537
		Population:Treatment	0.101	0.337	0.300	0.766

Table 5. 4 Linear mixed-effects model (LMM) results of morphometric measurements from early developmental stages of *Chrysoblephus laticeps* at the yolk-sac, preflexion and flexion stages. Zero level contrasts are population: EP and Treatment: control (low pCO_2)

Stage	Growth metric	Effect	Estimate	Std. Error	*t-Value*	*p*
Yolk-sac	Total length	**Intercept**	**0.878**	**0.012**	**71.713**	**0.000**
		Population	**0.106**	**0.015**	**6.945**	**0.000**
		Treatment	0.009	0.016	0.545	0.587
		Population:Treatment	0.014	0.020	0.674	0.501
	Depth at vent	**Intercept**	**5.055**	**0.017**	**300.857**	**0.000**
		Population	**0.109**	**0.019**	**5.862**	**0.000**
		Treatment	0.019	0.023	0.836	0.415
		Population:Treatment	-0.016	0.025	-0.650	0.517
	Head depth	**Intercept**	**5.739**	**0.030**	**190.750**	**0.000**
		Population	**0.247**	**0.036**	**6.909**	**0.000**
		Treatment	0.003	0.040	0.077	0.939
		Population:Treatment	0.041	0.048	0.865	0.389
	Eye diameter	**Intercept**	**5.310**	**0.027**	**199.233**	**0.000**
		Population	**0.130**	**0.029**	**4.526**	**0.000**
		Treatment	0.004	0.036	0.110	0.913
		Population:Treatment	0.017	0.038	0.449	0.654
Preflexion	Total length	**Intercept**	**1.143**	**0.038**	**30.261**	**0.000**
		Population	-0.080	0.048	-1.645	0.106
		Treatment	-0.072	0.048	-1.504	0.139
		Population:Treatment	0.084	0.062	1.355	0.181
	Depth at vent	**Intercept**	**5.382**	**0.077**	**69.557**	**0.000**
		Population	-0.160	0.099	-1.616	0.112
		Treatment	-0.122	0.098	-1.245	0.218
		Population:Treatment	0.040	0.128	0.314	0.755
	Head depth	**Intercept**	**6.336**	**0.063**	**100.359**	**0.000**
		Population	0.045	0.081	0.552	0.583
		Treatment	-0.055	0.080	-0.683	0.498
		Population:Treatment	-0.016	0.104	-0.150	0.881
	Eye diameter	**Intercept**	**5.596**	**0.049**	**115.174**	**0.000**
		Population	-0.052	0.056	-0.925	0.359
		Treatment	-0.083	0.063	-1.320	0.214
		Population:Treatment	0.049	0.072	0.676	0.502
Flexion	Total length	**Intercept**	**1.257**	**0.061**	**20.589**	**0.000**
		Population	**0.265**	**0.068**	**3.888**	**0.000**
		Treatment	0.107	0.082	1.303	0.201
		Population:Treatment	-0.103	0.093	-1.110	0.275
	Depth at vent	**Intercept**	**5.623**	**0.126**	**44.782**	**0.000**
		Population	**0.469**	**0.140**	**3.338**	**0.002**
		Treatment	**0.483**	**0.168**	**2.865**	**0.007**
		Population:Treatment	**-0.422**	**0.191**	**-2.206**	**0.034**
	Head depth	**Intercept**	**6.578**	**0.082**	**80.158**	**0.000**
		Population	**0.384**	**0.092**	**4.190**	**0.000**
		Treatment	**0.325**	**0.110**	**2.952**	**0.006**
		Population:Treatment	**-0.312**	**0.125**	**-2.499**	**0.017**
	Eye diameter	**Intercept**	**5.817**	**0.055**	**104.814**	**0.000**
		Population	**0.236**	**0.059**	**3.997**	**0.000**
		Treatment	**0.198**	**0.075**	**2.619**	**0.015**
		Population:Treatment	**-0.187**	**0.082**	**-2.273**	**0.029**

5.4 Discussion

Larvae spawned from adults of the protected population (PP) typically displayed greater VO_2min, VO_2rout, VO_2max and AAS rates and were significantly larger at flexion than those spawned from adults of the exploited population (EP). In addition, while larvae from both populations were significantly affected by high pCO_2 conditions, EP larvae appeared to be more sensitive to high pCO_2, showing greater differences in metabolic rates and growth when compared with their control. At preflexion, treatment EP larvae exhibited lower average metabolic rates and size, while at flexion, they exhibited significantly higher metabolic rates and size when compared with control larvae. Conversely, treatment PP larvae did not exhibit size or growth-related differences when compared to their controls. While metabolic rates were significantly elevated at flexion, it was not on the same scale as EP larvae. Although these findings should be evaluated with caution due to the absence of additional trials from the same and other populations, the current results do suggest that offspring from a protected population appear to exhibit greater resilience to an environmental stressor when compared with offspring from an exploited population. Furthermore, the selectivity of passive fisheries alters the physiology of the adult fish populations and may also be transferred to the next generation (i.e. FIE). This may have deleterious consequences, particularly when larvae are subjected to environmental stressors during sensitive developmental stages.

The evidence of FIE for *C. laticeps* may not be too surprising given previous research conducted on adults. Comparisons between areas within and outside of MPA‘s have found that densities, mean size, age-at-maturity and age-at-sex-change of *C. laticeps* were all higher within the confines of protected area‘s (Buxton, 1993; Götz *et al.*, 2008). Due to the highly resident nature of the species, with post-recruit home ranges as little as 100m, MPA‘s provide an effective means of protection for *C. laticeps* (Kerwath *et al*., 2007), while the species provides an ideal model organism to examine the effects of FIE. The offspring used in this study came from populations experiencing starkly contrasting exploitation pressure; EP larvae came from adults in a region experiencing consistent fishing pressure over decades, which has led to declining catches in commercial and recreational fisheries (Kerwath *et al*., 2013; Mann, 2013). Conversely, PP larvae were offspring from the Tsitsikamma National Park, one of South Africa‘s oldest and largest no-take, marine protected areas (Brouwer *et al*., 2003; Cowley *et al*., 2010). Duncan *et al.* (2019b) found shifts in physiological phenotypes between these two protected and exploited populations with the EP being composed of fewer high-performance phenotypes compared with the PP. As the aerobic scope of PP adult *C. laticeps* was greater over a range of environmentally relevant extreme temperatures, it was suggested that *C. laticeps* from the PP would exhibit

greater resilience to increasing climate variability than those from the EP (Duncan *et al.*, 2019b).

Exploited population preflexion larvae reared in hypercapnic conditions displayed VO_2min rates 36% lower, along with 7% smaller size-at-age, relative to their controls (Figure 5.1D and E). These differences were indicative of metabolic and growth depression under environmental stress (Reipschläger and Pörtner, 1996; Guppy and Withers, 1999b; Baumann *et al.*, 2012; Gobler *et al.*, 2018) and suggested that EP preflexion larvae underwent moderate levels of physiological stress imposed by environmental hypercapnia. Although these larvae were, on average, larger than controls by the onset of flexion, this compensatory growth, which has been shown to occur after periods of growth depression, may be associated with growth abnormalities (Ali *et al.*, 2003). However, none were observed in this study. Conversely, PP larvae displayed no evidence of metabolic depression relative to their controls, and at no stage were there any notable morphometric differences. PP larvae reared in hypercapnic conditions throughout early development appeared to maintain growth rates similar to those of controls. However, by the onset of flexion, this required significantly elevated rates of VO_2.

The limited energetic budget of fish larvae must be partitioned among a range of functions. As mortality declines rapidly with increasing size (Peterson and Wroblewski, 1984; Sogard, 1997), growth during early development is vital. Available energy may be channelled away from some processes and reassigned towards maintaining high growth rates (Rombough, 1994; Wieser, 1995). Significant increases in the metabolic rates of larvae in hypercapnic conditions, particularly VO_2min, suggest substantial increases in their energetic requirements by the onset of flexion. This is consistent with elevated protein turnover and maintenance costs associated with metabolic compensation during exposure to moderate stress (Sokolova *et al.*, 2012). Maintenance of high growth rates during environmental stress may have been facilitated by increased hormone production or hyperphagia, but also potentially at the expense of normal organ development, possibly in an attempt to reduce size-dependent mortality (Ali *et al.*, 2003; Frommel *et al.*, 2012; Zeng *et al.*, 2017; Hardy *et al.*, 2021). The tolerances to environmental stress and variation are inevitably constrained by a species‘ physiology, as well as the severity of variation (Sokolova, 2013), which depend on genetic make-up and therefore may differ amongst populations (De Almeida-Val *et al.*, 2005; Hollins *et al.*, 2018; Petitjean *et al.*, 2019). Through the process of FIE, exploitation may exacerbate the effects of a changing climate by reducing phenotypic variability within exploited populations (Harley *et al.*, 2006; Hollins *et al.*, 2018) and constrain tolerance to environmental variation (Morrongiello *et al.*, 2019). The persistence of marine species with complex life histories requires successfully completing all ontogenetic stages (Byrne, 2012).

As early development, from embryo to metamorphosis, clearly characterises a series of bottlenecks to this persistence, maintaining physiological diversity may represent an important strategy for mitigating the effects of climate change.

Ocean acidification and the concomitant reduction in pH will challenge the physiological tolerance limits of many marine organisms (Doney *et al.*, 2008; Wittmann and Pörtner, 2013). Moreover, among these organisms, fish larvae have shown varying responses to environmental hypercapnia from none at all through to changes in resting metabolic rate, yolk utilisation, growth, behaviour and increased mortality (Cattano *et al.*, 2018; Munday *et al.*, 2019). However, ocean acidification is only one of the stressors faced by fish and their larvae in the Anthropocene. They will become increasingly challenged by rising sea temperatures, expansion of oxygen dead zones, altered circulation patterns, thermal variability and increasing frequencies and severity of extreme weather events (Diaz and Rosenberg, 2008; Hoegh-Guldberg and Bruno, 2010; Oliver *et al.*, 2018). Changing temperature regimes may impose the greatest threat to populations and their replenishment as temperature influences size at hatching, development rate, pelagic larval duration and survival (Blaxter, 1991; Pankhurst and Munday, 2011).

A lack of repeated experiments within other exploited and protected population pairs remains a shortcoming of this study. Moreover, comparisons between populations conducted in this study could have been improved by running experiments simultaneously; this, however, proved to be a logistic constraint. Subsequently, experiments were conducted one year apart, during the same peak spawning period and season. The quality of eggs and gametes, in general, can vary throughout the reproductive season and may be related to changes in, among other factors, temperature and diet (Bobe and Labbé, 2010). While it is difficult to determine the potential consequences of the dissimilar timing of experiments, it was promising to see that larvae from both populations reared in control (low pCO_2/normocapnic) conditions generally displayed similar metabolic and growth trajectories (see Chapter 6, Figure 6.1). One other caveat for our results is that larvae in this experiment had unlimited access to food, which could have offset energetic constraints, facilitated greater metabolic rates and growth, and reduced potential rates of mortality. Food limitation has been shown to exacerbate the effects of hypercapnia. This has reduced the survival rates of *M. beryllina* (inland silverside) (Gobler *et al.*, 2018) and the growth rates of *Dicentrarchus labrax* (European sea bass) (Cominassi *et al.*, 2020). It also leads to liver, pancreas, and kidney abnormalities in *Gadus morhua* (Atlantic cod) larvae (Stiasny *et al.*, 2019).

In conclusion, the lack of replication in and among study sites means that results need be interpreted with caution. Nevertheless, there was evidence for differences in population-level

responses to the stress of environmental hypercapnia during the early development of *C. laticeps,* suggesting that physiological traits from the parents may indeed be heritable. In general, EP larvae displayed greater sensitivity to the stressor than did PP larvae. In particular, although not significant, EP preflexion larvae appeared to undergo metabolic and growth depression, which later shifted toward compensatory levels at the onset of flexion. Accelerated or compensatory growth in response to a period of depression has been found to have adverse effects on future development, growth or swimming performance (Ali *et al.*, 2003), while developmental and organ abnormalities have been reported in the literature related to ocean acidification (Frommel *et al.*, 2012, 2014; Stiasny *et al.*, 2019). In contrast, PP larvae exhibited increased maintenance costs through physiological adjustments with no evidence of reduced metabolic or growth rates. Though both populations likely experienced important energetic allocation trade-offs, PP larvae displayed greater resilience toward the stressor. This may indicate a greater physiological diversity of offspring from a population comprised of individuals of relatively higher-performance metabolic phenotypes (Duncan *et al.*, 2019b).

Our findings demonstrate that early developmental stages exposed to hypercapnic conditions may not be doomed because of increased maintenance costs, as this was accompanied by increased VO_2max and thus maintained AS. However, ocean acidification may cause effects on multiple physiological systems, suggesting that physiological adjustments and subsequent pH compensation do not necessarily confer tolerance as downstream consequences and trade-offs occur (Heuer and Grosell, 2014). Additionally, the introduction of additional stressors and particularly an irregular food supply are likely to have substantial effects on metabolic rates as well as negatively impact physiological adaptive capacity (Zeng *et al.*, 2017; Boyd *et al.*, 2018; Lange *et al.*, 2018; Petitjean *et al.*, 2019). Evidence of FIE on the physiological attributes of fishes and its potential effect on resilience to climate change highlights the need for an evolutionary adaptive management approach to fisheries, which may be achieved through spatial protection (Hollins *et al.*, 2018). Protection from fishing maintains larger populations that conserve age structure and genetic diversity, enhancing population stability and the raw material necessary for adaptation (Roberts *et al.*, 2017).

Chapter 6

General discussion

Roman seabream *Chrysoblephus laticeps* before the onset of flexion, approximately 20 days after hatching

6.1 Overview

6.1.1 Synthesis of findings

This thesis presents the first research exploring the relationship between fisheries-induced evolution (FIE) and physiological stress on metabolism and growth for a marine sparid's early life history. This thesis also demonstrates and utilises a novel approach for estimating the minimum and maximum oxygen consumption rates (VO_2) of small marine fish larvae collected using static respirometry. Subsetting VO_2 datasets from individuals into behaviourally relevant time periods to estimate rates of minimum (VO_2min) and maximum (VO_2max) VO_2 associated with quiescent and active periods provided high estimates of repeatability or test-retest reliability. These metabolic rate estimates identified the physiological effects of exposure to hypercapnic (high pCO_2) conditions and suggested that larval *Chrysoblephus laticeps* from the exploited Noordhoek (NHK) population may be vulnerable to ocean acidification conditions expected by the year 2100.

Although newly hatched, yolk-sac larvae displayed no adverse effects associated with environmental hypercapnia, preflexion stages underwent a period of metabolic and growth depression relative to control larvae. While all metabolic rates were depressed, average VO_2min, in particular, was ~30% lower than control counterparts and length at age was ~7% smaller. By the flexion stage, metabolic rates and the length of larvae reared in hypercapnic conditions were considerably greater than controls. Average rates for VO_2min of hypercapnic larvae were ~200% greater than controls, and length at age was nearly 10% greater. Conversely, *C. laticeps* larvae from the unexploited Tsitsikamma National Park (TNP) MPA, reared in hypercapnic conditions, did not appear to undergo a metabolic or growth depression period at any time during the preflexion stage. They did exhibit increased metabolic rates at flexion, like those from the exploited population; however, VO_2min was far less elevated (~90%) than their respective controls. There was no difference in size between treatment and control flexion larvae.

These results provide two critical findings. Firstly, the physiological response of fish larvae to hypercapnic conditions varies by life stage. Results from short-duration studies that examine only one developmental stage may draw different conclusions when a series of stages are examined. Secondly, protected populations may prove invaluable for the maintenance of resilient fish populations in the coming decades. As fish larvae experience varying environmental conditions during their planktonic duration, those which prove most tolerant to variable conditions and resume feeding more readily are those which will likely fare better.

6.1.2 Using an energy-limited concept to explain environmental stress on larval physiology

Early studies into the relationship between metabolism and growth assumed an additive growth model during embryonic and larval stages, whereby metabolic rate related directly to specific growth rate based on findings from juveniles and adults (Smith, 1957; Fauconneau, 1984). Later studies found a lack of correlation between metabolism and growth for larval stages, suggesting that there may be a compensatory allocation of energy towards growth (Wieser *et al.*, 1988; Rombough, 1994; Wieser, 1995; Moyano *et al.*, 2018). Essentially, given the high rates of growth during early larval stages, up to 50% per day (Kamler and Kamler, 1992), and the changing costs of other metabolic activities, available energy may be suppressed from some processes and reassigned towards maintaining high growth rates (Rombough, 1994; Wieser, 1995).

More recent investigations into fish energetics have shifted toward understanding the effects of stressors on a limited energy budget (Sadoul and Vijayan, 2016; Anacleto *et al.*, 2018), though few studies have focussed on early life stages. As mortality declines rapidly with increasing size (Peterson and Wroblewski, 1984; Sogard, 1997), growth during early development is paramount and, as such, it may be prioritised even though somatic growth is energetically costly and will require a large fraction of the energy available (Pedersen, 1997). Under periods of stress, an energy-limited model of tolerance proposes that maintenance costs take priority over other processes, including growth, to ensure survival (Van Weerd and Komen, 1998; Sokolova, 2013). Therefore, when environmental conditions deviate from optimal, aerobic scope or energy above maintenance is reduced due to increases of the standard metabolic rate and associated costs of maintenance (Sokolova *et al.*, 2012; Sokolova, 2013).

Through increasing CO_2 partial pressures and the associated decline in pH, ocean acidification is anticipated to increase the energetic costs of ionoregulation, potentially leading to energetic reallocations away from other processes for marine organisms (Heuer and Grosell, 2016). However, as these regulatory mechanisms and the tissues which aid these processes, such as gills, have not yet fully developed in embryonic and larval stages, these early life stages are considered to be the most sensitive to CO_2 and pH irregularities (Pörtner and Peck, 2010; Hurst *et al.*, 2013). Indeed several studies have reported decreased growth, developmental abnormalities and ultimately mortality (Baumann *et al.*, 2012; Munday *et al.*, 2019; Stiasny *et al.*, 2019). Likewise, warming or thermal variation in aquatic systems is a significant environmental stressor, where eggs and larvae have been observed to have a narrower range of tolerable temperatures than other life stages (Rijnsdorp *et al.*, 2009). This sensitivity to increased and fluctuating temperatures may result

from limited oxygen supply due to rudimentary ventilation and circulatory systems (Pörtner and Farrell, 2008). As conditions deteriorate from optimum through moderate (pejus) and up to severe (pessimum), the aerobic scope is further reduced as maintenance costs increase, leading to reallocations of energy from storage, development, growth and activity (Sokolova, 2013).

Sokolova (2013) used an energy-limited framework to assess the tolerance of an organism to an environmental stressor. While this may provide a valuable tool for understanding the physiological and growth results found in this study, the framework may require modification for the early life stages. In particular, larval marine fishes where pelagic eggs and larvae are exceedingly small, poorly developed at hatching and have limited endogenous resource supplies (Figure 6.1 modified from Sokolova (2013)). The primary modification to the concept is that of an energetic compensatory budget (Figure 6.1). Mass-specific metabolic rates of larvae are high, and subsequently, the aerobic scope is so low that energy-consuming functions such as growth, activity and storage cannot always be accommodated over and above maintenance due to a limited energy budget (Wieser *et al.*, 1988; Rombough, 1994; Ali *et al.*, 2003; Smith and Ottema, 2006). The available energetic budget is therefore below its total requirements. The second modification is the absence of anaerobic contributions (Figure 6.1) to the energetic budget, as the metabolism of larval stages is almost entirely aerobic (Wieser, 1991). The third and final modification is physiological adjustment or acclimation to an environmental stressor and energetic or metabolic adjustments to maintain aerobic scope (Figure 6.1). This physiological adjustment can be applied through plasticity or acclimation (i.e. phenotypic adjustment) to tolerable but otherwise adverse conditions. Examples include protein and enzyme upregulation (gene expression), mitochondrial and ionocyte densities or gill surface area (Huey and Bennett, 1990; Deigweiher *et al.*, 2008; Horng *et al.*, 2009; Li *et al.*, 2015), which may occur over prolonged exposure times.

Maintaining high growth rates is critical for the survival of planktonic marine fish larvae (Peterson and Wroblewski, 1984; Johnson *et al.*, 2014). As such, Peck and Moyano (2016) suggested that, because of the intrinsic value of growth, it may be impossible to separate growth costs from costs of maintenance when estimating standard or maintenance metabolic rates of fish larvae. Nevertheless, metabolic rates (most importantly SMR) and length measurements (as proxies for growth) are useful indicators when evaluating stress responsiveness (Sopinka *et al.*, 2016), mainly when used on a comparative physiological basis (Wikelski and Cooke, 2006). Sokolova's (2013) energy-limited model posits that reductions to organismal aerobic scope reflect a progressive decline in condition associated with increasing levels of environmental stress. However, reductions can be alleviated by

adaptation or acclimatisation. For example, short-term or acute exposure to hypercapnic conditions has been shown to reduce the aerobic scope (Munday *et al.*, 2009), typically through increased SMR, but prolonged exposure may result in increased MMR and thus maintained AS (Lefevre, 2019).

Adaptation to hypercapnic conditions may be related to changes in gill morphology (Goss *et al.*, 1998) or, alternatively, in the expression of proteins (bicarbonate transporters) and enzymes (NA^+-K^+-ATPase) (Deigweiher *et al.*, 2008) that are responsible for the maintenance of internal pH levels. In the current study, there were no significant differences in aerobic scope between control and high pCO_2 reared larvae but significant differences in metabolic rates at the onset of flexion, particularly in VO_2 min, indicating a substantial increase in energetic requirements associated with hypercapnic conditions. This is consistent with elevated protein turnover and maintenance costs associated with metabolic compensation during exposure to moderate stress (Sokolova *et al.*, 2012). Surprisingly, physiological adjustments (possibly by gene expression, enzyme activities and increased ionocyte density (Deigweiher *et al.*, 2008; Horng *et al.*, 2009)) by the onset of flexion allowed for a new metabolic ‗steady state' under hypercapnic conditions, as illustrated in Figure 6.1 condition B2, and therefore maintenance of AS. If a new steady state was achieved by flexion, then the necessary transient period was demonstrated by preflexion larvae where hypercapnic conditions resulted in a non-significant though comparatively reduced AS (Figure 6.1 condition B1 or C). Evidence for differing responses by population to hypercapnia was most apparent in the VO_2min of preflexion larvae (Figure 6.2, Table A.3). Larvae from the NHK exploited population underwent a period of metabolic depression associated with growth depression. This is represented in Figure 6.1 condition D and suggests that NHK EP larvae were more severely affected by hypercapnic stress during preflexion than were TNP PP larvae. In contrast, the reduced AS of PP preflexion larvae reared under hypercapnic conditions was constrained by an increased VO_2min but otherwise similar VO_2max with respect to control counterparts (Figure 6.1 conditions B1). Aerobic scope was therefore constrained by increased maintenance costs yet no physiological adjustment capacity to increase VO_2max. Notably, PP preflexion larvae from hypercapnic conditions did not show any size difference compared to corresponding control larvae, suggesting growth rates were either unaffected by the stressor or, more likely, compensatory allocations may have already been taking place. By the onset of flexion, metabolic rates of PP hypercapnic reared larvae were significantly greater than controls, yet AS was not significantly different. Additionally, length differences between treatments were negligible, indicating that physiological adjustments or phenotypic plasticity modified energetic budgets and maintained normal growth and development rates.

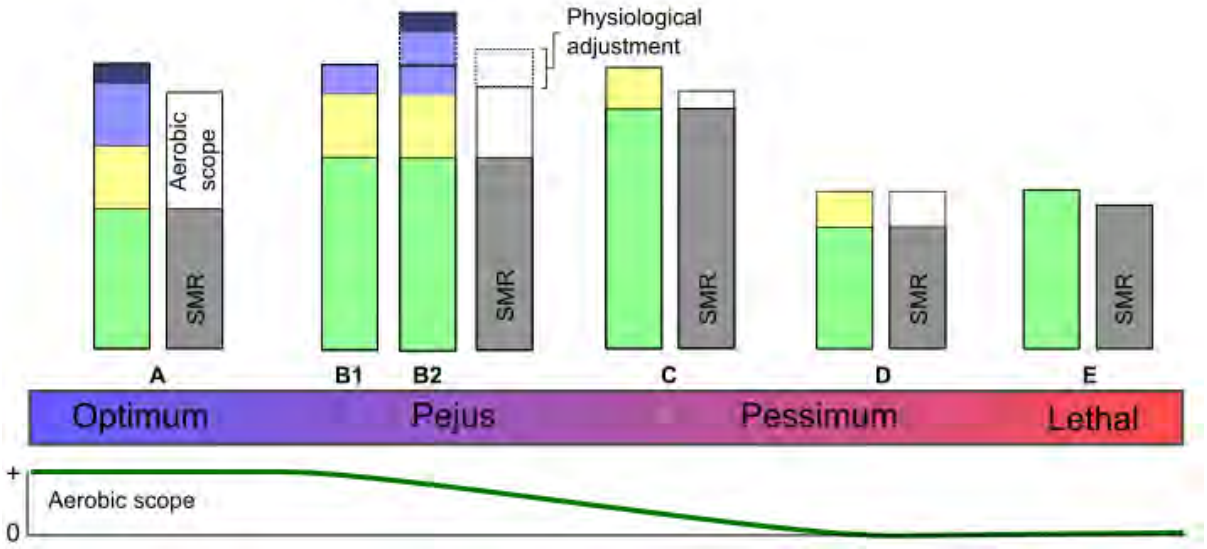


Figure 6. 1 Concept of energy-limited tolerance for assessing the effects of stressors. Sokolova (2013) is modified for early-developmental stages where anaerobic capacity is limited, and energy partitioning follows compensatory allocation. Energy demanding functions are shown on the left. They are composed of (from bottom to top): maintenance – which includes an inherent but limited growth factor (green), activity (yellow), growth and development (blue), storage (dark blue). These energetic costs are traded off, i.e. they are not additive. Aerobic metabolism is shown in the adjacent bar, the energy available above maintenance as aerobic scope (white) may be reduced with increasing severity of stressor/s. Anaerobic metabolism is not included due to the limited scope for anaerobiosis during early development. Additions to aerobic metabolism (physiological adjustment) are provided by phenotypic plasticity or compensatory mechanisms where a transition from B1 to B2 includes a period of exposure sufficient to induce acclimation

The onset of flexion has been identified as an energetically taxing period which may represent a critical bottleneck to survival (Edworthy *et al.*, 2018; Joly *et al.*, 2021). However, current results suggest that under environmental hypercapnia (pCO_2 ≈ 1600 µatm), a bottleneck to survival may begin at preflexion and be exacerbated at flexion. Environmental hypercapnia is expected to lead to acidosis (high blood pCO_2). The implication is that acidosis must be regulated by bicarbonate buffering and active ion transport at an increased energetic cost. Otherwise, it is expected to result in metabolic depression with a reduction in growth, survival and negative effects on development (Pörtner *et al.*, 2004; Munday *et al.*, 2009; Pimentel *et al.*, 2020). The dissimilar response of the two populations reflected these varying consequences as preflexion larvae from the PP increased VO_2min by ~14% relative to controls, while EP larvae decreased VO_2min by more than 35%. The PP larvae‘s ability to avoid metabolic and growth depression may be related to their increased size at hatch. This meant that they were able to increase feeding rates at the onset of exogenous feeding. More likely, it may relate to the PP larvae‘s inheritance of a relatively greater phenotypic diversity

and, therefore, increased resilience to environmental stress (Morrongiello *et al.*, 2019). The latter may be more likely because EP larvae at preflexion were of a similar average size to PP larvae and that the PP has already been shown to be composed of relatively more high-performance adults (Duncan *et al.*, 2019b).

Increased metabolic rates of flexion stage larvae are undoubtedly a consequence of the cost as mentioned above of ion-regulation, but high rates of feeding naturally facilitate this. Under food deprivation or irregularities, which would occur naturally, recovery from metabolic depression would be increasingly compromised, as would the maintenance of high metabolic

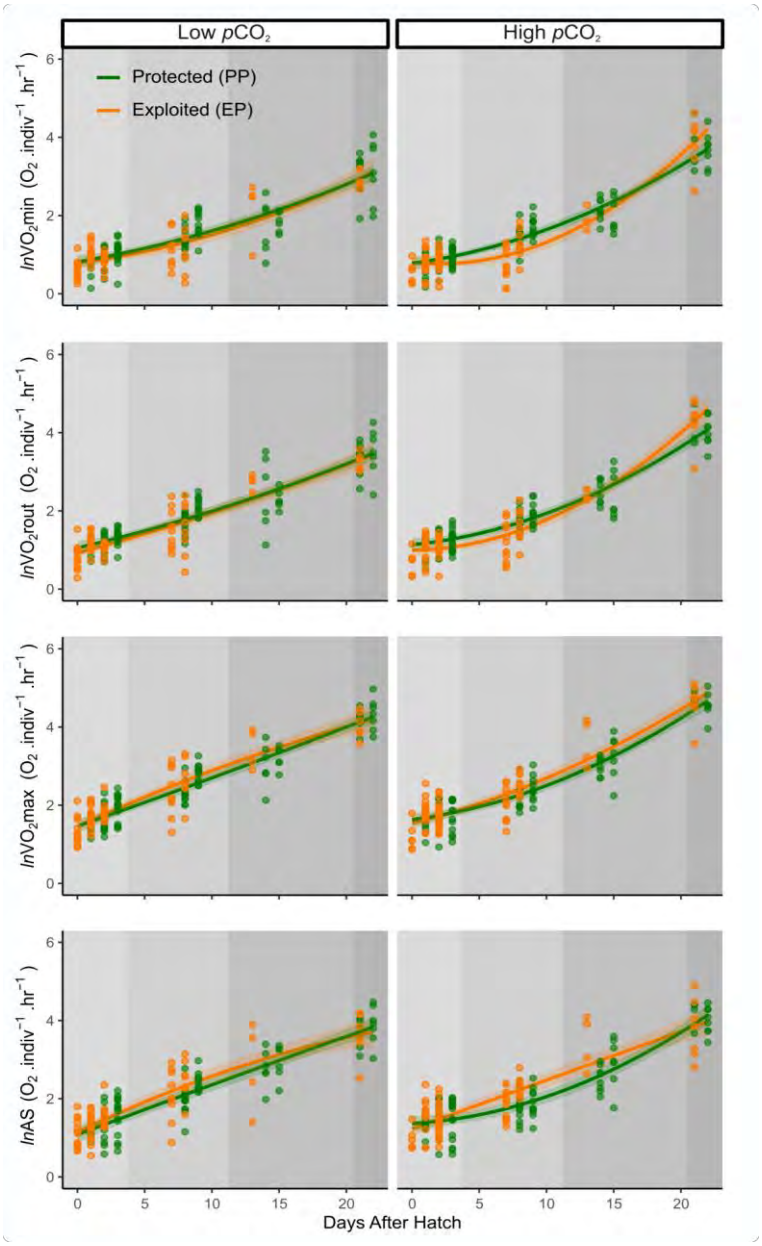


Figure 6. 2 Interaction plots for early-stage *Chrysoblephus laticeps* from TNP, protected (PP - green) and NHK, exploited (EP - orange) populations, comparing controls/low pCO_2 (left) and treatments/high pCO_2 exposure (right). Shaded area indicates from lightest to darkest: Yolk-sac, Early preflexion, Late preflexion and flexion

rates. Given these findings, the ability to withstand given environmental stress does not just appear to be species and ontogenetic stage-specific (Sokolova, 2013; Vagner *et al.*, 2019) but also, with regards to exploitation history, population-specific.

6.3 Caveats of the study

Egg quality is typically defined as the ability of an egg to be fertilised and develop into a normal embryo (Bobe and Labbé, 2010). However, egg quality can also affect the development and survival of embryos and early larvae (Bobe, 2015). The condition of eggs is affected by extrinsic and intrinsic factors. Quality can vary throughout the spawning period, which is particularly evident for batch spawners, like *C. laticeps*. Repeated spawning and rearing of *C. laticeps* was not possible due to practical and logistical constraints. The lack of expertise in rearing larval sparids created a substantial boundary to overcome before progressing with experimental trials.

Environmental conditions, feeding conditions, and associated egg quality vary across space and time, which adds uncertainty to comparisons between populations. However, in the case of batch spawning, the fact that ovaries contain eggs at several stages of development (Buxton, 1990; Bourque and Phelps, 2007) indicates that the standardized artificial stimulation of ovulation in both populations (using Aquaspawn) likely ensured that the eggs were similarly developed. Nevertheless, replication is necessary for spawning or sampling events and treatments to reduce the uncertainty around variable egg quality when conducting comparative research. As tank replicates were from the same experimental unit, statistical independence issues must be considered. A lack of replication at the population level invites the potential for random variation or error and reduces the precision of estimates (Hurlbert, 1984; Quinn and Keough, 2002). Likewise, reduced precision in estimates may be introduced with inappropriate methodological practices. Mixing devices within static respirometry chambers may have altered oxygen consumption measurements (Rodgers *et al.*, 2016) just as changes in measurement techniques, approaches, and data analysis can lead to significantly different estimates of metabolic rates (Chabot *et al.*, 2016).

Similarly, the practicality of an experiment must also be considered and focusing on a clear experimental response, such as a substantial physiological stressor, to reduce background variation assists low replication in studies (Oksanen, 2001). In opposition to Hurlbert's (1984) argument, Oksanen (2001) notes that appropriate inferential statistics should accompany repeated experiments to support patterns in results, although limitations must be acknowledged. Measuring the mass of larvae would have provided mass-specific metabolic rates, providing more robust results and greater effort should be placed in generating these outputs in future work. It is noteworthy and should support findings that differences between

population controls were minor, while on the other hand, differences between population treatments were considerable. Nevertheless, while extensive effort was made to avoid random variation amongst treatments and populations, the potential for ~~n~~ondemonic" intrusion always exists.

6.4 Recommendations for future work

Climate change simultaneously affects many environmental factors, which affect various processes at different levels of biological organisation (Harley *et al.*, 2006; Lehodey *et al.*, 2006). For example, temperature strongly regulates reproductive processes at the individual level, from gamete development and maturation, ovulation and spermiation, spawning, embryogenesis and hatching, to larval and juvenile development and survival (Pankhurst and Munday, 2011; Pankhurst, 2016). Comparatively, at the ecosystem level, temperature regulates population distributions, species interactions and food availability (Brierley and Kingsford, 2009). While temperature was considered a potential stressor for this study, our limited knowledge of the temperature tolerance of the larvae of this species precluded us from attempting to understand the impact of FIE on the thermal tolerance of larvae. Since there was clear evidence that exploitation impacted the metabolic response of adult *C. laticeps* to changing temperatures (Duncan *et al.*, 2019b), changing thermal conditions will likely have a contrasting impact on the physiological response of larvae from the EP and PPs. This represents a critical research gap. Resolving the effects of multiple climate change impacts will be exceedingly complicated, with multiple stressors having unpredictable effects compared with single stressors (Piggott *et al.*, 2015; Nagelkerken and Munday, 2016; Lange *et al.*, 2018). Despite the unpredictability, it does appear that multiple abiotic stressors more commonly result in synergistic rather than additive interactions, and this is likely to have negative impacts on larvae, which are typically more sensitive than embryos or juveniles (Przeslawski *et al.*, 2015). For example, *Menidia menidia* (Atlantic silverside), reared in hypercapnic conditions under varying temperature treatments, were significantly smaller at 17°C and 28°C than their normocapnic controls but showed no difference with controls at 24°C (Murrayid and Baumann, 2020). Furthermore, food limitation has been shown to exacerbate the effects of temperature and/or hypercapnia combinations, significantly reducing survival rates of *M. beryllina* (inland silverside) (Gobler *et al.*, 2018) and growth rates of *Dicentrarchus labrax* (European sea bass) (Cominassi *et al.*, 2020). In another study, Stiasny *et al*. (2019) found that *Gadus morhua* (Atlantic cod) larvae which were reared in hypercapnic, food-limited conditions, showed greater liver, pancreas, and kidney impairment rates than those reared in either normocapnic or high-food environments.

Adaptation or acclimation to multiple stressors may prove to be considerably more challenging than exposure to single-stressors (Gobler *et al.*, 2018). There is an urgent need to assess the impact of multiple stressors at differing levels of food availability on ecologically and economically important species with a focus on early developmental stages (Vagner *et al.*, 2019). Two divergent approaches would provide valuable insight. On the one hand, mesocosm experiments may add valuable information as they provide insight into overall community effects in response to ocean change (Baumann, 2019). Alternately, investigations of additional stress response indicators such as heat shock proteins, immediate early genes, metabolites, ion concentrations, bioenergetics, condition indices and reproduction and fitness will provide physiological insight into stress exposures from acute to prolonged exposure durations, respectively, and will aid in determining tolerance and adaptive capacity to various combinations of stressors (Sopinka *et al.*, 2016).

Evidence for interactions between exploitation and climate sensitivity, such as by Duncan *et al.*(2019b) and Morrongiello *et al.* (2019), are concerning and require further investigation. However, they are focused on changes to populations by the removal of more resilient phenotypes. Findings from this thesis suggest that there may be progenitor effects on populations through the heritability of resilient characteristics. Further research should be aimed at quantifying the heritability of metabolic or physiological phenotypes, and variation among offspring, particularly as self-recruitment or larval retention may rapidly accelerate climate sensitivity. On that note, the quantification of larval dispersal and recruitment dynamics should be explored further and increasingly used as a resource by marine spatial planners and resource managers. If larval dispersal is low or encounters consecutively poor recruitment seasons, as may occur during the La Nina southern oscillation with increased upwelling frequencies (Duncan *et al.*, 2019a), MPA‘s may become increasingly important as resources for genetic variability.

6.5 Conclusion

Increased pCO_2 did present a significant environmental stress to early-stage *Chrysoblephus laticeps*, which appeared to necessitate physiological adjustments to maintain normal rates of growth and (most likely) survival. It is pertinent to note that these adjustments are not ~~fr~~ee”. They require significant energetic allocations, which may at the very least be facilitated by increased feeding (hyperphagia), which may not be sustainable in a natural setting and may also increase foraging-related mortality (Holt and Jørgensen, 2015). On the opposite end, they may be associated with shifting from an anabolic to a catabolic state. If normal feeding is not resumed, the physical condition will deteriorate and result in mortality (Sopinka *et al*., 2016).

Altogether, this thesis found evidence that *C. laticeps* larvae from two populations with different levels of exploitation exhibited dissimilar sensitivities to environmental hypercapnia. By not displaying metabolic depression or growth dissimilarities relative to control counterparts, PP larvae displayed greater resilience to the environmental stressor than EP larvae. Several confounding factors may lead to the results obtained in this study, including differing periods of gamete collection. Nevertheless, these populations have already been shown to differ in their distribution of metabolic phenotypes, with the protected population composed of a greater proportion of high-performance individuals than the exploited population, owing to fisheries selection (Duncan *et al.*, 2019b). Furthermore, metabolism is a heritable trait (Burton *et al.*, 2011; Crespel *et al.*, 2011), with fitness and adaptive implications on offspring (Long *et al.*, 2021). When these findings are considered together, it appears that fisheries selection for a heritable trait may form the foundation of the fisheries-induced evolution concept (Biro et al., 2008; Heino et al., 2015; Hollins *et al.*, 2018). As such, it is reasonable to suggest that the underlying contributor to the differing physiological sensitivities to the environmental stressor examined here is due to the inheritance of differing physiological attributes or, in essence, fisheries-induced evolution. The trajectory and variability of marine populations are profoundly affected by exploitation and climate change (Harley *et al.*, 2006). The effects of these two drivers have, however, traditionally been considered in isolation or when in concert at the population or ecosystem level (e.g. Perry *et al.* (2010); Planque *et al.* (2010); Waples and Audzijonyte, (2016) and Hollins *et al.* (2018), but see Duncan *et al.* (2019b) and Morrongiello *et al.* (2019)).

This thesis provides the first evidence that exploitation can impact the climate resilience of offspring from fished populations by removing high-performance progenitors. This provides further support to the notion that the removal of high-performance individuals by fishing can erode the adaptive capacity of a population to deal with climate change by selecting against those phenotypes that can adapt to increasing climate variability. The loss of these high-performing individuals, with subsequent impact on progeny, may well be removing important buffers against the impact of climate variability and change and highlighting the critical importance of no-take MPA‘s in mitigating future uncertainties.

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Appendix

Table A. 1 ANOVA results from Linear mixed effects model (LMM) for VO_2min, VO_2rout, VO_2max and absolute aerobic scope (AAS) of larval *Chrysoblephus laticeps* comparing the effects of Developmental stage, Trial and their interaction on VO_2 rates for triplicate observations only

	Fixed Effect	F	Df	P-value
VO_2min	Development	11.729	1,14	0.004
	Trial	0.351	2,28	0.273
	Development:Trial	0.172	2,28	0.520
VO_2rout	Development	25.272	1,14	<0.001
	Trial	0.929	2,28	0.407
	Development:Trial	2.575	2,28	0.094
VO_2max	Development	68.992	1,14	<0.001
	Trial	1.217	2,28	0.311
	Development:Trial	0.826	2,28	0.448
AAS	Development	70.796	1,14	<0.001
	Trial	0.872	2,28	0.429
	Development:Trial	1.009	2,28	0.377

Table A. 2 Life stage grouped mean (±SD) rates of VO_2 per trial measured for *Chrysoblephus laticeps* in nanomole O_2. Individual^{-1}. Hour^{-1}

Trial	Lifestage	n	Mean ± SD VO_2min	VO_2rout	VO_2max
1	Early Preflexion	6	1.64 0.47	3.15 1.36	6.79 2.72
2			1.69 0.64	2.35 0.62	8.05 3.27
1	Late Preflexion	10	8.87 4.01	13.71 4.16	22.02 4.58
2			6.58 2.58	9.66 3.25	16.42 2.55
3			6.72 2.06	9.01 2.39	15.26 4.34
1	Flexion	7	21.95 13.23	31.92 15.48	73.32 22.48
2			24.29 11.03	34.32 16.52	65.87 26.21
3			20.57 17.85	36.93 24.72	75.52 4941

Table A. 3 Results of linear mixed effects models (LMM) on the metabolic rate estimates across early development for *Chrysoblephus laticeps* from both treatment (low and high pCO_2 exposure) and population (exploited and protected) crosses. Development is presented as linear (DAH) and quadratic (DAH^2) function, with treatment and population included as interaction term. Zero level for contrasts was as follows: Population exploited and Treatment control/low pCO_2. Significant values in bold

Metabolic rate	Effect	Estimate	Std. Error	t Value	p-Value
VO_2min	**Intercept**	**1.368**	**0.051**	**26.932**	**0**
	Population	0.057	0.066	0.864	0.389
	Treatment	0.017	0.069	0.25	0.803
	DAH	**12.812**	**0.99**	**12.946**	**0**
	DAH2	**2.196**	**0.905**	**2.425**	**0.016**
	Population:Treatment	0.044	0.09	0.493	0.623
	Population:DAH	-0.297	1.225	-0.242	0.809
	Popuation:DAH2	-0.689	1.17	-0.589	0.556
	Treatment:DAH	**5.22**	**1.36**	**3.839**	**0**
	Treatment:DAH2	**4.334**	**1.266**	**3.424**	**0.001**
	Pop:Treat:DAH	-1.766	1.703	-1.037	0.301
	Pop:Treat:DAH2	-2.895	1.641	-1.764	0.079
VO_2rout	**Intercept**	**1.674**	**0.052**	**32.462**	**0**
	Population	0.078	0.063	1.245	0.214
	Treatment	0.054	0.07	0.773	0.447
	DAH	**14.083**	**0.942**	**14.957**	**0**
	DAH2	0.759	0.866	0.877	0.381
	Population:Treatment	0.011	0.086	0.124	0.902
	Population:DAH	-0.584	1.165	-0.501	0.617
	Popuation:DAH2	0.25	1.121	0.223	0.824
	Treatment:DAH	**5.376**	**1.296**	**4.148**	**0**
	Treatment:DAH2	**4.722**	**1.208**	**3.909**	**0**
	Pop:Treat:DAH	-2.844	1.626	-1.749	0.081
	Pop:Treat:DAH2	-2.315	1.566	-1.478	0.14
VO_2max	**Intercept**	**2.429**	**0.057**	**42.774**	**0**
	Population	-0.083	0.062	-1.331	0.184
	Treatment	0.006	0.078	0.073	0.943
	DAH	**15.649**	**0.929**	**16.851**	**0**
	DAH2	-1.15	0.858	-1.34	0.181
	Population:Treatment	0.007	0.085	0.077	0.939
	Population:DAH	0.113	1.147	0.098	0.922
	Popuation:DAH2	1.368	1.112	1.23	0.219
	Treatment:DAH	**2.729**	**1.281**	**2.131**	**0.034**
	Treatment:DAH2	**3.443**	**1.195**	**2.882**	**0.004**
	Pop:Treat:DAH	-1.92	1.609	-1.194	0.233
	Pop:Treat:DAH2	-0.423	1.549	-0.273	0.785
Aerobic scope	**Intercept**	**2.099**	**0.071**	**29.543**	**0**
	Population	-0.12	0.074	-1.628	0.104

	Treatment	0.012	0.098	0.119	0.907
	DAH	**14.793**	**1.099**	**13.458**	**0**
	DAH2	-1.739	1.017	-1.711	0.088
	Population:Treatment	-0.016	0.101	-0.162	0.871
	Population:DAH	0.74	1.357	0.545	0.586
	Popuation:DAH2	1.598	1.319	1.212	0.226
	Treatment:DAH	0.695	1.516	0.458	0.647
	Treatment:DAH2	1.723	1.415	1.218	0.224
	Pop:Treat:DAH	-1.223	1.906	-0.641	0.522
	Pop:Treat:DAH2	2.046	1.835	1.115	0.266