The effect of cross-generational plasticity on the development of coral reef fish under multiple environmental stressors

Thesis submitted by

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Statement of contribution of others

This thesis includes collaborative work with my supervisors Dr. Jennifer Donelson and Prof. Andrew Hoey, as well as Dr. Shannon McMahon and Yogi Yasutake. I was responsible for project design, animal husbandry, performed the experiments, data collection, analysis and interpretation of results, and writing. Dr. Jennifer Donelson provided creative and intellectual guidance on all aspects of the thesis, project design, experiment technical support, data analysis assistance, considerable editorial guidance, and financial support. Prof. Andrew Hoey and Dr. Shannon McMahon provided intellectual guidance on the project and experimental design, and editorial guidance. Yogi Yasutake performed the behaviour video experiments in **Chapter 2**, and temporarily supported with animal husbandry.

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General abstract

Marine ecosystems are being increasingly impacted by global and localised anthropogenic activities. Foremost among these activities is the emission of greenhouse gases into the atmosphere that has resulted in both ocean warming and ocean acidification, with further increases in ocean temperatures of 1.42 to 3.47°C and pH reductions of 0.16 to 0.44 units projected by the end of the century. In addition, localised anthropogenic stressors such as agricultural, urban, and coastal development, catchment modification, and dredging, are increasing the input and resuspension of sediments into coastal marine systems. These global and local environmental stressors can occur simultaneously, sequentially within a lifetime, or in succession over multiple generations, making the response to future environmental conditions difficult to predict. A shift in environmental conditions beyond those that species have historically experienced, can cause a range of impacts on population dynamics and ecosystem health.

Environmental conditions experienced by current and previous generations can induce phenotypic plasticity in physiological, morphological, and behavioural attributes. Research to date shows that both within- and cross-generational thermal plasticity can result in improved thermal performance, however the response of individuals when exposed to additional environmental stressors is poorly understood. We also know that the timing of thermal experience in past generations can influence the phenotypic change observed in the current generation. Using a multi-generational crossed design, this thesis investigates how experience of +1.5°C warming at various timings (during development and post-maturation) in the two previous generations, influences the development and performance of juvenile *Acanthochromis polyacanthus* under multiple environmental stressors. This research focuses on environmental change during early life stages as they are generally more sensitive to abiotic changes, but they also have a greater potential to produce plasticity due to epigenetic sensitivity.

In **Chapter 2**, I examine how exposure to warm temperatures (i.e., +1.5°C above present-day average), seasonally and diurnally cycling, in the grandparent (F_1) and parent (F_2) generations influences the sensitivity of F_3 juveniles to warm temperature (summer Control: 28.5°C; Warm: 30°C) and CO_2 (Control: 490 µatm; Elevated: 825 µatm). Juvenile F_3 *A. polyacanthus* were maintained in these orthogonally crossed environmental conditions for 16 weeks post-hatching, after which their aerobic physiology (resting oxygen consumption, maximum oxygen consumption, and absolute aerobic scope), behaviour (boldness and activity), and growth (standard length and physical condition) were measured. I found that the exposure to warm temperatures in the parental or grandparental generations produced juveniles that were longer and in better condition irrespective of juvenile F_3 developmental temperatures (carry-over effect), and this did not alter with exposure to elevated CO_2, a stressor to which they hadn't been previously exposed. Overall, the developmental environment of F_3 juvenile *A. polyacanthus* had the greatest effect on performance, with warm temperature resulting in shorter and bolder fish that were in better physical condition, and elevated CO_2 resulted in shorter fish with a greater resting metabolic rate. The observed carry-over effects and additive nature of juvenile temperature and CO_2 conditions indicates that multiple stressor and generational responses could be informed by single stressor experiments under scenarios of projected future climate change.

In **Chapter 3**, I explore juvenile performance under ocean warming and elevated suspended sediment concentrations. Specifically, I tested whether warm temperature (+1.5°C above present-day average conditions) experienced during parental (F_2) or grandparental (F_1) development, compared to present-day control, influences the sensitivity of F_3 juveniles to warm temperature (summer Control: 28.5°C; Warm: 30°C) and elevated suspended sediment (No sediment: 0 mg L^{-1}; Suspended sediment: 50 mg L^{-1}) independently and combined. Juvenile F_3 *A. polyacanthus* were reared in these four environmental conditions for 11 weeks

(53-81 days post-hatch; dph) before a sub-sample was taken from each treatment and body morphology (standard length, and physical condition), gill morphology (lamellae length, width and perimeter, and filament width), and the presence of gill remodelling (epithelial lifting, hyperplasia, mucus, aneurysms, and lamellae fusion) was quantified. All sediment was then removed from the aquaria system and the fish maintained for additional 5 weeks under their initial temperature treatments. Body morphology metrics were again taken from a sub-sample 16 to 17 days following sediment removal (74-102 dph), and from the remaining juveniles after a further 17 to 20 days (34 to 37 total day post-removal; 92-122 dph). Overall, I found that juvenile fish exposed to both warm temperature and suspended sediment had significant gill remodelling and were shorter in body length but in better physical condition. Juvenile performance traits were also influenced by the thermal experience of both previous generations, and the F_3 juvenile environment. At 92 to 122 dph, In comparison to juveniles from the control and grandparental development cross-generation, juveniles from the parental development cross-generation did not undergo extensive gill remodelling under Warm temperature and Suspended sediment combined but they did have more mucus. Approximately 2 -5 weeks after sediment removal the significant effects of suspended sediment on body morphology were no longer present indicating that juvenile *A. polyacanthus* can recover following a short-term sediment event.

This thesis has provided a unique investigation into how environmental change sequentially across generations can affect phenotypic outcomes. Developmental exposure to ocean warming enhanced physical condition bold activities, and aerobic scope (**Chapter 2**), and influenced gill morphology (**Chapter 3**). The effect of cross-generational thermal exposure was more complex and trait specific, but there was consistent evidence for carry-over effects on body morphology. The results of this thesis also build our knowledge on how environmental stressors interact. While ocean warming and acidification interacted additively on all

performance traits, the effects of ocean warming and suspended sediment combined varied among traits, acting additively for some, and synergistically or antagonistically for others. This research underscores the complexity of predicting how marine fish will respond to diverse environmental change sequentially over multiple generations and provides a first step in understanding if and when historical thermal experience matters.

Table of Contents

List of figures

List of tables

Chapter 1 - General introduction

Humans are increasingly seen as the primary cause of environmental change (IPCC 2014; IPBES 2019). Notably, the emission of anthropogenic greenhouse gases into the atmosphere has increased rapidly since the pre-industrial era, with approximately 2040 ± 310 $GtCO_2$ (carbon dioxide; CO_2) being emitted between 1750-2011 (IPCC 2014). These emissions have resulted in an annual average atmospheric CO_2 reaching 410 ppm in 2017 (IPCC 2021), with further increases predicted under current government policies (IPBES 2019) and a growing world population (Gerland et al. 2014). Increased greenhouse gas emissions cause an enhancement of the greenhouse effect; with more heat radiation trapped within the atmosphere, resulting in warming of the land and oceans (i.e., global warming; Houghton 2005). As climate change advances, more physical and biochemical changes are expected within the next century (Kwiatkowski et al. 2020).

Marine systems are one of the most vulnerable to climate change as the ocean absorbs about 20% of anthropogenic CO_2 emissions per year (IPCC 2021). Together with increasing ocean temperatures, this dissolved CO_2 decreases ocean pH and the availability of dissolved carbonate and bicarbonate ions (commonly termed ocean acidification; IPCC 2014). It is estimated that ocean warming and acidification has already increased ocean surface temperature by 0.06°C per decade (observed 1901-2012) and reduced surface ocean pH by 0.018 units per decade (observed 1991-2011; Kwiatkowski et al. 2020). Under the most likely future scenarios (SSP1-2.6 and SSP2-4.5) it is predicted that ocean sea surface temperature will increase a further 1.42-2.1°C and pH reduce a further 0.16-0.26 (CMIP6 model end-of-century projections; Kwiatkowski et al. 2020; IPCC 2021). The tropics and subtropics are also projected to experience short-term climate stressors such as the increased frequency and intensity of flood events, tropical cyclones, and marine heatwaves (Kwiatkowski et al. 2020; IPCC 2021) adding sequential and compounding stress to an already changed environment.

Importantly, these climate change stressors can have significant impacts on marine ecosystems by shifting environmental conditions beyond historical levels, consequently inducing stress and mortality to resident organisms.

Humans are also affecting marine ecosystems through local activities such as agricultural and urban development, dredging, catchment modification, and deforestation, all of which, impact coastal marine ecosystems through inputs of sediment, nutrients, pollutants, and freshwater (Kroon et al. 2012; Rodgers et al. 2021). While inputs of suspended sediment from terrestrial sources or river run off is an important natural process that connects terrestrial and marine systems (Milliman and Meade 1967) through the influx of particulate and dissolved matter which increases plankton productivity (Thorrold and McKinnon 1995), elevated inputs of sediments and nutrients can have negative impacts on marine ecosystems. Elevated sediment and nutrient inputs into coastal waters increases turbidity and hence light attenuation, reduces visibility for animals (Fabricius 2005; Wenger and McCormick 2013), and can also cause direct mechanical damage or stress (Lake and Hinch 1999; Wong et al. 2013; Hess et al. 2017). While most suspended sediment from terrestrial sources is deposited within a few kilometres of river mouths, fine sediment can be carried up to 30 km offshore, and be continually resuspended via hydrodynamic forces such as winds, tides, currents, and upwelling (Devlin and Brodie 2005). Sediment can also be resuspended through anthropogenic activities such as dredging, shipping activities, and coastal infrastructure, which has been found to impact areas 20 km away from the site and last weeks to months (Fisher et al. 2015; Jones et al. 2015).

Coral reefs are one of the most sensitive ecosystems to human induced environmental change. Ocean warming (Hughes et al. 2017, 2018, 2019; Sully et al. 2019; van Woesik et al. 2022), especially when coupled with local anthropogenic activities that increase terrestrial run-off (Brodie et al. 2012; Gissi et al. 2021), have been found to lead to the mortality of corals, and the degradation of reef communities. This habitat degradation can indirectly affect the

abundance and diversity of reef-associated fish and invertebrates (Jones et al. 2004; Wenger et al. 2012; Wen et al. 2016; Rodgers et al. 2021). In addition, fishes and other reef associated organisms are directly impacted by global and localized environmental change as it causes the abiotic factors (e.g., temperature, pH, suspended sediments) to shift beyond what is historically naturally observed (Hughes et al. 2003; Hoegh-Guldberg et al. 2007; Hoegh-Guldberg and Bruno 2010; Poloczanska 2013; IPCC 2021; Schunter et al. 2022). These effects may be especially pronounced during early life stages, when mortality is high (e.g., Hoey and McCormick 2004; Almany and Webster 2006) and variation in survivorship can shape future population size (e.g., Doherty et al. 2004). For marine fish, early life is a critical developmental period during which survival often determines the recruitment rate and population persistence (Sogard 1997; Almany and Webster 2006; Nash and Geffen 2012; Burton and Metcalfe 2014), and is often highly sensitive to environmental conditions (Pörtner and Peck 2010; Byrne 2011; Pankhurst and Munday 2011; Brauner et al. 2019).

1.1 Ocean warming

Temperature governs nearly all rates of biological activity (Huey and Kingsolver 1989; Clarke and Johnston 1999; Brown et al. 2004; Deutsch et al. 2008). As temperature rises, cellular kinetic energy increases, increasing the speed of metabolic processes and the demand for energy and oxygen (Clarke and Fraser 2004). As the vast majority of reef species are ectothermic, and lack internal temperature regulation, changes in environmental temperature directly relate to changes in the rate of cellular physiological processes (Huey and Stevenson 1979; Huey and Kingsolver 1989). As environmental temperature continues to increase a maximum threshold for physiological performance is surpassed (e.g., enzyme denaturing, cardio-vascular system) and aerobic energy production is unable to keep pace with cellular energy demand (Clarke and Fraser 2004). This generally results in a left-skewed performance

response for ectotherms, whereby as temperature rises, performance increases up to an optimal point and then decreases rapidly thereafter (thermal performance curve; Huey and Stevenson 1979).

In the case of aerobic performance, the energy intake to maintain internal biological processes (i.e., basal or resting metabolic rate; Figure 1.1) increases with rising environmental temperature until a point (Clarke and Fraser 2004; Lefevre 2019). Past a threshold temperature the decline in maximum metabolic (Figure 1.1) is believed to be due to the inability for the circulatory and ventilatory systems to support the increased oxygen demand (Pörtner 2001, 2010; Pörtner and Knust 2007; Pörtner and Farrell 2014), and because there is reduced available oxygen in water at high temperatures (Neubauer and Andersen 2019). The difference between resting and maximum metabolic rate, termed aerobic scope/capacity, is often used to construct thermal performance curves (Pörtner 2010; Clark et al. 2013; Verberk et al. 2016). Aerobic scope is expected to indicate the capacity to preform higher level functions like swimming, growth and reproduction (oxygen and capacity limited thermal tolerance hypothesis: OCLTT; Pörtner 2001; Clarke and Fraser 2004; Lefevre 2016), although the reliability for application to all fish species is debated (Clark et al. 2013; Gräns et al. 2014).

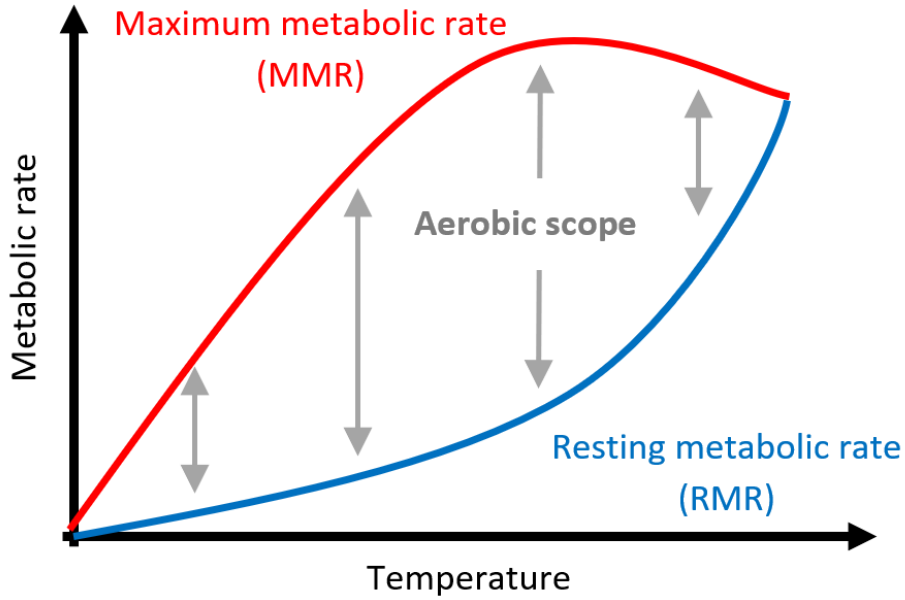

Figure 1.1. Generalization of effects of temperature on aerobic scope. Where resting metabolic rate is predicted to increase exponentially with temperature, maximum metabolic rate reaches an optimum and then declines, and aerobic scope is the difference between resting and maximum metabolic rate.

Tropical species are especially sensitive to changes in their thermal environment as they have evolved under relatively stable thermal conditions, and are thus presumed to have a narrow thermal tolerance, and live close to their thermal maximum (Deutsch et al. 2008; Tewksbury et al. 2008). Conversely, temperate species have broader thermal ranges (both seasonally and spatially), greater intraspecific variability, and a lower critical thermal maximum which has often been found to correlate with a greater thermal tolerance (Vinagre et al. 2016; Nati et al. 2021). The effects of ocean warming are therefore likely to be evident sooner in tropical species as a relatively small increase in seawater temperature may exceed their thermal optimum and cause declines in the performance. For example, elevated resting metabolic rate has been consistently recorded for early life stages of a range of coral reef fish species when exposed to temperatures 1.5 to 4°C above their current average summer temperature (Donelson et al. 2012; Miller et al. 2012; Motson and Donelson 2017; McMahon et al. 2020). Across studies there is variation in the level of thermal sensitivity, with the

subtropical snapper *Chrysophrys auratus* the least sensitive (~5.5% increase in resting metabolic rate $1°C^{-1}$; McMahon et al. 2020), the tropical damselfish and anemonefish (*Acanthochromis polyacanthus*, *Amphiprion melanopus*) moderately sensitive (7-10% increase $1°C^{-1}$; Donelson and Munday 2012; Miller et al. 2012), and wrasses (*Halichoeres melanurus*, *Halichoeres miniatus*, *Thalassoma amblycephalum*) the most sensitive (20-24% increase $1°C^{-1}$; Motson and Donelson 2017). While there seems to be some consistency within taxonomic groups, one species of damselfish, *Pomacentrus moluccensis*, exhibited very high thermal sensitivity with a 33% increase for warming of 1°C (Grenchik et al. 2013). Even the lowest observed increase in resting metabolic rate (i.e., 5.5% $1°C^{-1}$) is still a substantial amount of additional energy required for general cell maintenance in future warming.

The additional energy required to fuel essential cellular processes leaves less energy available for non-essential processes including growth, reproduction, and energy storage. Alternatively, the Gill-Oxygen Limitation Theory (GOLT) proposes that the reduction in body size under elevated water temperature is thought to be a consequence of an insufficient gill surface area, and thus inability to meet increased oxygen demands (Pauly and Cheung 2018). For the spiny chromis (*A. polyacanthus*), a common coral reef damselfish, growth is generally greatest at temperatures that correspond to their current average summer temperature of 28-29°, with higher temperatures leading to slower growth in terms of length (3.5-9.7% decrease $1°C^{-1}$) and mass (8-18.3% decrease $1°C^{-1}$; Rodgers et al. 2017; Munday et al. 2008; Zarco-Perelló et al. 2012). Similar reductions in the growth of three juvenile wrasse species were also found in response to elevated water temperature (Motson and Donelson 2017), while growth was unaffected in two rabbitfish species (LaMonica et al. 2021) and the damselfish *P. moluccensis* (Grenchik et al. 2013), and even improved growth for juvenile *Premnas biaculeatus* (Donelson 2015). The diversity of growth response in coral reef fish indicates that understanding where species are currently living in relation to their thermal optimum (and their

geographic range) is important (McLeod et al. 2015). In addition, the growth-temperature relationship is further variable across fish guild, asymptotic length (van Denderen et al. 2020), and life stage (Munday et al. 2008b) making growth a key morphological trait to investigate further under predicted environmental change scenarios.

As proposed by the OCLTT hypothesis, increasing temperature above the optimum has been found to reduce oxygen transport and aerobic capacity (Pörtner and Knust 2007; Donelson 2015; Pörtner et al. 2017). Various studies using the same species show that aerobic scope can decline due to an increase in resting metabolic rate (Nilsson et al. 2009), a decrease in maximum metabolic rate (Munday et al. 2017; Laubenstein et al. 2019), or both (Donelson et al. 2012b; Rodgers et al. 2019). Specifically, with acute warming (~7 days) of +2°C above the summer average there was little to no effect on maximum metabolic rate, but a significant increase in resting metabolic rate that led to aerobic scope falling by 61% in *A. polyacanthus* (Nilsson et al. 2009). While, Laubenstein et al. (2019) found +3°C above the summer average during development had no effect on resting metabolic rate but reduced maximum metabolic rate by 18.2% and led to a 20% reduction in aerobic scope. Reduced aerobic capacity has been found to occur alongside reduced swimming performance in some cases (Johansen and Jones 2011; Motson and Donelson 2017) as is predicted by OCLTT hypothesis. However, reductions in swimming ability have also been observed even when aerobic capacity is maintained (e.g., McMahon et al. 2020a). Highlighting that swimming capacity is a combination of an individual's scope for activity (energy fluxes and metabolism), as well as the capabilities of its functional structures (muscles, gills, and biochemical pathways) all of which can be negatively impacted by elevated temperature (Claireaux et al. 1995; Taylor et al. 1997; Green and Fisher 2004; Johnston and Hall 2004).

Individuals may be able to buffer the physiological impacts of warming through shifts in their distribution, phenology, or changes in behaviour (Bailey et al. 2022). Alternatively

behaviour may be impacted due to underlying physiological responses to ocean warming (Wong and Candolin 2015; Bailey et al. 2022). Using a startling stimulus (to demonstrate a physical/visual cue of a predator) the escape direction of juvenile reef fish can be negatively impacted with warming of only 1°C above the current summer average (Allan et al. 2017; Warren et al. 2017; Spinks et al. 2019). Ocean warming of 3°C has also been shown to limit the capacity of juvenile damselfish (*Pomacentrus chrysurus*) to detect chemical alarm cues, and thereby hinder eliciting an antipredator response (Lienart et al. 2014). As predation is a key driver of early life mortality, the inability to detect or respond to predator cues (as seen by the impaired escape speed and escape direction) can have major population implications. For example, a population bottleneck may occur if the inability to detect and respond to predator cues reduces the number of fish surviving to enter the juvenile, and ultimately adult, population (Lima and Dill 1990; Almany and Webster 2006; Nash and Geffen 2012; Hughes et al. 2017). Other non-predator-prey behaviours, such as aggression between juvenile conspecifics (though this varied across species and the duration of exposure: Biro et al. 2010; Warren et al. 2016), and habitat preferences of juvenile coral reef damselfish (Matis et al. 2018), have been shown to be altered under elevated temperatures.

1.2 Ocean acidification

To avoid acidosis that can occur at high levels of CO_2 in the surrounding water, marine fish expend energy to regulate their acid base balance by accumulating HCO_3^-, and excreting Cl^- and H^+ ions (Ishimatsu et al. 2008; Brauner and Baker 2009; Brauner et al. 2019). The metabolic cost of active ion regulation is often observed by an increase in resting metabolic rate (Lefevre 2016) and can flow on to reduce the available energy for non-essential processes such as growth and energy storage (Brauner et al. 2019), however, this is not always the case. Ecological differences in ion exchange methods, starting blood CO_2 levels, and consequently

the relative cost of acid-base regulation between species, may provide physiological benefits under elevated CO_2 (Brauner et al. 2019).

Differences in fish ecology means that the effects of elevated CO_2 on the metabolism and morphology of juvenile coral reef fish are varied. For example, juvenile blackback anemonefish, *A. melanopus*, reared under elevated CO_2 (1000 µatm) were on average shorter and lighter, and had an increased resting metabolic rate compared to those reared under control (430 µatm) conditions (Miller et al. 2012). While the resting metabolic rate of the Australasian snapper, *C. auratus*, increased by 9-10% under similar CO_2 concentration (1000 µatm; McMahon et al. 2020a), no difference in juvenile growth was observed (McMahon et al. 2020b). Reduced growth (by 9%) and weight (by 11%) have also be observed in larvae of the clown anemonefish (*Amphiprion percula)* larvae exposed to ~1000 µatm CO_2 (McMahon et al. 2019). Yet, for other coral reef fish resting metabolic rate and growth can be unaffected by elevated CO_2 (e.g., *A. polyacanthus* ; Munday et al. 2011; Laubenstein et al. 2019). These differences across reef fish species may be related to numerous factors, including their developmental environment. For example, *C. auratus* and *A. polyacanthus* larvae/juveniles develop in inshore and reef locations that naturally experience CO_2 fluctuations (Hannan et al. 2020), whereas the larvae of other species like *A. percula* and *A. melanopus* develop in the pelagic environment where they are likely to experience more stable CO_2 (Munday et al. 2011).

Ocean acidification is also likely to affect the maximum oxygen consumption resulting in shifts in aerobic scope (Lefevre 2016). For the snapper, *C. auratus*, aerobic scope of juveniles developing in 1000 µatm condition was substantially reduced (31-35%) driven mostly by the 14-15% reduction in maximum oxygen consumption (McMahon et al. 2020). While elevated CO_2 (860-1000 µatm) had little to no effect on the maximum metabolic rate and aerobic scope of juvenile *A. polyacanthus* (Laubenstein et al. 2019), *Pomacentrus amboinensis* (Couturier et al. 2013), *P. moluccensis* (Couturier et al. 2013; Munday et al. 2014),

and *Dascyllus aruanus* (Munday et al. 2014). Further, most of these early studies investigating the effects of elevated CO_2 used relatively consistent CO_2 concentrations, yet dissolved CO_2 is known to exhibit significant diel variation in natural environments. When elevated CO_2 of 1000 µatm has been tested with the addition of diel fluctuation, no effect on the aerobic scope of juvenile *A. polyacanthus*, or the components of resting and maximum oxygen consumption, were observed (Laubenstein et al. 2020). Variation in the aerobic response to elevated CO_2 has also been observed across life stages, with adults showing both positive and negative respiration responses to elevated CO_2, but earlier life stages were mostly unaffected (Lefevre 2016).

The effect of elevated CO_2 on fish behaviour is the most consistent and ecologically concerning. Fish rely on a range of sensory cues for critical processes, including the detection of reefs, suitable settlement habitats, and potential predators (Lima and Dill 1990). However, the ability to detect and/or respond to the sensory cues is altered under conditions of elevated CO_2. For example, juvenile *A. percula* exposed to elevated (600-900 µatm) CO_2 levels are unable to perceive auditory (Simpson et al. 2011) and olfactory cues (Munday et al. 2009), both of which reduces the homing ability, habitat selection, and settlement timing for early life stages (Munday et al. 2012). Juvenile reef fish exposed to elevated (570-1087 µatm) CO_2 levels have also been shown to exhibit compromised anti-predator responses, such as a reduction in boldness (Munday et al. 2013a), reduction in feeding rate (McMahon et al. 2018; Laubenstein et al. 2019), and an increase in swimming speed and distance moved in response to a stimulus (*A. melanopus*: Allan et al. 2014). Some juvenile reef fish not only appeared to lose the ability to detect and avoid areas with predator odours, but were attracted to a predator odour following exposure to elevated CO_2 (Munday et al. 2009; Dixson et al. 2010; Munday et al. 2013; Munday 2014). Behavioural lateralisation, a measure of brain function (Domenici et al. 2012), was also

impaired in *A. percula* larvae (Nilsson et al. 2012) and juvenile *A. polyacanthus* (Jarrold and Munday 2018) exposed to elevated CO_2 of 900-1000 µatm.

One of the underlying mechanisms of the behavioural changes we see across species, is believed to be because changes in HCO_3^- and Cl^- in acid-base regulation causes a reversal of the GABA-A receptor function (Nilsson et al. 2012). Some variation in sensitivity has been observed across reef fish species (Ferrari et al. 2011) and this may be due to varying ability to regulate ion exchange. However, the ubiquity and reserved function of GABA-A receptor may explain the consistent behavioural response to elevated CO_2 (Nilsson et al. 2012). Interestingly, when diurnal cycles in CO_2 were included in recent studies the previous effects of elevated CO_2 on the behaviour of juvenile *A. polyacanthus* disappeared (lateralization; Jarrold and Munday 2018), suggesting that previous experimental work using treatments with stable CO_2 might be over estimating the effects in nature.

1.3 Suspended sediment

Elevated levels of suspended sediment has consistently been found to alter the gill structures of early life stages of marine fish (Au et al. 2004; Wong et al. 2013; Hess et al. 2015, 2017; Cumming and Herbert 2016), likely due to the constant contact between gills and the surrounding seawater for gaseous exchange (Evans et al. 2005). While suspended sediment can cause mechanical abrasion and clogging of the gills (Lake and Hinch 1999) it can also induce morphological remodelling (structural changes) to protect the gill tissue (pillar system) and/or enhance the respiratory surface area (Mallatt 1985; Nilsson 2007). Protective gill remodelling observed include thickening of the epithelium (Hess et al. 2015; Cumming and Herbert 2016), epithelium lifting (Au et al. 2004), a shortening of gill lamellae (Hess et al. 2017), vascular congestion, lamellar blood sinus dilation (Wong et al. 2013) and hyperplasia (Au et al. 2004; Wong et al. 2013; Cumming and Herbert 2016). In some cases, epithelial thinning and

thickening of the pillar system has also been observed (Au et al. 2004; Hess et al. 2017). Changes to gill structure can have direct implications to organism function. For example, epithelial lifting, thickening, and mucus production increases the diffusion distance for waterborne pollutants to diffuse into the bloodstream and therefore reduces the gas exchange capacity of the lamellae and therefore oxygen consumption (Mallatt 1985). Despite extensive protective gill remodelling in response to elevated suspended sediment concentrations, the metabolic performance (oxygen consumption) of *A. percula*, *A. polyacanthus* (Hess et al. 2017) and *C. auratus* (previously *Pagrus auratus*: Cumming and Herbert 2016) was not affected, but was impaired in *A. melanopus* (Hess et al. 2017). More research is required to understand the physiological cost of morphological gill remodelling and what changes constitute evidence of acclimatory responses.

Suspended sediment can also affect fish through reductions in visual acuity due to the effect of sediment particles on light scatter. Visual impairment with suspended sediment levels as low as 45 mgL^{-1} has been found to increase the time for juvenile *A. polyacanthus* to locate food (Wenger et al. 2012), and reduced the overall feeding rate of larval *Chromis viridis* (O'Connor et al. 2016), both of which reduced the growth and body condition of these species. This contrasts to *A. percula* larvae who were longer and heavier at metamorphosis when exposed to elevated suspended sediment up to 45 mgL^{-1} compared to those held under control (i.e., 0 mgL^{-1}) conditions (Wenger et al. 2014). Elevated suspended sediment concentrations have also been found to influence predator-prey interactions with both enhanced predator-induced mortality (Wenger et al. 2013), and antipredator behaviour (Hess et al. 2019) observed under elevated suspended sediment. While thus far there have been limited studies and only relatively short exposure periods (7 to 42 days) for which to draw conclusions on the likely effect to the early life stages of reef fish, the studies to date indicate that suspended sediment

levels regularly experienced on inshore coral reefs will affect the morphology, physiology, and behaviour of juvenile reef fish.

Our ability to generalise the effects of suspended sediment on fishes is hampered by the small number of studies, the varied levels and type of sediment used across papers, and the methods of measurement. Studies commonly report suspended sediment levels as either nephelometric turbidity units (NTU) or total suspended sediment/suspended sediment concentration (TSS/SCC; e.g., Jones et al. 2015), however, these units are not directly comparable and switching between units requires NTU:TSS conversion factors. Water quality papers have often reported a relationship between turbidity (NTU) and total suspended sediment (TSS, mgL^{-1}) at a ratio between 1:1 to 1:1.8 (Larcombe et al. 1995, 2001; Davis-Colley and Smith 2002; Holliday et al. 2003; Daphne et al. 2011), with the ratio being dependent on the sediment type and particle size (Holliday et al. 2003). However, according to *in situ* data that record NTU and TSS individually, there is no correlation between the units (Tosic 2007). This disparity is likely due to the different attributes measured by each: NTU measures light scattering often caused by suspended material, whereas TSS is the amount of suspended sediment in a volume of water. For the community to gain a more holistic understanding of the impacts of suspended sediment, it is important for experiments to measure and document in terms of both NTU and TSS in addition to the type of sediment used.

1.4 Multiple stressor effects

While a large body of research has considered the effects of elevated temperature and CO_2, and a growing body of research has considered the effects of elevated suspended sediment on larval and juvenile coral reef fishes, only a limited number of studies have explored these stressors in unison. Yet, marine systems are often, and will be, simultaneously or sequentially exposed to

multiple components of environmental change (Gissi et al. 2021). Without directly testing multiple stressors, it is not known if single stressor data can be used to accurately inform multiple stressor outcomes in nature. For example, the interaction between multiple stressors can only be predicted by single stressor experiments if the interaction is additive or single stressor induced (Table 1.1). However, interactions between multiple stressors may be synergistic or antagonistic (Côté et al. 2016; Table 1.1).

Table 1.1. Definitions of multiple stressor interaction types. Modified from Côté et al. (2016)

Interaction	Definition	Example
Additive	The effect during combined exposure is the sum of the two isolated stressors	1+1=2 or 1+0=1
Synergistic	The effect from combined exposure is higher than expected from the sum of the two isolated stressors	1+1=3 or 1+0=3
Antagonistic	The effect during combined exposure is less than expected from either one of the stressors in isolation	1+1=1 or 1+0=0

The few studies to date that have investigated the effects of multiple stressors simultaneously, illustrate that the outcome of multiple stressors mixed (see Table 1.2 for details). To date, most multiple stressor studies have investigated the combined effect of elevated temperature and CO_2 and have found that the combined effect across performance categories is mostly additive (Table 1.2). In the one study on the combined effect of elevated temperature and suspended sediment on a coral reef fish (Hess 2019), the effects appear to be variable depending on the level of temperature and suspended sediments. To date, there have been no studies, that I am aware, that have investigated the combined effects of CO_2 and suspended sediment or all three stressors on juvenile reef fish. The lack of studies investigating these multiple stressors may be related to the challenges of manipulating these different conditions in aquaria, with CO_2 requiring large open/connected systems while suspended sediment requires small closed or partially-closed systems, and the logistical challenges of maintaining multiple treatments levels for multiple stressors simultaneously. Considering the

diverse responses to single stressors described above, and the likely effect on different species, stressor magnitude, and methodology, further research is required to understand the effects of multiple stressors, and in particular those including elevated suspended sediments, on juvenile reef fish.

Table 1.2. Summary of the response each environmental change has on the physiology, morphology, and behaviour on reef fish early life development.

Abiotic conditions	Interaction type	Trait type	Findings
Temperature & CO_2	Additive	Physiology	Resting metabolic rate of *C. auratus* increased at +4°C, 1,000 µatm, and when combined (McMahon et al. 2020). Maximum metabolic rate of *C. auratus* increased at +4°C, decreased at 1,000 µatm, and no different to the controls when combined (McMahon et al. 2020). Aerobic scope of *C. auratus* increased at +4°C, and decreased at 1,000 µatm, and when combined (McMahon et al. 2020). Resting metabolic rate of *P. amboinensis* was not affected by 995 µatm, but increased at +3°C and when combined (Ferrari et al. 2015). Critical swimming speed of *C. auratus* increased at +4°C, decreased at 1,000 µatm, and when combined (McMahon et al. 2020). Critical swimming speed of *Rachycentron canadum* was not significantly affected at 1700–2100 µatm but increased at +5°C and when combined (Bignami et al. 2017).
		Morphology	Length and weight of *A. melanopus* decreased at +3°C, 1,000 µatm, and when combined (Miller et al. 2012). Morphology metrics (including standard length) of *C. auratus* was not affected by 1,000 µatm but increased at +4°C and when combined (McMahon et al. 2020b). Daily growth rate of *Rachycentron canadum* was not significantly affected at +5°C but decreased at 1700–2100 µatm and when combined (Bignami et al. 2017).
		Behaviour	The right-turning relative lateralisation bias of *Pomacentrus wardi* was reduced in +3°C, reversed (left-bias) at 930 µatm, and no bias when combined (Domenici et al. 2014). Anti-predator behaviour of *A. polyacanthus* was not affected at +3°C, but was impaired at 1,000 µatm and when combined (Laubenstein et al. 2019).
	Synergistic	Physiology	Resting metabolic rate of *A. melanopus* was not affected by 1,000 µatm, but increased at +3°C, and increased further when combined (Miller et al. 2012). Resting metabolic rate of *A. polyacanthus* was not affected by 1,000 µatm or +3°C, yet increased when combined (Laubenstein et al. 2019). Resting metabolic rate of *Pomacentrus nagasakiensis* was not affected by 995 µatm or +3°C, but increased when combined (Ferrari et al. 2015).
		Behaviour	Food consumption of *A. melanopus* was not affected by 960 µatm or +3°C, but increased when combined (Nowicki et al. 2012).

	Antagonistic	Morphology	Standard length of *R. canadum* 20dph decreased at 1700–2100 µatm, but increased at +5°C and when combined (Bignami et al. 2017).
		Physiology	Maximum metabolic rate and aerobic scope of *A. polyacanthus* was reduced in +3°C, but was not affected by 1,000 µatm or combined (Laubenstein et al. 2019).
Temperature & Sediment	Additive	Morphology	Gas diffusion distance of *A. polyacanthus* was not affected by 90 mgL^{-1}, but decreased at +3°C and combined (Hess 2019). Filament thickness of *A. polyacanthus* was not affected by 90 mgL^{-1} or 130 mgL^{-1}, but decreased at +3°C and combined (Hess 2019).
		Behaviour	Predator escape performance (speed and distance) of *A. polyacanthus* was not affected by +3°C, but increased at 90 mgL^{-1} and combined (Hess 2019). Predator escape performance (response latency) of *A. polyacanthus* was not affected by +3°C, but was shorter at 130 mgL^{-1} and combined (Hess 2019).
	Synergistic	Morphology	Lamellae total length of *A. polyacanthus* was not affected by 90 mgL^{-1} or +3°C, but increased when combined (Hess 2019).
		Behaviour	Predator escape performance (speed and distance) of *A. polyacanthus* was not affected by +3°C, but increased at 90 mgL^{-1} and combined (Hess 2019). Predator escape performance (response latency) of *A. polyacanthus* was not affected by +3°C, but was shorter at 130 mgL^{-1} and combined (Hess 2019).
	Antagonistic	Morphology	Gas diffusion distance of *A. polyacanthus* decreased at +3°C, 130 mgL^{-1} and a similar decrease was observed when combined (Hess 2019).
		Physiology	Maximum metabolic rate of *A. polyacanthus* was reduced at +3°C but was not affected by 90 mgL^{-1}, 135 mgL^{-1}, or combined (Hess 2019). Aerobic scope of *A. polyacanthus* was not affected by +3°C or 90 mgL^{-1}, but was higher when combined with no interaction (Hess 2019)
CO_2 & Sediment			No studies to date
Temperature, CO_2, & Sediment			No studies to date

1.5 Phenotypic plasticity

There is little doubt that the early life development of coral reef fish is being impacted by anthropogenic environmental stressors, both in isolation and in combination. To persist in this rapidly changing world, phenotypic plasticity is predicted to be especially important in enabling individuals to maintain performance as the rate of environmental change will likely exceed the capacity for genetic adaptation (Chevin et al. 2010; Hoffmann and Sgró 2011; Munday et al. 2013b; Merilä and Hendry 2014). Phenotypic plasticity is traditionally defined as the capacity of a given genotype to produce alternative phenotypes under different environmental conditions (Pigliucci 2001), but in relation to climate change is often investigated in terms of the ability of individuals to maintain or improve performance under altered environmental conditions (Hoffmann and Sgró 2011; Munday et al. 2013).

Phenotypic plasticity can occur within generations (reversible or developmental plasticity) or across generations (transgenerational, cross-generational plasticity) and the type of plasticity is expected to depend on the nature of environmental variation. Reversible plasticity is a controlled phenotypic response in relation to temporal (i.e., daily or seasonal) fluctuations, and is common in circumstances where large fluctuations are experienced over these timescales (Angilletta 2009). Whereas developmental plasticity is an irreversible response to conditions experienced during ontogeny and is expected to occur when the environment varies unpredictably between generations (Angilletta 2009). Transgenerational plasticity on the other hand is where the offspring's phenotype is a response to conditions experienced by previous generations, and is expected when conditions vary predictably across generations (Agrawal et al. 1999; Reed et al. 2010; Salinas et al. 2013; Herman et al. 2014). In the strictest definition of the term, transgenerational plasticity is reserved to describe differences in offspring performance due to the interaction between the offspring and previous generations environments (Salinas et al. 2013; Donelson et al. 2018). Individuals may also

exhibit phenotypic plasticity in the form of carry-over effects. Carry-over effects are when previous generation experience provides consistent change across offspring conditions (i.e., phenotypic plasticity is observed regardless of offspring environment; Jablonka et al. 1995; Bonduriansky and Crean 2018; Donelson et al. 2018). Both transgenerational plasticity and carry-over effects (collectively here as cross-generational plasticity) can induce adaptive plasticity where the phenotypic change is favoured by natural selection, and improve population persistence in the new environment (Ghalambor et al. 2007, 2015). In contrast, plasticity can also be maladaptive if the phenotype passed down reduces individual fitness and is far from the phenotypic optimum, leading to stronger directional selection and population declines (Ghalambor et al. 2007, 2015). Phenotypic plasticity therefore is a key mechanism responsible for evolutionary shifts under environmental change (Merilä and Hendry 2014). As tropical coral reef fish have evolved in a relatively stable environment (with little daily and seasonal variation) compared to their temperate counterparts, they have limited capacity for reversible thermal plasticity (Nilsson et al. 2009, 2010; Gardiner et al. 2010; Johansen and Jones 2011; Leimar and McNamara 2015). Instead, developmental plasticity (Donelson et al. 2011, 2014; Grenchik et al. 2013; Donelson 2015; Spinks et al. 2019, 2021; Yasutake 2019), and cross-generational plasticity (Donelson et al. 2012b, 2012a, 2016; Donelson and Munday 2015; Munday et al. 2017; Spinks et al. 2022) have been observed to mitigate the impairment of performance traits caused by changes in environmental conditions. Collectively this research has shown that negative effects of elevated temperature on reproduction and metabolic traits of coral reef fish can be mitigated (or reduced) when previous generations are exposed to these conditions, with greater beneficial changes possible across generations than within (Donelson et al. 2011; Donelson et al. 2012; Donelson et al. 2016; Munday et al. 2017).

The majority of previous research has found carry-over cross-generational effects, with development of parents and grandparents resulting in overall improved metabolic (Donelson

et al. 2012b) and reproductive (Donelson et al. 2012a; Spinks et al. 2022) performance across the testing range, irrespective of offspring conditions. However, not all parental exposures lead to improved performance of offspring. Both the length of exposure, timing of the environmental cue, and whether one or both parents experience the change, can influence the capacity for a plastic response (Donelson et al. 2018; Spinks et al. 2021). For example, when warming and elevated CO_2 is only experienced during post-maturation, beneficial impacts to early life development are found to be limited (CO_2: Miller et al. 2012; Allan et al. 2014; McMahon et al. 2019; Warming: Donelson and Munday 2015; Yasutake 2019; Spinks et al. 2022).

1.6 Thesis outline and research aims

Coral reef fish will continue to be exposed to multiple human-induced environmental stressors. Global environment stressors such as ocean warming and acidification often occur slowly over decades, whereas local stressors such as floods or nutrient inputs can occur over much smaller temporal and spatial scales as seen globally including in the Gulf of Mexico, East China Sea, North Atlantic Ocean, and the Western Pacific Ocean (including the GBR; Ghedini et al. 2013; Gissi et al. 2021). This highlights that environmental stressors can occur simultaneously, sequentially within a lifetime, or in succession over multiple generations, making the response to future environmental conditions difficult to predict with single stressor or within-generation research alone (Ghedini et al. 2013; Gissi et al. 2021). Although, to date, there is limited information on how cross-generational plasticity influences the response of reef fish to multiple environmental stressors. Seminal work by Miller and colleagues (2012), which included parental exposure to ocean acidification and offspring exposure to both ocean warming and acidification found direct effects of both environmental stressors and an additive interaction when combined. This experiment, however, does not allow investigation of cross-generational

plasticity effects, because of the limited parental exposure (only months before reproduction). Thus, how performance of juvenile reef fish is influenced by multiple stressors in succession over multiple generations is currently unknown. Additionally, exploring the effect of both climate change and localised stressors will better capture the complexity of natural systems, and allow more accurate predictions of the impacts to reef fish.

To investigate the role of phenotypic plasticity under multiple environmental stressors, this thesis examines the development and performance traits of the spiny chromis, *Acanthochromis polyacanthus,* a common coral reef damselfish. This species was selected for this study as it is a widespread Indo-Pacific species with populations ranging from the southern Coral Sea to the southern Philippines. *A. polyacanthus* form monogamous breeding pairs that last throughout the 4-month breeding season (most often between October and February; Robertson 1973). Both parents then care for and defend the eggs which are laid on (and attach to) the substrate (Kavanagh 2000). Unlike most marine fish, *A. polyacanthus* lack a dispersive pelagic larval phase and instead, juveniles (in tightly aggregated groups) remain with their parents for a few months after hatching (Kavanagh 2000). The limited dispersal and gene flow between *A. polyacanthus* populations as well as high rates of self-recruitment (Doherty et al. 1994) is expected to promote local adaptation. This genetic distinction between populations can also lead to variations in how populations respond (degree of plasticity) to environmental change such as ocean warming, and thus increase species variation over time (Gardiner et al. 2010; Donelson and Munday 2012). Utilising an available multi-generational orthogonal crossed design, this thesis investigates how phenotypic plasticity to +1.5°C warming in two previous generations influences the development and performance of juvenile *A. polyacanthus* under multiple environmental stressors.

In **Chapter 2**, I examine how exposure to +1.5°C above present-day average conditions in the grandparent (F_1) and parent (F_2) generations influences the sensitivity of F_3 juveniles to

elevated temperature (summer Control: 28.5°C; Warm: 30°C) and CO_2 (Control: 490 µatm; Elevated: 825 µatm) independently and combined. After developing in these environmental conditions for 16 weeks, aerobic physiology (resting oxygen consumption, maximum oxygen consumption, and absolute aerobic scope), behaviour (boldness and activity), and growth (length and physical condition) were measured in juveniles and the relationships between these performance traits.

In **Chapter 3**, I explore juvenile performance under ocean warming and a localised environmental stressor of elevated suspended sediment. Specifically, whether +1.5°C above present-day average conditions in the grandparent (F_1) and parent (F_2) generations influences the sensitivity of F_3 juveniles to warm temperature (summer Control: 28.5°C; Warm: 30°C) and elevated suspended sediment (present-day Control: 0 mgL^{-1}; Suspended sediment: 50 mgL^{-1}) independently and combined. Juvenile *A. polyacanthus* were reared in these environmental conditions for ~11 weeks (53-81 days post-hatch; dph) before a sub-sample was taken from each treatment and a variety body morphology (length, weight, and physical condition), gill morphology (lamellae length, width, perimeter), and the presence of gill remodelling (epithelial lifting, hyperplasia, mucus, aneurysms, and lamellae fusion) was quantified. All sediment was then removed from the aquaria system and the fish maintained for additional 5 weeks. Body morphology metrics were again taken from a sub-sample 17-18 days following sediment removal (74-102 dph), and from the remaining juveniles after a further 17-20 days (92-122 dph).

Together, these two chapters will advance our understanding of the potential impacts of global and local environmental change across generations. It will also elucidate the sensitivity early life stages of a coral reef fish to multiple stressors and how sequential and simultaneous environmental change can affect their phenotypic outcomes. This knowledge can

be used to inform reef ecosystem management practices that reduce anthropogenic pressures and support reef resilience and persistence into the future.

Chapter 2 - The role of cross-generational warming on the juvenile development of a coral reef fish under ocean warming and acidification

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2.1 Abstract

Marine ecosystems are increasingly being exposed to a plethora of environmental stressors, including ocean warming and ocean acidification. While numerous studies have quantified the effects of environmental stressors individually, few have considered how the combined effects of multiple stressors may be influenced by the order of exposure. Two generations of the coral reef damselfish (*Acanthochromis polyacanthus*) exposed to either present-day control (+0°C) or warm temperature (+1.5°C) during development and/or post-maturation were used to explore how thermal experience influenced their offspring performance in warm and acidified ocean conditions. Specifically, the developmental sensitivity of juveniles to Warm temperature (+1.5°C: 30°C) and Elevated CO_2 (825 µatm) compared to control conditions (+0°C: 28.5°C, 490 µatm) were tested in an orthogonal design. After developing in these environmental conditions for 16 weeks from hatching, aerobic physiology (resting oxygen consumption, maximum oxygen consumption, and aerobic scope), behaviour (boldness and activity), and growth (length and physical condition) were measured and the relationship between these performance traits was analysed. Utilising this complex multi-generational, multiple stressor experiment, I found that the exposure to warm temperatures in the parental or grandparental generations produced juveniles that were larger and in better condition (carry-over effects) and this did not alter under the novel environmental stressor of elevated CO_2. Although, developmental environment had the greatest effect on juvenile performance in which development in Warm temperature resulted in shorter and bolder fish that were in better

physical condition, and development in Elevated CO_2 resulted in shorter fish with a greater resting oxygen consumption. These finding illustrate the complex yet significant role of phenotypic plasticity under projected future climate change.

2.2 Introduction

Marine ecosystems are experiencing increased anthropogenic pressure as greenhouse gas emissions continue to warm and acidify the ocean at an unprecedented rate (IPCC 2021). This shift in environmental conditions beyond those that species have historically experienced, can cause a range of physiological, morphological, and behavioural changes, which could have significant impacts on population dynamics and ecosystem health (Munday et al. 2008a; Brierley and Kingsford 2009; Hoegh-Guldberg and Bruno 2010; Doney et al. 2012; Hoey et al. 2016; Rummer and Munday 2017). While many species have the capacity to genetically adapt to changing environmental conditions, there are concerns that the rate of environmental change under ongoing and future climate change may outpace the capacity for genetic adaptation in many species (Chevin et al. 2010; Merilä and Hendry 2014). This has led to the expectation that phenotypic plasticity (the capacity of a genotype to render alternate phenotypes; Pigliucci 2001) could be especially important in allowing individuals to maintain performance under altered environmental conditions (Hoffmann and Sgró 2011; Munday et al. 2013).

Phenotypic plasticity can be induced by environmental conditions experienced by both the current and past generations. Within a generation, developmental plasticity can occur in response to environmental conditions experience during early ontogeny and is generally considered to be permanent (West-Eberhard 2003; Angilletta 2009). While early life stages are generally more sensitive to abiotic changes (Pankhurst and Munday 2011; Brauner et al. 2019), experiences during this time also have a greater potential to produce plasticity due to epigenetic

sensitivity (Burton and Metcalfe 2014; Jonsson and Jonsson 2014; O'Dea et al. 2016). Additionally for most species, climate change will occur over multiple generations possibly allowing for transgenerational plasticity and carry-over effects (Hoffmann and Sgró 2011; Rummer and Munday 2017; Donelson et al. 2018; Schunter et al. 2022). While both terms are used to describe differences in offspring's phenotype in response to conditions experienced by previous generations, transgenerational plasticity is reserved for when offspring phenotype interacts with the current environment (Salinas et al. 2013; Donelson et al. 2018), while carry-over effects occur regardless of offspring environment (Jablonka et al. 1995; Bonduriansky and Crean 2018; Donelson et al. 2018). Whether plasticity is produced in relation to environmental change can depend on the costs of sensing and responding to change relative to the direct costs of being exposed to the stressor (Angilletta 2009). Disentangling the effects developmental and transgenerational plasticity while difficult, can be achieved if the current generation did not experience the parental environmental conditions during primordial germ cell development or embryogenesis (Donelson et al. 2018).

Ocean warming is considered one of the greatest threats to marine species due to the majority of species being ectothermic and lacking internal temperature regulation (Huey and Stevenson 1979; Huey and Kingsolver 1989). Temperatures increases as small as 1-3°C above the present-day summer average have been found to reduce the aerobic scope (Nilsson et al. 2009; Donelson et al. 2011; Johansen and Jones 2011; Motson and Donelson 2017; Slesinger et al. 2019), growth (Munday et al. 2008b; Rogers et al. 2011; Motson and Donelson 2017; Watson et al. 2018; Spinks et al. 2019), and reproduction (reviewed in Pankhurst and Munday 2011), as well as alter anti-predator behaviour (Lienart et al. 2014; Motson and Donelson 2017; Watson et al. 2018) in marine fish. These negative effects can be mitigated (or reduced) when the current generation is exposed to conditions from early life (Donelson et al. 2011; Grenchik et al. 2013; Shama et al. 2014; Donelson et al. 2016) or previous generations are exposed to

these conditions (Donelson et al. 2012; Shama et al. 2014; Donelson et al. 2016; Munday et al. 2017). However, the nature of the plasticity can depend on the timing and magnitude of thermal exposure (Donelson et al. 2016; Bernal et al. 2022; Spinks et al. 2022).

Compared to ocean warming, the effects of ocean acidification on marine fishes are generally lower and more variable, both within and among species (Lefevre 2016, 2019). For example, the effect of ocean acidification on metabolic rate of marine fishes has been shown to vary among species, with some species exhibiting no change (Couturier et al. 2013; Munday et al. 2014; Laubenstein et al. 2019, 2020), while others had increased (Munday et al. 2009a; Miller et al. 2012; Couturier et al. 2013; Rummer et al. 2013; Laubenstein et al. 2018; McMahon et al. 2020a), or reduced metabolic rate (Rummer et al. 2013; Pimentel et al. 2014; McMahon et al. 2020a). Growth of marine fish was also not affected (Munday et al. 2011), increased (Munday et al. 2009), or reduced (Baumann et al. 2012; Miller et al. 2012; McMahon et al. 2019) by elevated CO_2. Consistent effects of elevated CO_2 has been observed on fish behaviour, with relatively high levels of dissolved CO_2 (750-1100 µatm), altering responses of fishes to auditory (Simpson et al. 2011; Rossi et al. 2016), olfactory (Munday et al. 2009), and physiochemical cues (Welch et al. 2014; Pistevos et al. 2017; McMahon et al. 2018). The variable response to ocean acidification across species and life stages highlights that predicting for the likely future impacts is challenging without direct testing of species and scenarios (Harvey et al. 2013; Przeslawski et al. 2015; Lefevre 2016; Cattano et al. 2018; Sampaio et al. 2021; Baag and Mandal 2022)

Knowledge to date on the capacity for plasticity in reef fishes in response to climate change is primarily based on a single environmental stressor, however, this is an oversimplification of the complexities organisms will face as climate change advances. The extent and timing of environmental change is not expected to be uniform across the world's oceans (IPCC 2014, 2021). As a result, marine species may be exposed to multiple

environmental stressors simultaneously or sequentially within a lifetime, or among over generations, making the response in future environmental conditions difficult to predict based on single stressor studies (Ghedini et al. 2013; Gissi et al. 2021). For instance, exposure to one stressor may prime an organisms system to handle another stressor (Gunderson et al. 2016). Alternatively, organisms may be more susceptible to a stressor when it is superimposed on an existing one (Nyström et al. 2001). When ocean warming and acidification have been experienced simultaneously during early life, the effects on aerobic physiology and growth have generally been found to be either additive (Munday et al. 2009; Miller et al. 2012; Flynn et al. 2015) or synergistic (Miller et al. 2012; Laubenstein et al. 2019). Work by Laubenstein and colleagues (2019) showed that development in elevated temperature and CO_2 not only affected aerobic physiology and behaviour, but there was a negative correlation between beneficial behavioural and physiological traits resulting in a trade-off that may limit adaptive potential. Considering the complexities of multiple stressors impacts and the potential importance of plasticity to coping with climate change, testing of more complex scenarios that could occur in nature is required.

This study builds on previous mutigenerational work investigating thermal plasticity in the tropical damselfish *Acanthochromis polyacanthus*, by investigating whether thermal experience of previous generations influences the sensitivity to multiple environmental stressors in the current generation. Specifically, I used a multigeneration crossed design to examine how exposure to +1.5°C above present-day average conditions in the F_1 and F_2 generations influences the sensitivity of F_3 juveniles to elevated temperature (summer Control: 28.5°C; Warm: 30°C) and CO_2 (Control: 490 µatm; Elevated: 825 µatm) independently and combined. After developing in these environmental conditions for 16 weeks, aerobic physiology (resting oxygen consumption, maximum oxygen consumption, and absolute aerobic scope), behaviour (boldness and activity), and growth (length and physical condition)

were measured in juveniles and the relationships between these performance traits was explored. These various performance traits were selected to provide a holistic understanding of juvenile development and ecology. From previous work, I expect that warming in the F_1 grandparent and F_2 parent generations may improve thermal performance of F_3 juveniles when they also develop in warm conditions (Yasutake 2019; Spinks et al. 2021). However, whether this thermal exposure impacts sensitivity to elevated CO_2 or alters the trade-offs between performance traits (as seen in Laubenstein et al. 2019) is unknown.

2.3 Materials and Methods

2.3.1. Study species

The spiny chromis, *Acanthochromis polyacanthus,* is a widespread Indo-Pacific species with populations ranging from the southern Coral Sea to the southern Philippines (15°N–26°S and 116°E–169°E). *A. polyacanthus* form monogamous breeding pairs that last throughout the Austral summer breeding season (most often between October and February; Robertson 1973). Both parents care for and defend the eggs which are laid on the substrate (Kavanagh 2000). *A. polyacanthus* lack a dispersive pelagic larval phase and instead, juveniles remain with their parents up to 45 days after hatching (Kavanagh 2000). Wild adult *A. polyacanthus* used in this experiment were collected from the Palm Island region (18°40-45’S, 146°34-41'E) in 2014, and from Bramble Reef (18°24′S, 146°42′E) in 2015 and transported to the Marine and Aquaculture Research Facility at James Cook University, Townsville, Australia (F_0 generation; see Spinks et al. 2021 for more details).

2.3.2 Cross-generational experimental design

Two temperature treatments were used in this 3-generation experiment: 1) Control treatment in which water conditions simulated seasonal temperature cycles (winter: 23.2°C, summer:

28.5°C) for the Palm Islands region of the GBR (AIMS 2016), and 2) Warm treatment in which water conditions simulated +1.5°C than present-day (winter: 24.7°C, summer: 30°C, as per Spinks et al. 2021) to represent predicted temperatures for 2050-2100 under climate change (Collins et al. 2013; IPCC 2021). Both temperature treatments included a diurnal temperature cycle (0300 hrs -0.6°C, 1500 hrs +0.6°C) matching the natural daily temperature cycle of the Palm Island region (shallow reef; Spinks et al. 2021). Research was conducted inside environmentally controlled laboratories for which photoperiod was simulated to reflect sunrise and sunset times at the collection location (e.g., 13 h 15 min light in December (summer) and 11 h 01 min light in June (winter); as per Spinks et al. 2021). All temperature-controlled systems were partially connected to each other and joined to form one larger closed system (~25,000 L). Water quality was maintained with mechanical, biological, and ultraviolet filtration, as well as protein skimming, and partial water changes. Across all generations of the experiment fish were fed according to their development and size. From hatching, juveniles were fed a mixture of live *Artemia* nauplii and were slowly weaned onto 0.2-0.4 mm aquaculture NRD pellet (INVE Aquaculture O.range Wean). As fish grow and develop the aquaculture NRD pellet size increased to 0.5-0.8 mm and 1.2-2.0 mm (INVE Aquaculture O.range Grow and O.range Nurse). At all ages fish were fed ~2-4% of body weight daily (see Spinks et al. 2021 and Yasutake 2019 for more details).

F_0 *A. polyacanthus* adults were housed in breeding pairs within 60 L aquaria with a half terracotta pot for shelter and egg deposition. Pairs were maintained at seasonally fluctuating present-day Control conditions. In the Austral summer of 2015-2016 the first clutch (F_1) from the six wild-caught pairs were produced at Control conditions (~28.5°C) and split at hatching into the two temperature treatments Control and Warm +1.5°C (Figure 2.1). These F_1 fish were maintained in sibling groups at these 2 temperature treatments throughout development until 1.5 years of age (development period), at which time each sibling group was divided further

into Control or Warm temperature conditions creating 4 treatments throughout post-maturation (Figure 2.1; Spinks et al. 2021). As the F_1 fish approached 3 years of age non-sibling pairs were made from male and female fish maintained at the same treatment conditions during both development (i.e., hatching to 1.5 years) and post-maturation (1.5 to 3 years), in September 2018. In each of the 4 treatments, 3 to 5 F_1 adult pairs contributed a clutch to the F_2 generation. The first clutch of offspring (F_2) produced by F_1 breeding pairs in the Austral summer of 2018-2019, were split at hatching into both Control and Warm temperature conditions and reared to 1.5 years as described above for the F_1 generation, creating 8 treatments (Figure 2.1; Yasutake 2019). These F_2 fish were maintained at their respective treatment conditions until 1.5 years of age (post-maturation) when all F_2 adults were transferred to Control temperature conditions and maintained until 3 years of age for the current experiment.

Adult F_2 fish were allocated into non-sibling pairs with fish from the same treatment beginning in late August 2020 (at ~2 years of age) and where possible, further pairs were made in December 2021 for the current experiment. Of the possible 8 cross-generational thermal experiences (Figure 2.1), 5 were used in this experiment: **Control** (present-day conditions throughout F_1 and F_2 generations, n=5 pairs); **Parental development** in Warm temperature (developmental exposure of F_2 parents to +1.5°C from hatching to 1.5 years, n=5 pairs); **Grandparent post-maturation** in Warm temperature (post-maturation exposure of F_1 grandparents to +1.5°C from 1.5 to 3 years, n=5 pairs); **Grandparental development** in Warm temperature (developmental exposure of F_1 grandparents to +1.5°C from hatching to 1.5 years, n=5 pairs); **Continuous grandparent** in Warm temperature (continual exposure of F_1 grandparents to +1.5°C from hatching to 3 years, n=3 pairs) (n's indicate the number of unique pairs that produced F_3 offspring utilized in this study).

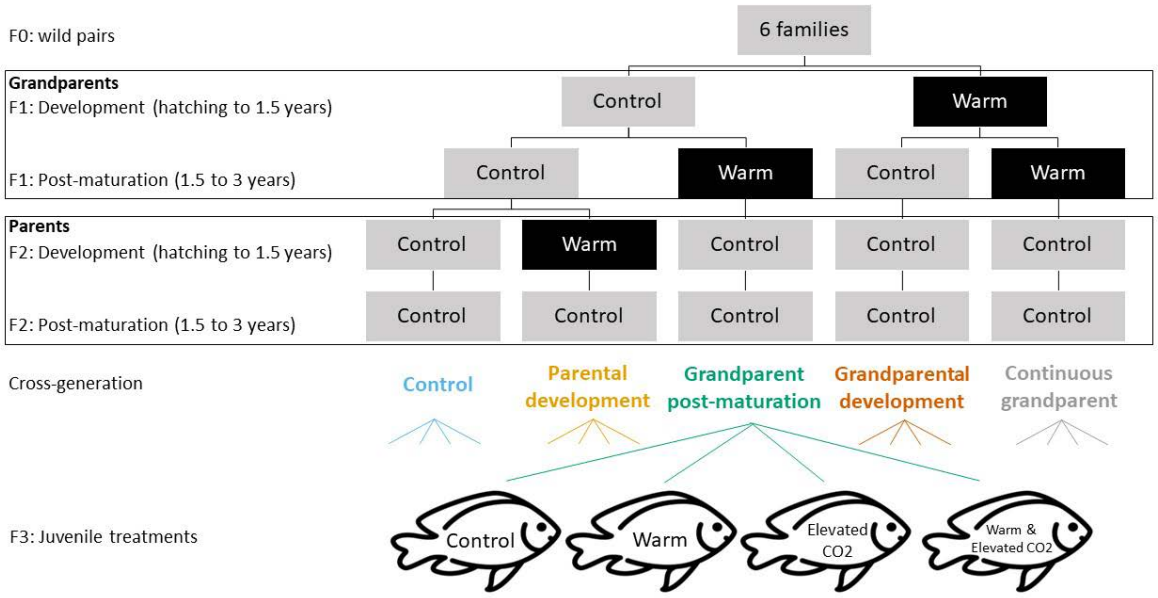

Figure 2.1. Cross-generation experimental design outlining the thermal experience of F_1 grandparent and F_2 parent generations. F_3 offspring from the five cross-generations were split orthogonally between 4 development treatments including Control (28.5°C, 490 µatm); Warm temperature (30°C, 490 µatm); Elevated CO_2 (28.5°C, 825 µatm); and Warm temperature and Elevated CO_2 (30°C, 825 µatm).

2.3.3 F_3 juvenile experimental design

When summer average water temperature (Control: 28.5°C) was reached in November 2021, terracotta pots were checked daily for newly laid egg clutches. Once an egg clutch was recorded, it was checked daily for the presence of hatched offspring (F_3), which generally occurs in the afternoon for this species around 9 days after eggs are laid (Donelson et al. 2010). Newly hatched offspring from all clutches were randomly divided into groups of 20 individuals and placed in aerated holding containers within 32 L tanks which were maintained at one of 4 juvenile treatments. To facilitate a slow transition to the treatment conditions, water from the tank was gradually added into the holding containers over 4 to 12 hours after which juveniles were then released into the tank. If there was any mortality during this transition time, those individuals were replaced to achieve a starting tank density of 20 individuals. Juvenile F_3 treatments included **1) Control:** in which water temperature was the present-day summer average 28.5°C and control atmospheric CO_2 of 490 µatm; **2) Warm temperature:** in which

temperature was +1.5°C warmer than Control (i.e., 30°C) and present-day atmospheric CO_2 of 490 µatm; **3) Elevated CO_2:** in which temperature was the present-day summer average 28.5°C and projected elevated CO_2 by 2100 of 825 µatm; **4) Warm temperature and Elevated CO_2:** in which temperature was +1.5°C warmer than Control (30°C) and projected elevated CO_2 of 825 µatm). These values were selected to reflect the most likely future scenarios (SSP1-2.6 and SSP2-4.5) under the CMIP6 model end-of-century projections (Kwiatkowski et al. 2020; IPCC 2021). For all five cross-generational clutches from up to n=5 pairs were used for this experiment. In the case of Continuous grandparent cross-generation only 3 pairs reproduced, and 2 clutches were used from one pair. For each clutch n=2 replicate tanks were made per each of the 4 juvenile treatments.

Water for this juvenile experiment was supplied from four 3,000 L recirculating seawater systems that were partially connected via Belimo valve actuators. Two of the recirculating systems contained Control CO_2 seawater and two contained Elevated CO_2 seawater. In all 4 systems the Control temperature treatment was maintained by a heat pump (Toyesi TET600SSD) and Warm temperature treatments were created though an inline heater (3kW Toyesi WHIL 3000 or 3.8kW Gecko In Therm 3.8kW) controlled by Innotech C40 PLC. The control of treatment conditions with the external recirculating system is congruent with previous elevated temperature and CO_2 studies (McMahon et al. 2018; Laubenstein et al. 2019). In the case of temperature treatments in the F_3 generation, no diurnal cycle was included, and temperatures varied ±0.2°C around the desired set-point (Table 2.1). CO_2 treatments were created using a pH computer (Endress and Hauser Liquiline CM442) connected to a pH probe and a solenoid valve which allows within sump dosing of CO_2 to the desired setpoint. To adjust the setpoint for fluctuation in total alkalinity, pH_{total} was measured daily, and total alkalinity was measured weekly. Both pH_{total} and total alkalinity was measured using gran titration (Metrohm 888 titrando) to within 1% of certified reference material (Mettler Toledo technical

buffer solution at pH 4.01 and 7.00). Details on the water parameters during the experiment from January to July 2022 are provided in Table 2.1.

Juveniles were fed a high food ration; ~2-4% of body weight, once per day. From 1-3 days post-hatching, juveniles were fed newly hatched *Artemia* nauplii at a concentration of 1 individual ml^{-1} (~35,000 individual *Artemia* per tank), and from days 4-6, juveniles were fed *Artemia* nauplii at 2 individual ml^{-1} and 40 mg of 0.2-0.4 mm sized INVE Aquaculture Nutrition NRD pellets. Subsequently, juveniles were fed only INVE Aquaculture Nutrition NRD pellets which increased in volume and pellet size from 40 mg of 0.2-0.4 mm sized pellets on day 7-15, 80 mg of 0.2-0.4 mm sized pellets on day 16-29, 100 mg of 0.5-0.8 mm sized pellets on day 30-60, to 200 mg of 0.5-0.8 mm sized pellets on day 60-end of the experiment. Fish were grown in sibling groups under the 4 juvenile treatment conditions until ~120 days old. After developing in these environmental conditions, various performance traits were then measured to provide a holistic understanding of juvenile development and ecology. For example, aerobic physiology is a measure of the energy available for both internal biological process and higher level functions like swimming, growth and reproduction (Pörtner 2001; Clarke and Fraser 2004; Lefevre 2016), behaviour may indicate juvenile survival through foraging success and predation evasion (Metcalfe et al. 2016), and lastly body size (growth) is a key trait related to competitive ability against conspecifics and predators (Booth and Beretta 2004; Hoey and McCormick 2004; Poulos and McCormick 2015).

Table 2.1. Mean (±1 SD) seawater chemistry parameters for each F_3 juvenile treatment. Tank temperature was measured every second day for the first 3 months, then weekly during experimental trials. pH_{total} was measured daily, and total alkalinity was measured weekly. These parameters were used to estimate pCO_2 in CO2SYS at the lower (34.5ppt) and upper (36ppt) range of salinity.

Juvenile treatment	Temperature (°C)	Total alkalinity (µmol/kg SW)	pH (total)	pCO_2 (µatm) salinity 34.5ppt	pCO_2 (µatm) salinity 36ppt
Control	28.5 ± 0.11	2586.67 ± 212.98	8.15 ± 0.04	498.09 ± 33.22	489.66 ± 32.91
Warm Temperature	29.9 ± 0.08	2586.67 ± 212.98	8.15 ± 0.04	500.98 ± 33.68	492.48 ± 33.37
Elevated CO_2	28.5 ± 0.10	2584.66 ± 216.06	7.96 ± 0.03	833.13 ± 44.84	821.40 ± 44.34
Warm Temperature and Elevated CO_2	29.9 ± 0.09	2584.66 ± 216.06	7.96 ± 0.03	840.40 ± 45.22	828.57 ± 44.71

2.3.4 Aerobic physiology

Between 101-137 days post-hatching (dph), the aerobic physiology of 4 juveniles from each tank (n=8 juveniles per clutch per F_3 treatment; Table 2.3) was measured using intermittent flow respirometry under their juvenile treatment's conditions. Prior to the respirometry, fish were not fed for 12-24 hours to ensure that measurements were not affected by additional metabolic functions such as digestion (Niimi and Beamish 1974). Oxygen consumption at rest and at maximum was tested and used as a proxy for metabolic performance. To obtain maximal oxygen rate (MO_{2max}), fish were placed in a circular swim chamber (120 mm diameter, filled with ~650 ml treatment water) situated in an empty glass tank above a magnetic stir plate (Nilsson et al. 2007). Water was set in motion using a 70 mm stir bar at the bottom of the chamber (protected by a mesh covering situated just off the bottom of the chamber) and water speed was increased until the juvenile was seen to switch from pectoral to caudal swimming (aerobic swimming). A circular swimming motion was created around the mesh cylinder located in the middle of the swim chamber. Juveniles remained in the swim chamber for 3 minutes of aerobic swimming which was then followed by 1 minutes of air exposure (Clark et al. 2013). Fish were then immediate placed in a randomly allocated glass or clear plastic

chamber respirometry chamber purposely built for juvenile fish of this size (between 32-70 ml). Respirometry chambers, run in groups of 4, were submerged in 52 L aquaria which received constant flow from the system with the respective juvenile treatment conditions and aeration. Each chamber was connected to its own reticulating pump by gas impermeable hosing, and all four chambers were connected to the same flush pump via a hose with 4 outflow holes (one for each chamber) and an open valve on the opposite end. The testing aquaria was then covered with a black tarp to shield chambers from external disturbances throughout the duration of the trial. Juveniles remained in the chambers while a purpose-built python program (AquaResp v3.0) was used to control the timing measurement cycle. This consisted of a 1 min wait period, a 3 min measurement period, and a 3 min flushing period to return oxygen ~100% O_2. Oxygen concentration was measured at an average rate of once per second during the measurement period using a PyroScience Optical Oxygen Meter (Firesting Pro, FSPRO-4) which was connected to fibre-optic sensors focused on 5 mm O_2 sensor spots (PyroScience, OXSP5) fixed on the inside of each chamber. This 5-min cycle was repeated continuously during the 3 h trial duration allowing the fish to come to rest for resting oxygen rate; MO_{2rest} (Clark et al. 2013; Killen et al. 2014; Laubenstein et al. 2019). Before each trial, each oxygen probe was calibrated to 100% (0% calibration were calibrated at the start of the experiment) and background microbial respiration was measured for 2-3 cycles. Two aquaria were run simultaneously (one Control and one Elevated CO_2), twice per day between the hours of 08:00 to 18:00. The time of day (am or pm) that each of the 4 treatments was run alternated over the 2 days it took to complete the trials for a single clutch. The aquaria and all respiration equipment were beached at the end of each day to prevent bacterial build up.

Maximum oxygen consumption, MO_{2max}, was calculated manually from the steepest rate of oxygen decline in 60 sec, from the periods 1-60, 31-90, 61-120, 91-150 and 121-180 s in either of the first two 3 min measurement cycles. This was put into the equation:

$$MO_2 = K * V * \beta / M,$$

where K is the linear rate of decline (kPah−1) in the oxygen content over time (h); V is the volume (L) of the chamber; β is the solubility of oxygen in water at a specific temperature (mg O_2 L^{-1} kPa^{-1}); and M is the body mass of the fish (kg). Average background respiration for each chamber was then subtracted to generate $MO_{2max.}$ Resting oxygen consumption was calculated using the MO_2 values generated from the python program (AquaResp V3.0) during the 3-hour trial. After average background respiration was subtracted, MO_2 values below 0.9 R^2, and outside 2 standard deviations (SD) of the average were removed and only the lowest 10 values (of the 3 h trial) were then averaged to generate MO_{2rest} for each fish (Clark et al. 2013; Hess et al. 2017; Laubenstein et al. 2020). After all data was collated, individuals ±2 SD of the mean MO_{2rest} and MO_{2max} were excluded from the data set. Absolute aerobic scope (MO_{2max} - MO_{2rest}) was also calculated for each fish.

2.3.5 Behavioural tests

Behaviour traits were measured for all individuals that underwent respirometry trails. Directly after the completion of their respirometry trial, juveniles were placed in 75 L holding tanks that were set at their respective treatment conditions. To keep track of individual fish, they were maintained within fine mesh 3 L breeding baskets within each of the holding tanks. If juveniles finished respiratory trials before 2 pm (and required >18 h holding before behaviour trials) juveniles were fed newly hatched *Artemia* nauplii at a concentration of 1 individual 5 ml^{-1} to prevent starvation. For a single clutch, respirometry treatment timing (am or pm) alternated over the 2 days to balance any effect of feeding prior to the behaviour trials. The following day, fish were placed into one of 3 identical square white behaviour arenas (300 x 300 x 150 mm) filled with 7 L of water from of their respective treatment conditions (water height 75 mm), which contained newly hatched *Artemia* nauplii at a concentration of 1 individual 5 ml^{-1}

to encourage movement. Larval fish are known to quickly recover from activities and as such, behavioural tests have commonly been conducted the day following respirometry trials (Killen et al. 2014). To commence the trial, fish were placed into a shelter (5-way 25 mm PVC joint) in the centre of the arena that was surrounded by circular tube (100 mm diameter PVC) and allowed to habituate for 10 min. At the start of the behaviour trial, the habituation cover was removed, and movement of the fish was recorded for 15 min using a digital video camera that was mounted above the tank for an aerial view (GoPro hero: 3 or session). Through the trial period the behaviour experimental conductor (YCY) was absent from the room. Videos were analysed by the primary investigator blinded to the cross-generational and juvenile treatments (JSC). From the videos, the combination of boldness and activity behaviour were scored on a scale from 1-5, with 1 being the least and 5 being the most bold and active (hereafter known as behaviour score; Table 2.2).

Table 2.2. Combined boldness and activity (behaviour) score given to each individual juvenile during the 15min behaviour trial with description of the behaviour.

Score	Description	Details
1	Stationary in tunnel/shelter or in corner	Little to no movement, stays in same spot for the entirety of the video recording
2	Stationary in multiple places along walls	<5 moves to another spot on the wall, sticks very close to the wall
3	Swimming along walls	Always close to the walls; may swimming up and down or to change spots (include swimming along the wall at up to one body length distance)
4	Ventures to the middle	Comes far off the wall for exploration (greater than one body length). Or swimming to the other side of the tank, crossing close to or above the tunnel
5	Often swimming around in the middle	Swimming around for the majority of the video recording

2.3.6 Morphological metrics

Morphometric traits of standard length and wet weight were measured for all F_3 juveniles (n=2955; Table 2.3). Following the physiological and behavioural testing outlined above, all tested juveniles were euthanised with an overdose of clove oil and sea water (1:20) and then preserved in 75% ethanol. For all remaining juveniles that did not undergo respirometry and

behavioural testing, they were euthanised and preserved as above at a slightly later age (+1 to 13 days). Standard length to the nearest 0.01 mm (digital callipers) and weight to the nearest 0.0001 g (Shimandzu ATX224) were measured for each fish post-preservation. In this study, physical condition (a ratio of weight to length) was calculated as weight for a given standard length which is a common alternative to the criticised Fulton's K condition index (Jones et al. 1999; Froese 2006; Nash et al. 2006; Spinks et al. 2022).

Table 2.3. Total number of individuals in each data set within each treatment group (used in the final statistical analysis and output).

Physiology	**Control**	**Warm**	**CO_2**	**Warm and CO_2**
Control	32	30	28	28
Parental development	35	26	34	27
Grandparent development	37	31	29	32
Grandparent post-maturity	31	32	25	25
Continuous grandparent	25	24	24	25

Behaviour	**Control**	**Warm**	**CO_2**	**Warm and CO_2**
Control	32	30	26	24
Parental development	34	23	25	26
Grandparent development	35	31	29	30
Grandparent post-maturity	31	31	25	24
Continuous grandparent	24	19	22	23

Morphology	**Control**	**Warm**	**CO_2**	**Warm and CO_2**
Control	162	152	171	150
Parental development	169	144	170	176
Grandparent development	124	138	177	159
Grandparent post-maturity	175	177	187	179
Continuous grandparent	73	79	104	89

2.3.7 Statistical analysis

The effect of cross-generational thermal experience and juvenile treatment conditions on the physiology of juvenile coral reef fish was modelled in R (version 4.2.2) with linear mixed

effects model (using lmer within the LME4 package; Bates et al. 2014). Prior to model analysis, three significant outliers that were disproportionality heavier (>75% mean weight) than the rest of the data set, and one outlier that was deemed a significant outlier from raw data qqplots, were removed the data set. Resting oxygen consumption, maximum oxygen consumption, and aerobic scope were separately modelled as the dependant variable with cross-generation, temperature, and CO_2 treatments entered the model as fixed factors. Respirometry chamber ID, maternal linage (maternal grandfather and grandmother code A-F), and paternal linage (paternal grandfather and grandmother code A-F) were also included into the models as random factors. The inclusion of additional model covariates (density, tank number, parental clutch ID, or time of day (am/pm)) were sequentially explored and the model's goodness of fit was compared using analysis of variance (ANOVA; Pathak et al. 2013). It was concluded that these covariates did not improve model fit and were therefore not included in the final model (as per Fisher et al. 2015; LaMonica et al. 2021). Aerobic scope underwent a square root transformation to better adhere to the model assumptions.

Prior to model analysis of the behaviour data, the number of individuals under each behaviour score (1-5) was counted for each clutch . This count data was then analysed using a negative binomial regression model (glm.nb within the MASS package; Venables and Ripley 2002). The count value was used as the dependent variable with cross-generation, temperature, CO_2 treatment and the behaviour score (1-5) as fixed factors. Model assumptions of linearity, residual fit, and lack of over-dispersion and zero-inflation were met. Raw data values were plotted using ggplot2 to generate proportional count at each behaviour score.

The interrelationship between behaviour score and physiology (MO_{2rest} and aerobic scope) was also analysed with lmer. MO_{2rest} or aerobic scope were the dependant variable, and cross-generation, temperature, CO_2 treatments and behaviour score were entered into the model as fixed factors. Parental number (clutch ID; allowing identification of siblings) and

Respirometry chamber ID was included into both models as a random factor. Additional covariates of density, tank number, or time of day (am/pm) were explored through a stepwise process firstly using analysis of variance (ANOVA) and then looking at the Akaike's information criterion (AIC; Pathak et al. 2013). It was concluded that these covariates did not improve model fit and were therefore not included in the final model (as per Fisher et al. 2015; LaMonica et al. 2021).

The morphology of juveniles was analysed with lmer (as above). Firstly, significant kurtosis (5.25) was evident in the standard length data set, so data points outside the interquartile range were removed. However, this did not change the overall model results and significance. Standard length was then modelled as the dependant variable with cross-generation, temperature, and CO_2 treatments entered the model as fixed factors. Tank number, maternal linage, and paternal linage were also included into the models as random factors. Alongside all other model analysis, the additional covariates of density and parental clutch ID covariates were sequentially explored through a stepwise process using analysis of variance (ANOVA; Pathak et al. 2013). As while this comparison selected two models, the one including tank ID and density did not converge so the model including tank ID was next best and hence selected. Parental clutch ID did not improve model fit and was not selected for (as per Fisher et al. 2015; LaMonica et al. 2021). The physical condition model had log weight being the dependent variable with cross-generation, temperature, CO_2 treatments and log length entered into the model as fixed factors. Tank number, maternal linage, and paternal linage were also included into the models as random factors. Five influential outliers that were disproportionally impacting the model fit were examined using Cook's distance (Bochdansky et al. 2005; Bernal et al. 2022) and were removed from the final statistical analysis. Models were also run with all data included and did not change output significance.

During rearing there was natural mortality across treatment groups which is known to influence food availability and size structure (Holm et al. 1990; Brockmark and Johnsson 2010). Juvenile survival was analysed using an independent generalised linear mixed effect model glmer (Bates et al. 2015) with a binomial distribution and logit-link function (logistic regression). The number of fish present at the end of the experiment with versus the number of deaths was the dependent variable, while cross-generational thermal experience, temperature treatment, and CO_2 treatment were fixed factors. Maternal linage, and paternal linage were also included into the models as random factors. Covariates were not able to be included into the data set and were hence not included into the model.

For all lmer models, assumptions including linearity, normality and homogeneity of residuals were visually assessed with Q-Q plots and frequency distributions. Following construction of models, main effects were determined with a sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom. If there was a significant interaction between two or more factors, pairwise comparisons were made with estimated marginal means and Tukey method of p-value adjustment ($p<0.05$; lmer, glm.nb, and glmer models). Estimated marginal means and standard errors are depicted in the figures for physiological and morphological traits. The fitted data values are depicted in figures for the interrelationship between behaviour score and physiology.

2.4 Results

2.4.1 Physiology

The resting oxygen consumption (MO_{2rest}) of juvenile *A. polyacanthus* was significantly affected by juvenile CO_2 conditions ($F_{1,543}$= 4.22, P = 0.04; Figure 2.2a), but not juvenile temperature, cross-generation, or their interaction (Appendix 1: Table A1.1). MO_{2rest} was 2.61% lower for juveniles that developed in elevated CO_2 conditions than those reared in

present day control conditions (Control = 230 ± 7.96 mg O_2 kg^{-1} hr^{-1}; CO_2 = 224 ± 8.03 mg O_2 kg^{-1} hr^{-1}; mean ± SE).

Maximum oxygen consumption was greater for juveniles reared in warm temperatures compared to those reared in control temperatures ($F_{1,555}$ = 3.92, P = 0.05; Figure 2.2b; Control = 659 ± 45.2 mg O_2 kg^{-1} hr^{-1}; Warm = 680 ± 45.2 mg O_2 kg^{-1} hr^{-1}). However, the increase in maximum oxygen consumption was influenced by the cross-generation thermal experience of previous generations (Cross-generation*Temperature*CO_2: $F_{4,555}$ = 3.45, P = 0.008; Appendix 1: Table A1.2). Irrespective of juvenile treatment, the general pattern was that the Parent development, Grandparent post-maturation, and the Continuous grandparent cross-generation all had similar MO_{2max} across the 4 juvenile treatments, while Grandparent development and Control cross-generation showed variation. However, within the Warm temperature and Control CO_2 juvenile treatment, the Continuous grandparent cross-generation appeared to have a larger $MO_{2max,}$ but it was not significantly different to those within the same development treatment (all post-hocs P>0.05).

Juvenile aerobic scope was also higher for juveniles reared in warm temperatures compared to those reared in control temperatures ($F_{1,555}$ = 3.96, P = 0.047; Figure 2.2c; Control= 420.25, + 39.68 and – 37.89 mg O_2 kg^{-1} hr^{-1}; Warm = 441.00 + 40.58 and – 38.80 mg O_2 kg^{-1} hr^{-1}). However, the level of increase was dependant on the cross-generation thermal experience (Cross-generation*Temperature*CO_2: $F_{4,555}$ = 2.66, P = 0.032; Appendix 1: Table A1.3). This pattern was consistent with the pattern seen for MO_{2max} whereby juveniles from the Continuous grandparent cross-generation had a greater aerobic Scope than the Control lineage in Warm temperature and Control CO_2, however, this was not significant from pairwise comparisons (P = 0.16).

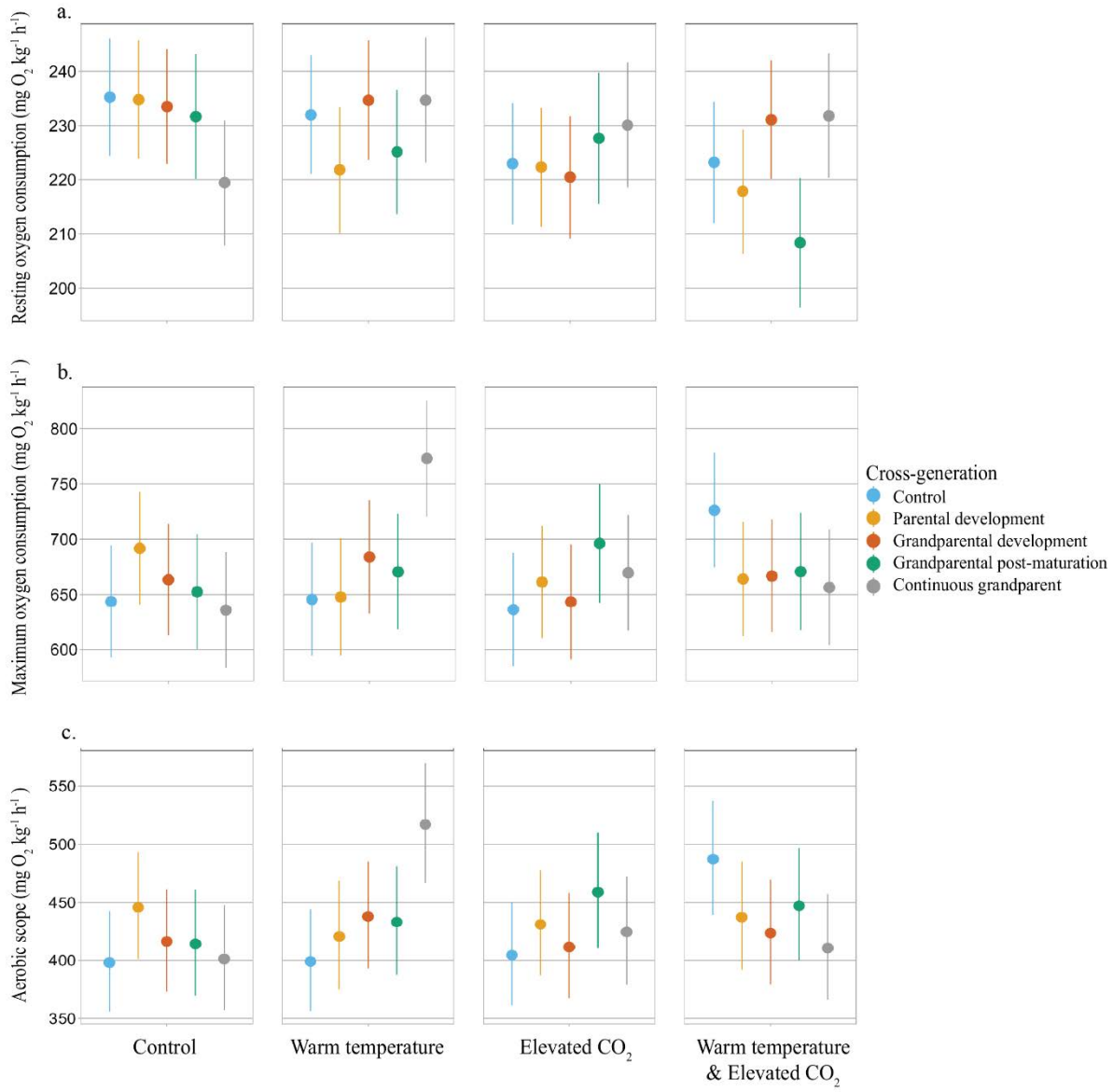

Figure 2.2. Resting oxygen consumption (a), maximum oxygen consumption (b) and absolute aerobic scope (c) of juvenile *A. polyacanthus* maintained at Control (28.5°C, 490 µatm); Warm temperature (30°C, 490 µatm); Elevated CO_2 (28.5°C, 825 µatm); or Warm temperature and Elevated CO_2 (30°C, 825 µatm) for 101-137 days post-hatching. All data is estimated marginal means in mg O_2 kg^{-1} hr^{-1}.

2.4.2 Behaviour

The behaviour score of juvenile *A. polyacanthus* was affected by juvenile developmental temperature (P = 0.02; Appendix 1: Table A1.4). In the Control treatment, juveniles predominantly had a behaviour score of 3 whereas in the Warm treatment, juveniles predominantly had a score of 4 (Appendix 1: Figure A1.1). Neither juvenile CO_2 treatment,

cross-generation, or interactions among treatments affected the behaviour score of juvenile *A. polyacanthus* (Figure 2.3).

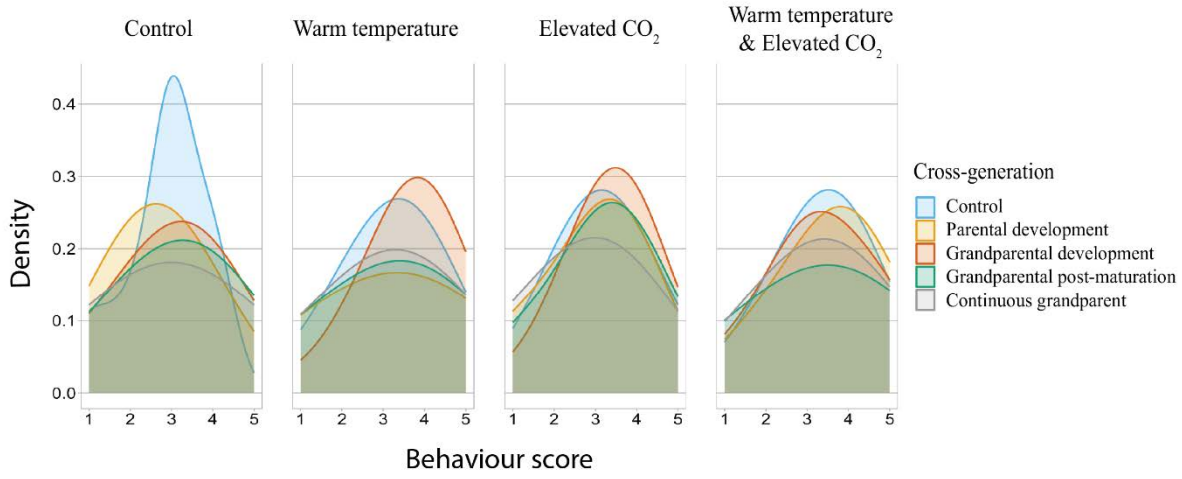

Figure 2.3. Proportional density juvenile *A. polyacanthus* at each combination behaviour (boldness and activity) score. Juveniles were maintained at Control (28.5°C, 490 µatm); Warm temperature (30°C, 490 µatm); Elevated CO_2 (28.5°C, 825 µatm); or Warm temperature and Elevated CO_2 (30°C, 825 µatm) for 101-137 days post-hatching.

2.4.3 Physiology and behaviour interrelationship

The potential for relationships between individual behaviour score and both MO_{2rest} and aerobic scope were explored. Overall, there was no relationship between score and MO_{2rest} found, however, juvenile CO_2 conditions did affect this relationship ($F_{1,501}$ = 6.45, P = 0.01). Specifically, under Control CO_2 conditions, shy (low score) fish had a higher MO_{2rest} than bold (high score) fish, whereas under Elevated CO_2 conditions shy fish had a lower MO_{2rest} than bold fish (Figure 2.4). Bold and active fish, regardless of development CO_2 conditions, had similar MO_{2rest}. There was also an interaction between behaviour score, cross-generational experience and juvenile CO_2 treatment ($F_{4,495}$ = 2.82, P = 0.025; Appendix 1: Table A1.5) due to the juveniles from Control cross-generation exhibiting the strongest interaction between MO_{2rest} and behaviour score depending on CO_2 conditions, and thus the overall pattern of CO_2 significance is driven largely by this cross-generational experience (Appendix 1: Figure A1.2).

The relationship between aerobic scope and behaviour score was significantly influenced by juvenile temperature ($F_{1,500}$ = 5.31, P = 0.02; Figure 2.5; Appendix 1: Table A1.6). Specifically, under Warm temperature, shy (low score) fish had a higher aerobic scope than bold (high score) fish, whereas under Control temperature aerobic scope was similar for shy and bold fish. Bold and active fish (with a high behaviour score) had similar aerobic scope regardless of developmental temperature.

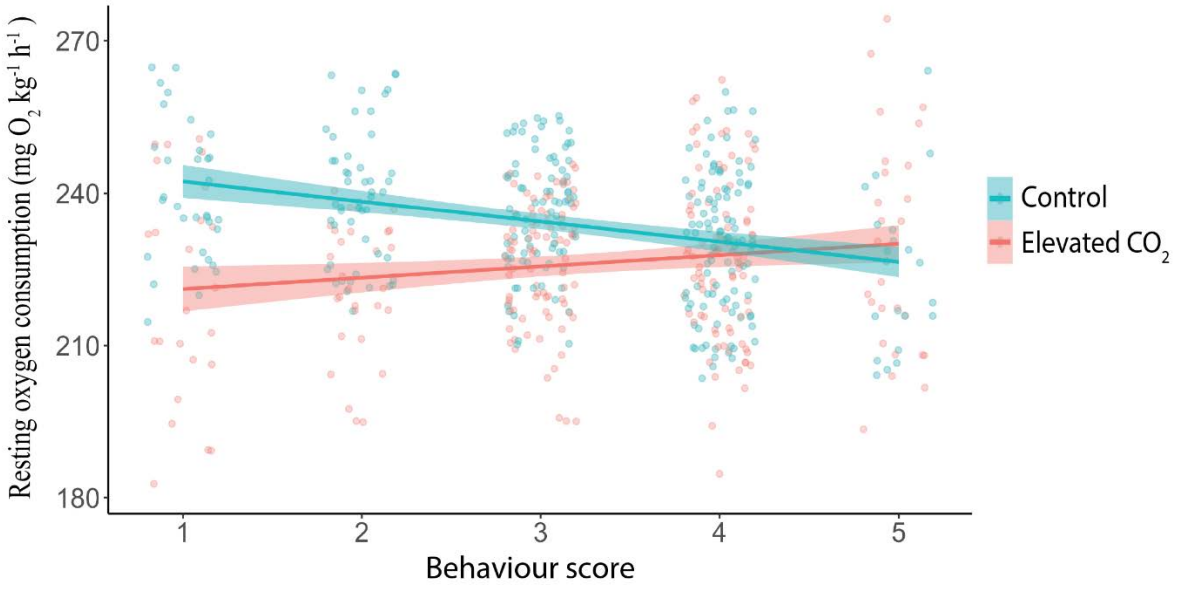

Figure 2.4. Resting oxygen consumption of juvenile *A. polyacanthus* maintained at Control (490 µatm, 28.5°C or 30°C); Elevated CO_2 (825 µatm, 28.5°C or 30°C) for 101-137 days post-hatching at each behaviour (boldness and activity) score. Fitted data points are displayed with a linear trendline (Control: y = 250 – 4x, R^2 = 0.10; Elevated CO_2: y = 220 + 2.2x, R^2 = 0.02).

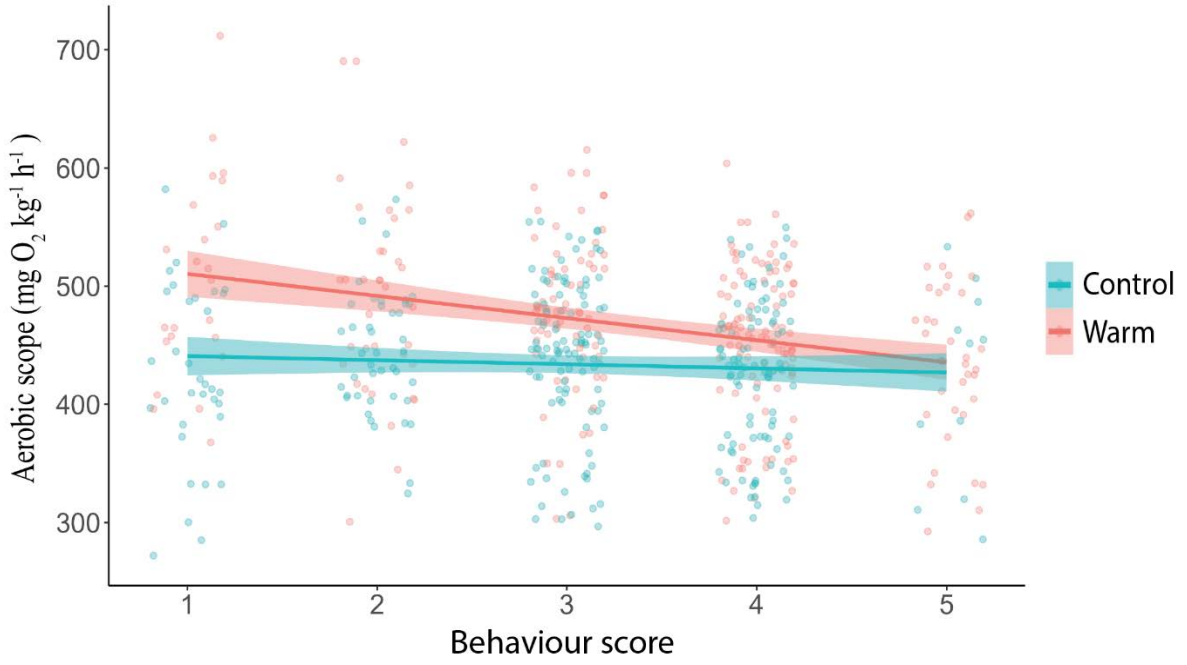

Figure 2.5. Aerobic scope of juvenile *A. polyacanthus* maintained at Control (28.5°C); Warm temperature (30°C) for 101-137 days post-hatching at each behaviour (boldness and activity) score. Fitted data points are displayed with a linear trendline (Control: y = 440 – 3.5x, R^2 = 0.003; Warm: y = 530 + 19x, R^2 = 0.086).

2.4.4 Morphology

Linear growth (standard length) of juvenile *A. polyacanthus* was affected by juvenile temperature ($F_{1,128}$ = 8.15, P < 0.005), CO_2 ($F_{1,123}$ = 6.80, P = 0.01), and cross-generational thermal experience ($F_{4,93}$ = 8.44, P < 0.001; Figure 2.6a), but not their interactions. Juveniles that developed in the Warm temperature were on average 1.86% shorter than those in the Control temperature (Control = 32.2 ± 1.35 mm; Warm = 31.6 ± 1.35 mm; mean ± SE). Juveniles reared in Elevated CO_2 were also shorter than those reared in Control conditions by 1.56% (Control = 32.1 ± 1.36 mm; CO_2 = 31.6 ± 1.35 mm; mean ± SE). Irrespective of juvenile treatment conditions, juveniles from the Control cross-generation (30.5 ± 1.38 mm; mean ± SE) were 6.73 and 6.15% shorter compared to the Grandparental development and Continuous grandparent cross-generation respectively (P < 0.001). Cross-generational thermal experience did not interact with temperature or CO_2, nor was there a three-way interaction between Cross-generation, juvenile temperature, and CO_2 (Appendix 1: Table A1.7).

Juvenile physical condition (weight for a given standard length) was also influenced by juvenile temperature ($F_{1,154}$ = 23.61, P < 0.001), CO_2 ($F_{1,149}$ = 4.28, P = 0.04), and cross-generational thermal experience ($F_{4,157}$ = 3.15, P = 0.02) but there were no interactions among factors (Figure 2.6b). Contrary to standard length, juveniles that developed in Warm temperature were on average in better condition (heavier for a given weight) than those in present day Control temperature (Control =1.108 ± 0.03; Warm = 1.134 ± 0.03). Juveniles reared in Elevated CO_2 were also in better condition than those reared in Control conditions (Control = 1.115 ± 0.03 mm; CO_2 = 1.127 ± 0.03 mm; mean ± SE). The effect of cross-generationthermal experince on juveniles was seen in those from Parental development cross-generation being in better condition than those fish from the Grandparental development cross-generation (P = 0.04). There were no interactions were observed between any of the factors (Appendix 1: Table A1.8).

Survival was influenced by juvenile treatment and cross-generational experience (Cross-generation*Temperature*CO_2: X^2 = 11.45, df = 4, P = 0.022; Appendix Figure A1.3). Within the Control juvenile treatment, the probability of survival for juveniles from the Grandparental development cross-generation was lower than juveniles from the Control or Parental development cross-generation (Grandparental development = 0.70 ± 0.09; Control = 0.85 ± 0.05; Parental development = 0.85 ± 0.05; probability ± SE; P < 0.01) . In addition, the probability of survival for juveniles from the Grandparental development cross-generation was lower (P < 0.002) in the Control juvenile treatment (0.70 ± 0.09) and Warm temperature treatment (0.70 ± 0.09) compared to the Elevated CO_2 treatment (0.90 ± 0.05).

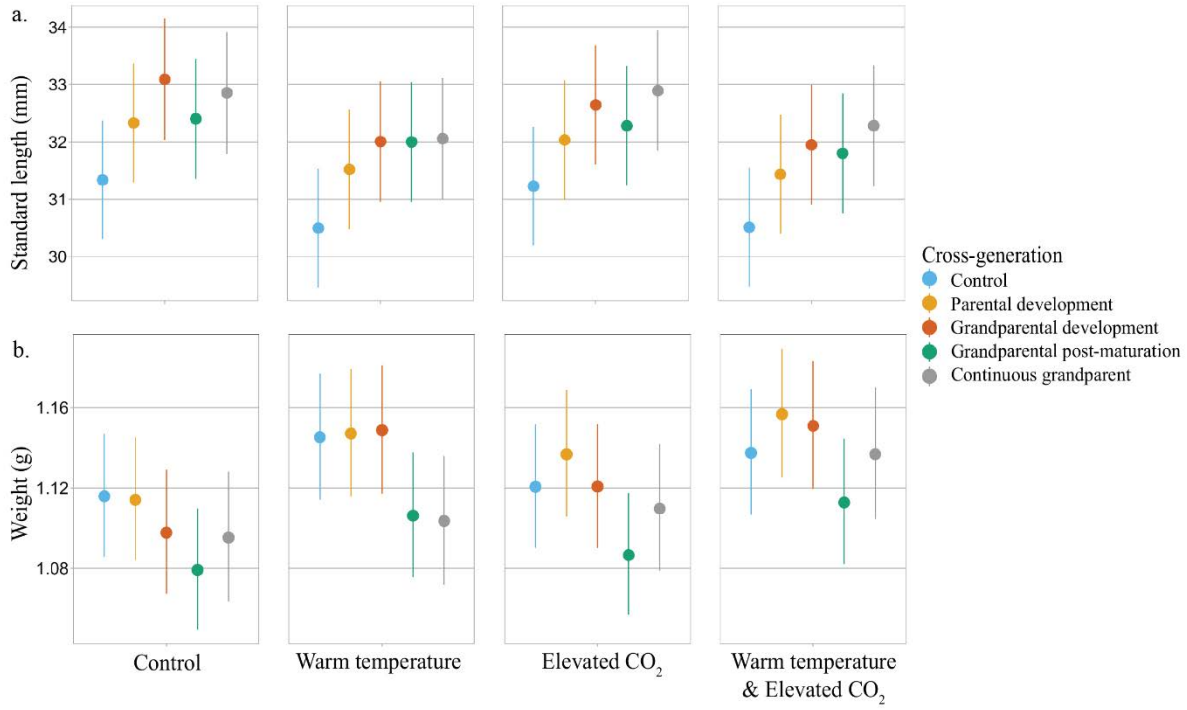

Figure 2.6. Standard length (a) and physical condition in terms of weight for a given standard length (b) of juvenile *A. polyacanthus* maintained at Control (28.5°C, 490 µatm); Warm temperature (30°C, 490 µatm); Elevated CO_2 (28.5°C, 825 µatm); or Warm temperature and Elevated CO2 (30°C, 825 µatm) for 101-137 days post-hatching. All data is the estimated marginal means ± SE. In the case of physical condition this estimated marginal mean of weight is for the average standard length of 31.2 mm.

2.5 Discussion

Phenotypic plasticity was an important mechanism that *A. polyacanthus* employed to maintain or improve performance under altered environmental conditions such as rapid climate change (Chevin et al. 2010; Hoffmann and Sgró 2011; Munday et al. 2013b; Donelson et al. 2018; Fox et al. 2019). This study found that developmental conditions, specifically Warm temperature, influenced the largest number of performance metrics. Compared to juveniles from the Control juvenile treatment, juvenile *A. polyacanthus* that developed in Warm temperature were shorter, bolder, and in better physical condition, whereas juveniles that developed in Elevated CO_2 were shorter but had a lower resting oxygen consumption rate. Environmental conditions experienced by previous generations also influenced juvenile performance. Exposure to warm temperatures in the parental or grandparental generations produced juveniles that were larger and in better condition (carry-over effects) and this did not alter under the novel environmental

stressor of elevated CO_2. There was also some limited evidence for transgenerational plasticity with enhanced maximum oxygen consumption (and subsequently aerobic scope) by juveniles from the Continuous grandparent cross-generation. Interestingly, there was no interaction between ocean warming and acidification for any of the performance traits measured, suggesting that future climate change conditions will likely act additively.

2.5.1 Developmental temperature effects

Development in warm temperature resulted in shorter and bolder fish that were in better physical condition, regardless of developmental CO_2 and previous cross-generational thermal experience. All these phenotypic changes have the potential to provide enhanced survival in nature. For example, bolder and more active individuals can have increased foraging success (Metcalfe et al. 2016) and individuals with enhanced physical condition may be selected for in nature through reduced predation (Booth and Beretta 2004; Hoey and McCormick 2004; Poulos and McCormick 2015). Theory suggests that behavioural, physiological, and life history traits are expected to covary, and due to trade-offs combinations of traits will exist along a fast-slow continuum (Pace-of-life theory; Réale et al. 2010; Binder et al. 2016; Montiglio 2018; Tüzün and Stoks 2022). The average composition of traits, including increased behaviour score, growth in terms of weight for a given standard length and the aerobic metabolism for the Continuous grandparent cross-generation at Warm development temperature suggests a shift towards fast pace-of-life. This risk-prone, fast-paced life strategy would enable individuals to outcompete conspecifics enabling greater access to resources such as food, habitat, and mates (Goulet et al. 2017; Hämäläinen et al. 2021). This covariance of life history traits with thermal exposure is expected to be stronger for early-life stages and in predictable stable environments (Polverino et al. 2018; Hämäläinen et al. 2021). However, this theory has

only been explored in a limited number of studies and support has been mixed (reviewed in Gopal et al. 2023).

Under Warm temperature there was an overall relationship that fish with higher behaviour score had lower aerobic scope, but this pattern was not seen under Control temperatures. Often it is expected that individuals with a greater aerobic scope have a greater capacity for bold and highly active behaviours (Biro and Stamps 2010; Metcalfe et al. 2016). Our results may suggest a trade-off in these performance traits in which individuals can generally either have a high aerobic capacity or be bold and active but not have the energetic capacity for both. In contrast, Laubenstein et al. (2019) found no clear relationship between aerobic capacity and behaviour measured in terms of an anti-predator response, until juvenile *A. polyacanthus* were reared under warm temperature and elevated CO_2 combined. In this case, individuals with a lower antipredator response had a higher aerobic scope under combined stressor conditions (Laubenstein et al. 2019). This may indicate that a greater aerobic capacity is beneficial for survival behaviours such as anti-predator response, and that defensive behaviours may be more sensitive to environmental change compared to bold and active exploratory behaviours as tested in this experiment (Metcalfe et al. 2016). In any case, boldness is not considered to be without risks and costs and while it can increase access to resources it also can increase the risk of predation (Nash and Geffen 2012).

The shift in aerobic metabolic traits depending on water temperatures experienced during early life development has been well studies in this species and other coral reef fish. A common pattern from developmental acclimation to temperatures 1-3°C above summer temperature is an improved (lower) resting metabolic rate (Donelson et al. 2011; Donelson and Munday 2012; Grenchik et al. 2013), which I did not observe. Comparatively, performance (physiology and behaviour) in this study was only measured under their respective developmental treatments. Therefore, unlike previous studies (Donelson et al. 2011; Donelson

and Munday 2012; Grenchik et al. 2013), conclusions cannot be made about how juveniles would perform under a range of thermal conditions (e.g. the other juvenile treatments). Broader thermal testing would be required to gain a better understanding of thermal performance.

2.5.2 Developmental CO_2 effects

The effect of elevated CO_2 on resting metabolic rate has been variable, with species showing no change (Lefevre 2016), increases (Munday et al. 2009; Miller et al. 2012; Couturier et al. 2013; Laubenstein et al. 2018; McMahon et al. 2020), and decreases (Rummer et al. 2013; Pimentel et al. 2014) as was found in this study. The reduced resting metabolic rate under elevated CO_2 conditions observed in this study and previously (Rummer et al. 2013) could be due to *A. polyacanthus* living their entire life on the reef where CO_2 fluctuations are naturally experienced (Hannan et al. 2020) and consequently might be adapted to moderate increases in CO_2. This reduction in resting metabolic rate would be expected to provide energetic savings that can be directed to other activities such as growth, and while there was some improvement in physical condition (~1%), I also observed a reduction in standard length (~1.56%) with elevated CO_2. Reductions in juvenile growth following developmental exposure to elevated CO_2 coincides with previous studies (Baumann et al. 2012; Miller et al. 2012; McMahon et al. 2019) and concurrent shift in metabolic traits have not always been observed when measured (Miller et al. 2012). Energetic trade-offs may have occurred in elevated CO_2 between producing phenotypic plasticity of reduced resting metabolic rate and improved physical condition, at a cost to standard length.

While elevated CO_2 had no significant effect on juvenile behaviour in isolation, it did significantly influence the interaction between resting oxygen consumption and behaviour score. Juveniles that were bolder and more active tended to have similar resting oxygen consumption regardless of CO_2 conditions, however, shyer individuals in elevated CO_2 had a

lower resting oxygen consumption than those in control conditions. This pattern perhaps suggests that the reduced resting oxygen consumption observed in elevated CO_2 is driven by the plasticity of shy individuals. Additionally, under elevated CO_2 there is stronger support for the "performance model" whereby resting oxygen consumption determines the energy available to invest in activities such as bold and active behaviours (Careau et al. 2008), which is also supported by previous research (Biro and Stamps 2010; Laubenstein et al. 2018).

No interactions between ocean warming and acidification was observed on the performance traits measured in this study. This absence of an interaction for standard length, which was significantly affected by both temperature and CO_2, suggests that future climate change conditions will not act synergistically, but rather additively. Additive effects of warm temperature and elevated CO_2 coincides with previous studies on juvenile reef fish (Miller et al. 2012), adult coral reef fish (Munday et al. 2009), and more broadly in marine ectotherms (Lefevre 2016). Additive effects likely mean a predictable burden of multiple stressors on coral reef fish under future climate change conditions from single stressors experiments (Lefevre 2016; McMahon et al. 2019).

2.5.3 Cross-generational effects

Morphological traits were not only influenced by juvenile developmental temperature (i.e. Warm temperature reduced standard length while maintaining physical condition) but also by the historic cross-generational temperature conditions. I found that juveniles from the Control cross-generation were smaller than juveniles from the Grandparental development and Continuous grandparent cross-generation, regardless of juvenile developmental treatment. These results suggest that prior exposure to warm temperatures in the grandparental generation during development or post-maturation was beneficial, resulting in longer fish within the F_3 generation. However, there was a general trend that Grandparental development cross-

generation had lower survival (although this varied across juvenile treatment conditions). Interestingly, across all developmental treatments, juveniles from the Parental development cross-generation tended to be in better condition than those from the Grandparental development cross-generation but were not different in standard length. The transfer of high condition or greater standard length through carry-over effects can be adaptive in nature as it enhances offspring performance regardless of the offspring environment and therefore does not require complex machinery to assess environmental conditions (Jablonka et al. 1995; Bonduriansky and Crean 2018). Spinks et al. (2022) on the other hand found negative carry-over effects in the previous generation of this experiment whereby prior exposure to warming in the F_1 generation decreased offspring length and condition in the F_2 generation across all offspring developmental temperatures. Both lower body size (Pörtner and Knust 2007; Forster et al. 2012; Leiva et al. 2019) and increased physical condition (Robinson et al. 2008) has been found to correlate to improved thermal tolerance of aquatic organisms. Variation in which morphological trait is selected for and carried over to the next generation can arise from differences in the micro-environments such as diurnal temperature fluctuations, metabolic costs, or other intrinsic factors (Bonduriansky and Crean 2018).

Evidence for transgenerational plasticity was found with enhanced aerobic performance by juveniles from the Continuous grandparent cross-generation that developed at Warm temperature and Control CO_2. However, it was not significantly different to other cross-generations within the same juvenile developmental treatment. Bernal et al. (2022) also found enhanced aerobic capacity (maximum oxygen consumption and aerobic scope) in juvenile *A. polyacanthus* following grandparental exposure to Warm temperature (+1.5°C), but this was observed to be a carry-over effect with benefits in both juvenile developmental temperatures tested. These differing results may be explained by a difference in how these experiments were conducted across generations; in Bernal et al. (2022) the grandparent warm treatment also

reproduced at warm conditions in the F_2 generation, while in the present study F_2 fish were returned to control conditions post-maturation at 1.5 years. This means that F_3 offspring in Bernal et al. (2022) experience embryogenesis at +1.5°C and the enhanced metabolic traits may be developmental plasticity in addition to cross-generational effects.

Unexpectedly, the enhanced aerobic capacity produced by Continuous grandparent cross-generation did not occur in juveniles that developed at Warm temperature with and without CO_2 exposure. One possible explanation is that elevated CO_2 induced developmental plasticity that reduced resting metabolic rate and this outweighed any transgenerational plasticity on maximum metabolic rate or aerobic scope (Shama and Wegner 2014; Burggren 2015). Individual and species with higher maximum metabolic rate and aerobic capacity tend to have a higher standard metabolic rate (and hence resting metabolic rate to support the required physiological machinery (Careau et al. 2008; Biro and Stamps 2010; Killen et al. 2016; Metcalfe et al. 2016). This pattern highlights the importance of directly testing plastic outcomes in a range of conditions as phenotypic outcome are context dependent with transgenerational plasticity.

By reflecting on the cross-generational experiences that produce carry-over effects and transgenerational plasticity in this study I aim to better understand the circumstances under which various types of plasticity occur (Reed et al. 2010; Herman et al. 2014; Leimar and McNamara 2015). The result showing transgenerational plasticity in aerobic performance only when Continuous grandparent cross-generation conditions and juvenile conditions matched are in line with the theory that transgenerational plasticity will manifest when conditions are changing predictability between generations (Salinas et al. 2013; Donelson et al. 2018). All other cross-generations in this study did not produce the same effect, perhaps due them only containing one 1.5 year warming exposure period after which conditions returned to control. Yet, these same thermal exposure periods resulted in carry-over effects to produce larger

offspring. These parental carry-over effects may arise from a range of mechanisms including the transfer and inheritance non-genetic epigenetic cues (DNA methylation), hormones, or nutritional resources (Jablonka et al. 1995; Bonduriansky and Day 2009; Miller et al. 2012). Regardless of the mechanism, the phenotype of a larger body size is expected to be beneficial under all environmental conditions (Sogard 1997) and perhaps any costs of production are outweighed by benefits. In contrast, developing and an enhanced physiological system could either be costly to develop if not required in the juvenile environment (e.g., a high maximum oxygen consumption requires more energy and resources).

Due to the complex nature of marine environments, multiple stressor experiments are important to determining if unpredictable interactions occur with single stressors alone. This research takes a novel step forward and shows that the impact of elevated CO_2 did not differ depending on whether previous generations experienced ocean warming. This meant that any potential benefits of historical thermal plasticity were realised regardless of elevated CO_2 conditions (length and condition), but equally any likely beneficial (reduced resting metabolic rate), or negative effects (reduced length), also remained. Overall, the largest diversity of F_3 juvenile traits were influenced by developmental temperature implying that the thermal environment experienced in early life has a greater influence on juvenile phenotype than that of previous generations. For a tropical reef species that already lives close to its thermal maximum and are typically non-dispersive and site-attached, this is critical to understanding how the long-term effects of multiple environmental stressors across multiple generations will affect the performance and persistence in the future.

Chapter 3 – The interactive effects of ocean warming, suspended sediment, and the role of cross-generational thermal plasticity

Proposed authors: Jasmine S. Cane, Shannon J. McMahon, Andrew S. Hoey and Jennifer M. Donelson

3.1 Abstract

Environmental change is a major driver of contemporary coral reef communities. Both global and local environmental change has and will continue to occur over multiple generations, which may allow for cross-generational plasticity across a range of performance traits. Yet few studies have considered how long-term exposure of global ocean warming might influence the response and recovery of a marine fish following a localised pulse suspended sediment which are occurring more frequently with increasing coastal development. Three generations of the tropical damselfish *Acanthochromis polyacanthus* were each exposed to +1.5°C above present-day average during their early development. The third generation *A. polyacanthus* was additionally exposed to a suspended sediment treatment (0 or 50 mg L^{-1}) for 53 to 81 days, before being returned to no sediment conditions for a further 16 to 37 days. Exposure of *A. polyacanthus* to elevated suspended sediments for 53 to 81 days post-hatching led to considerable gill remodelling, characterised by increases in filament and lamellae width, a greater incidence of hyperplasia and epithelial lifting, and a lower incidence of mucus. The combined effects of warm temperature and suspended sediment were varied and dependent on the trait of interest, being synergistic or antagonistic on gill morphology and physical condition, but additive on standard length. Several traits also differed depending on the cross-generational thermal experience of parent and grandparent generations. Once suspended sediment was removed, differences in standard length and physical condition diminished within 2-5 weeks. These results suggest that juvenile coral reef fish may be able to recover relatively quickly following a pulse suspended sediment event.

3.2 Introduction

Anthropogenic activities are impacting marine systems globally (Devlin and Brodie 2005; Foley et al. 2005; Gissi et al. 2021). The release of CO_2 into the atmosphere through the burning of fossil fuels is causing an enhancement of the greenhouse effect such that ocean temperature has already increased by around 1°C and is predicted increase a further 1-3°C by 2100 across the world's ocean (IPCC 2014, 2021). Ectothermic marine organisms are especially sensitive to ocean warming as they lack internal temperature regulation (Huey and Stevenson 1979; Huey and Kingsolver 1989). Consequently, elevated temperature has been found to impair the physiological (Nilsson et al. 2009; Donelson et al. 2011; Johansen and Jones 2011; Motson and Donelson 2017; Slesinger et al. 2019), morphological (Munday et al. 2008b; Rogers et al. 2011; Motson and Donelson 2017; Watson et al. 2018; Spinks et al. 2019), and behavioural (Lienart et al. 2014; Motson and Donelson 2017; Watson et al. 2018) performance of marine fish. However, few studies have considered how ocean warming interacts with local anthropogenic activities such as elevated suspended sediment.

While the input of terrestrial sediment into marine systems is a natural process, coastal development has resulted in significant increases in the input of terrestrial sediments, and subsequently rates of sedimentation and suspended sediment loads in coastal marine ecosystems (Kroon et al. 2012). Further, the inputs of terrestrial sediments are likely to increase further with predicted increases in extreme rainfall events under future climate change models (IPCC 2021) and ongoing agricultural, urban, and coastal development, catchment modification, and dredging (Foley et al. 2005; Brodie et al. 2012; Jones et al. 2016). Several studies have reported declines in species richness, composition, and abundance of coral reef fish communities in areas with elevated suspended sediment conditions (Fabricius et al. 2005; Mallela et al. 2007; Moustaka et al. 2018). While this may in part be due to the indirect effects of habitat loss (Fabricius 2005), sediment can reduce visual acuity (Utne-Palm 2002; Wenger

et al. 2012; O'Connor et al. 2016), cause direct mechanical gill damage (Lake and Hinch 1999; Wong et al. 2013; Hess et al. 2017), and reduce the aerobic capacity (Hess et al. 2017) in some reef fish.

Uptake of oxygen via the gills is critical to support aerobic metabolism in fishes and can be adversely impacted by both acute to short-term suspended sediment exposure and chronic ocean warming. For example, elevated environmental temperatures increase the rate of metabolic processes, and hence the demand for oxygen increases while less oxygen is available in the surrounding water (Clarke and Fraser 2004; Pörtner and Knust 2007). To compensate for this increase oxygen demand, several freshwater and marine fishes have been shown to remodel the morphology of their gills (freshwater: Sollid et al. 2005; Sollid and Nilsson 2006; Nilsson 2007; Phuong et al. 2017; Gibbons et al. 2018; Foyle et al. 2020; Mohamad et al. 2021; marine: Bowden et al. 2014; Hess 2019; Johansen et al. 2021). For example, a decrease in gill epithelium thickness, which reduces gas diffusion distance, and an increase in gill surface area have been reported as modifications to increase gas exchange efficiency following exposure to elevated temperatures (e.g., Sollid et al. 2005; Phuong et al. 2017). Elevated levels of suspended sediment have also been found to induce gill remodelling, with the changes linked to providing protection from mechanical abrasion (Agamy 2013) and to increase gas exchange (Mallatt 1985). Additionally, epithelial lifting (Au et al. 2004), thickening (hyperplasia/hypertrophy) of the lamellae and filament epithelium (Hess et al. 2015, 2017; Cumming and Herbert 2016), and excess mucus production (Hess et al. 2015) have all been observed as likely forms of protective remodelling and defence responses to suspended sediments. However, the role of these protective mechanisms can also result in reduced gas exchange capacity of the lamellae (Mallatt 1985), as well as reduced interlamellar space and water flow across the gill surface (Au et al. 2004).

Developing in elevated water temperature (Munday et al. 2008b; Rogers et al. 2011; Motson and Donelson 2017; Spinks et al. 2019) and suspended sediment (Utne-Palm 2002; Wenger et al. 2012; O'Connor et al. 2016) conditions can result in reduced growth rate of juvenile fishes. This reduction in growth has generally been related to reduced aerobic scope under elevated temperatures (Nilsson et al. 2009; Donelson et al. 2011; Johansen and Jones 2011; Motson and Donelson 2017; Slesinger et al. 2019), and elevated suspended sediment (Hess et al. 2017), supporting the theory that less energy is available for non-essential processes (Pörtner and Peck 2010; Brauner et al. 2019). Oxygen is also a key mechanisms driving temperature-size rule (Forster et al. 2012) whereby under conditions of high temperature, ectothermic organisms often grow and develop faster but are smaller at maturation (Arendt 2011; Trip et al. 2014; Álvarez-Noriega et al. 2023). Alternatively, the Gill-Oxygen Limitation Theory (GOLT) proposes that the reduction in body size under elevated water temperature is thought to be a consequence of an insufficient gill surface area, and thus inability to meet increased oxygen demands (Pauly and Cheung 2018). However, both theories have had contrasting levels of support. For example, gill remodelling that increases gill surface area may support the metabolic requirements under elevated temperatures (Johansen et al. 2021), but modification may also be energetically costly with negative flow on effects to growth performance (Pauly 1979; Hughes 1984).

Marine systems may be exposed to multiple environmental stressors simultaneously or sequentially making the response to future environmental conditions difficult to predict based on single stressor studies (Ghedini et al. 2013; Gissi et al. 2021). For instance, exposure to one stressor may prime an organism's system (Gunderson et al. 2016), or make them more susceptible (Nyström et al. 2001), to a second superimposed stressor. As a result, a limited number of studies have considered the combined impacts of ocean warming and suspended sediment, and these stressors have been found to interact synergistically (Mari et al. 2021),

additively, and antagonistically (Hess 2019). For example, Mari et al. (2021) found that exposure to warm temperature and sediment during the embryo development of four Arctic char (*Salvelinus alpinus*) populations, reduced and significantly interacted resulting in a synergistic reduction in size at hatchling. On the other hand, Hess (2019) found that for juvenile coral reef damselfish (*Acanthachromis polyacanthus*), warm temperature and sediment acted independently on gill morphology, antagonistically on aerobic performance, and only suspended sediment influenced predator escape performance. These complex interactions highlight the need for multi-stressor research to fully comprehend the complexities of environmental change.

As well as a limited understanding of the combined effects of elevated temperature and suspended sediment, little is known if the effects of elevated suspended sediments persist or may be reversed if sediment levels return to ambient levels. Exposure to elevated suspended sediments tend to be acute (e.g., following extreme rain and flooding events, Devlin and Brodie 2005; Fabricius et al. 2013; or dredging and shipping activities, Jones et al. 2016). Following exposure to suspended sediments, one temperate marine fish has been found to recover from reduced osmotic activity (within 10 days; Li and Shen 2012). Whether coral reef fish are able to recover and over what time frame following extensive gill changes, and physiological and morphological impacts due to suspended sediment exposure is currently unknown (Sutherland and Meyer 2007). However, we do know that fish are capable of accelerated growth following a period of stress when more favourable conditions arise (Nicieza and Metcalfe 1997; Metcalfe and Monaghan 2001; Ali et al. 2003; Donelson et al. 2012a; Spinks et al. 2019)

Environmental change has and will continue to occur over multiple generations, which provides the opportunity for cross-generation plasticity. This includes transgenerational plasticity (an offspring's phenotype is a response to the interaction between the current and previous generation's environmental conditions; Salinas et al. 2013; Donelson et al. 2018) and

carry-over effects (an offspring's phenotype is a response to the previous generation's environmental conditions regardless of offspring environment; Jablonka et al. 1995; Bonduriansky and Crean 2018; Donelson et al. 2018). To date, cross-generation plasticity to warm temperature has been found to improve the metabolic (Donelson et al. 2012b; Bernal et al. 2022), reproductive (Donelson et al. 2012a; Spinks et al. 2021), and growth performance (Shama et al. 2014; Spinks et al. 2022) of marine fish. There is also growing evidence that early-life exposure to an environmental stressor is more influential in the transfer of information across generations (Burton and Metcalfe 2014; Shama et al. 2014; Donelson and Munday 2015; Donelson et al. 2018; Spinks et al. 2021). However, the relative significance of warm temperature exposure during the development of parents or grandparents is not yet fully understood as it requires complex multigenerational experimental designs (West-Eberhard 2003; Angilletta 2009; Donelson et al. 2018; Spinks et al. 2021). Cross-generation plasticity may also influence how an organism responds to the sequential exposure to a novel stressor. Prior exposure to elevated temperature has previously been found to influence the tolerance to heavy-metals in a range of marine species (Nyström et al. 2001) and suggests that elevated temperature may also influence the interactive response with suspended sediment. Highlighting the importance of considering the complexities of multiple stressors impacts and the potential importance of plasticity to coping with climate change.

This study builds on previous muti-generational work to investigate the effect of developmental exposure to +1.5°C above present-day average in the F_1 and F_2 generations in the tropical damselfish *Acanthochromis polyacanthus*. Specifically, how this influences the morphology, and gill structure of juveniles exposed to Control temperature – No sediment (28.5°C, 0 mg L^{-1}), Warm temperature – No sediment (30°C, 0 mg L^{-1}), Control temperature – Suspended sediment (28.5°C, 50 mg L^{-1}), Warm temperature – Suspended sediment (30°C, 50 mg L^{-1}). Juvenile *A. polyacanthus* developed in these environmental conditions for 53 to 81

days post-hatching (dph) at which time a sub-sample of individuals were selected, and their body and gill morphology traits quantified. All suspended sediment was then removed from the system and juveniles were maintained for a further 16 to 37 days before body morphology traits were measured.

3.3 Materials and Methods

3.3.1. Study species

The spiny chromis, *Acanthochromis polyacanthus*, is a widespread Indo-Pacific coral reef damselfish with populations ranging from the southern Coral Sea to the southern Philippines. *A. polyacanthus* form monogamous breeding pairs that last throughout the Austral summer breeding season (most often between October and February; Robertson 1973) with both parents defending and caring for the eggs which are laid on the substrate (Kavanagh 2000). *A. polyacanthus* lack a dispersive pelagic larval phase and instead, juveniles remain with their parents up to 45 days after hatching (Kavanagh 2000).

3.3.2 Cross-generational experimental design

This project utilises the multigenerational experiment described in **Chapter 2**. Briefly, adult *A. polyacanthus* were collected from the Palm Island region (18°40-45'S, 146°34-41'E) in 2014, and from Bramble Reef (18°24′S, 146°42′E) in 2015 and transported to the Marine and Aquaculture Research Facility at James Cook University, Townsville, Australia (F_0 generation; see Spinks et al. 2021 for more details). The first clutch (F_1) from the six wild-caught pairs (F_0) were split at hatching into two temperature treatments: either 1) a 'Control' treatment in which water conditions simulated seasonal temperature cycles (winter: 23.2°C, summer: 28.5°C) for the Palm Islands region of the Great Barrier Reef (GBR; AIMS 2016), or 2) an elevated temperature treatment (i.e., 'Warm') in which water conditions simulated 1.5°C warmer than

present-day (winter: 24.7°C, summer: 30°C, as per Spinks et al. 2021). These F_1 fish were maintained at these 2 temperature treatments throughout development (hatching to 1.5 years), and at 1.5 years each group was divided further into the two temperature treatments for post-maturation (1.5 to 3 years) creating 4 treatments (Figure 3.1). The first clutch of offspring (F_2) produced by F_1 breeding pairs (made from male and female fish maintained at the same treatment conditions throughout the 3 years) were split at hatching into the 2 temperature treatments and reared to 1.5 years as described above for the F_1 generation, creating 8 treatments (Figure 3.1; Yasutake 2019). At 1.5 years they were transferred to Control temperature and maintained until 3 years of age. Further details of these two generations (F_1 and F_2), the aquarium set up, and environmental conditions can be found in **Chapter 2**.

Adult F_2 fish were allocated into non-sibling pairs with fish from the same treatment beginning in late August 2021 (at ~3 years of age) and where possible, further pairs were made in December 2021. Of the possible eight cross-generational thermal experiences from a fully-orthogonal design, three were used in this experiment (Figure 3.1): **Control** (present-day conditions throughout F_1 and F_2 generations, n = 4 pairs); **Parental development** in Warm temperature (developmental exposure of F_2 parents to +1.5°C from hatching to 1.5 years, n = 3 pairs); **Grandparental development** in Warm temperature (developmental exposure of F_1 grandparents to +1.5°C from hatching to 1.5 years, n = 4 pairs; Figure 3.1).

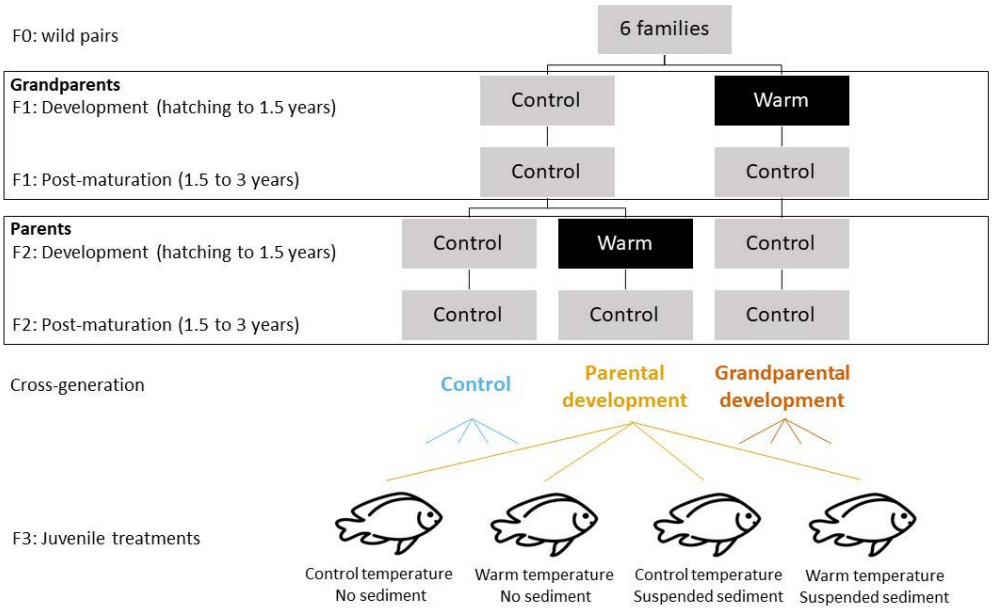

Figure 3.1. Cross-generation experimental design outlining the thermal experience of F_1 grandparent and F_2 parent generations. F_3 offspring from the three cross-generational thermal experiences were split orthogonally between 4 development treatments including at Control temperature – No sediment (28.5°C, 0 mg L^{-1}), Warm temperature – No sediment (30°C, 0 mg L^{-1}), Control temperature – Suspended sediment (28.5°C, 50 mg L^{-1}), Warm temperature – Suspended sediment (30°C, 50 mg L^{-1})

3.3.3 F_3 juvenile experimental design

When summer average water temperature (Control: 28.5°C) was reached in November 2021, terracotta pots of F_2 pairs were checked daily for newly laid egg clutches. Clutches produced early in the season (January-March 2022) were utilised for **Chapter 2**, and any clutches after March were used for this experiment. Once an egg clutch was recorded, it was checked daily for the presence of hatched offspring (F_3), which generally occurs in the afternoon for this species around 9 days later (Donelson et al. 2010). Newly hatched offspring from all clutches were randomly divided into groups of 20 individuals and placed in 11 L tanks which were maintained at one of 4 juvenile treatments. To facilitate a slow transition from natal to the treatment conditions, water from the juvenile treatment tank was added into the holding containers over 4 to 12 hours after which juveniles were then released into the tank. If there was any mortality during this transition time, those individuals were replaced to achieve a starting tank density of 20.

Juvenile F_3 treatments included **1) Control temperature – No sediment:** in which water temperature was the present-day summer average 28.5°C and 0 mg L^{-1} sediment; **2) Warm temperature – No sediment:** in which temperature was +1.5°C warmer than Control (i.e., 30°C) and 0 mg L^{-1} sediment; **3) Control temperature – Suspended sediment:** in which temperature was the present-day summer average 28.5°C and 50 mg L^{-1} Australian bentonite clay was added ; **4) Warm temperature – Suspended sediment:** in which temperature was +1.5°C warmer than Control (30°C) and 50 mg L^{-1} clay was added (Table 3.1). These suspended sediment concentrations reflect the conditions frequently experienced on the inshore reefs of the GBR (Larcombe et al. 1995, 2001; Wolanski et al. 2008; Bainbridge et al. 2012; Wenger et al. 2012; Jones et al. 2015; Rodgers et al. 2021). In the case of these 4 juvenile treatments there were 2 replicate sumps of each. For all three cross-generational thermal experiences, clutches from n = 3-4 pairs were used for this experiment. For each clutch n = 2 replicate tanks (one on each duplicate sump) were made per each of the 4 juvenile treatments. In cases where there was additional offspring, an extra tank was added (see Appendix 2: Table A2.1 for details).

Water for this experiment was supplied from a 4,500 L external seawater system which was maintained at the Control temperature (28.5°C) by a heat pump (Oasis). This external seawater system supplied water to eight ~240 L internal sumps, which were maintained at one of the four juvenile treatment conditions. Temperature in each system was maintained in each sump by a 1 kW heater that was controlled by a Full Gauge controller with a bandwidth around a desired setpoint ±0.2°C. For the first 11 weeks of the experiment, the 8 internal closed systems underwent a partial water exchange 3 times a week with the external sea water system. During week 12, partial water exchanges were conducted daily. During a partial exchange ~160 L (~68%) was drained from each internal sump and refilled at a rate of 5 L min^{-1}. Following this exchange, the Nephelometric Turbidity Unit (NTU) of each sump was measured using a

waterproof turbidimeter (Thermo Scientific Eutech TN-100). The turbidimeter was calibrated using the TN100CALKIT (containing the calibration standards 0.02, 20.0, 100 and 800 NTU) in accordance with the operation instructions before water samples were measured on the device. Prior to this experiment, a self-conducted a pilot study determined that 50 mg L^{-1} sediment in seawater equated to 10.28 ± 1.22 NTU (mean ± SD) on top of the baseline seawater NTU of 0.34 to 0.45. This ratio of sediment to turbidity is similar to a range of sediment studies (e.g., Macdonald et al. 2013; Fisher et al. 2015; Jones et al. 2015; Jones et al. 2016). Australian bentonite clay (Bentonite Trugel 100) was added to the suspended sediment treatment sumps to raise it to the desired 50 mg L^{-1} within 1 hour of the internal system finishing the water exchange (Table 3.1). Salinity and dissolved oxygen were spot checked and remained between 33-36 ppt and ~100% respectively (Table 3.1).

To maintain sediment suspended, specialised purpose built 11 L tanks were designed with cylindrical plastic tanks and a conical base (diameter: 290 mm, main tank height: 250 mm, funnel height: 185 mm). Each tank was provided constant aeration through an air stone at the centre of the conical base to maintain oxygen levels and keep the sediments in suspension. In addition, each sump that supplied water to 11 or 14 of these specialised tanks, contained two disturbance pumps and two air stones to further sediment suspension. Seawater removed from the internal system during a water exchange underwent particle filtration (1 micron bag filters) to return outflowing water to the external seawater system to control levels.

Juveniles remained in their respective treatments for 53 to 81 days post-hatching (dph) at which point the first sampling occurred (Sample time 1). All systems then underwent 5 partial water exchanges over 2 days to flush the sediment out of the system. On the following day, all systems were put on constant flow through with the external system at which time NTU was recorded at control levels across all systems. As a result, juveniles were exposed to their respective temperature treatments for the rest of the experiment. To investigate how juveniles

recovered following sediment removal, juveniles were sampled 16 to 17 days following the removal of sediment (Sample time 2: 74 to 102 dph), and again 17 to 20 days later (Sample time 3: 92 to 122 dph).

Table 3.1. Mean (±1 SD) seawater parameters for each F_3 juvenile treatment. Tank temperature was measured once to three times a week for the duration of the experiment. Turbidity (NTU) was measured daily.

Juvenile treatment	Control temperature – No sediment	Warm Temperature – No sediment	Control temperature - Suspended sediment	Warm temperature -Suspended sediment
Temperature (°C)	28.45 ± 0.08	29.97 ± 0.10	28.47 ± 0.04	29.91 ± 0.06
Turbidity (NTU)	0.39 ± 0.44	0.44 ± 0.42	10.68 ± 1.38	10.50 ± 1.51

Juvenile *A. polyacanthus* were fed a high food ration; ~2- 4% of body weight, once per day. From day 1-3 post hatching, juveniles were fed newly hatched *Artemia* nauplii at a concentration of 1 individual per ml (~15,000 individual *Artemia* nauplii per tank), and from day 4-6, juveniles were fed *Artemia* nauplii at 2 individual per ml and 40 mg of 0.2-0.4 mm sized INVE Aquaculture Nutrition NRD pellets (protein 55%, lipid 9%, fibre 1.9% moisture 8%). Subsequently, juveniles were fed only INVE Aquaculture Nutrition NRD pellets which increased in volume and pellet size from 40 mg of 0.2-0.4 mm sized pellets on day 7-15, 80 mg of 0.2-0.4 mm sized pellets on day 16-29, 100 mg of 0.5-0.8 mm sized pellets on day 30 to the end of the experiment.

3.3.4 Morphological metrics

At Sampling time 1 (i.e., following 53 to 81 days of exposure to sediment treatments), 6 juveniles were randomly selected from each tank and euthanised by severing the spinal cord anterior to the dorsal fin (van Dyk et al. 2009; McHugh et al. 2011) and the standard length (nearest 0.01 mm; digital callipers) and wet weight (nearest 0.0001 g; Shimandzu ATX224)

were measured. For three of these juveniles, the entire gill structure was dissected and placed in individual histology cassettes. Both the juvenile bodies and the gill arches were fixed in Sea Water Davidsons fixative (Bridle et al. 2005; Cadoret et al. 2013) for ~36 hours, then transferred to 70% ethanol.

At Sampling time 2 (i.e., 16 to 17 days after sediments were removed from the aquaria), 3 juveniles were sampled at random from each tank, euthanised, and their length and weight were measured using the protocol above. At Sampling time 3 (i.e., 34 to 37 days after sediments were removed from the aquaria), all remaining fish from this experiment were euthanised, and their length and weight were measured as described above (Appendix 2: Table A2.2).

3.3.6 Histological protocol

Preserved gill arches from Sampling time 1 (n=71; Appendix 2: Table A2.2) were serially dehydrated (Sakura Tissue-Tek VIP E300), embedded in paraffin wax blocks (Leica Acardia Embedding Station), and sectioned at a 5 µm thickness (Rotary Microtome Microm HM325). Tissue sections were stained with Haematoxylin and Eosin stain (Hess et al. 2015). A single observer, JSC, completed all gill assessments and measures outlined below.

To assess the presence of gill remodelling, slides were viewed at 20x magnification (Olympus BX43 Light Microscope with EP50 Camera). Five types of gill remodelling was observed: epithelium lifting (Au et al. 2004; Cumming and Herbert 2016; Mohamad et al. 2021), lamellae fusion (Agamy 2013; Lowe et al. 2015; Mohamad et al. 2021), mucus secretion (Jacobs et al. 1981; Prakash et al. 1998; Hess et al. 2015; Khieokhajonkhet et al. 2022), and basal hyperplasia (Au et al. 2004; Wong et al. 2013; Lowe et al. 2015; Cumming and Herbert 2016), as well as any signs of gill damage (e.g., aneurysm: Agamy 2013; Wong et al. 2013; Rodgers et al. 2019; Mohamad et al. 2021; Figure A2.1). To assess the incidence of the various types of gill remodelling, the total number of filaments on the gill arch was recorded alongside

the number of filaments with a remodelling type (i.e., a single filament could have multiple remodelling types). This presence/absence data is later used in statistical analysis to generate the likelihood of a filament (within a gill arch) containing a type of gill remodelling.

To measure lamellae characteristics, 3 intact filaments stratified across the gill arch (1 x dorsal, 1 x medial, and 1 x ventral) were selected for analysis and photographed at 60x magnification (Olympus BX43 Light Microscope with EP50 Camera). Multiple photographs (2 to 5) were taken to cover the length of each filament so that lamellae could be measured in detail. Specifically, 6 lamellae (3 on each side, left and right) from each of these 3 aforementioned filaments (18 measure per gill arch) were measured in Image J (V1.53 Wayne Rasband, National Institutes of Health, USA) blinded in respect to cross-generational thermal experience and juvenile treatments. Morphological measurements (Figure 3.2) taken included lamellae length (the maximum distance form tip of the lamellae to the edge of the filament epithelium, excluding filament width), and filament width (base of the lamellae/outer edge of filament epithelium, to the edge of the filament pillar; a measure of basal hyperplasia; Hess et al. 2015). Lamellae width (a measure of lamellae epithelial hyperplasia or hypertrophy) was calculated as lamellae area divided by lamellae length; as per Hess et al. 2015). Lamellae perimeter was also calculated using the equation:

$$= 2 \times (LL - [0.5 \times LW]) + (0.5 \times \pi \times LW)$$

where LL is the lamellae length and LW is the lamellae width (Wilson et al. 1994; Pane et al. 2004). While not all lamellae may be uniform in shape as assumed by this calculation, it provides an estimate of lamellae surface area for gas exchange. Gill lamellae morphometric measures for each individual (18 measures per gill arch) were averaged to generate one value to prevent pseudo-replication.

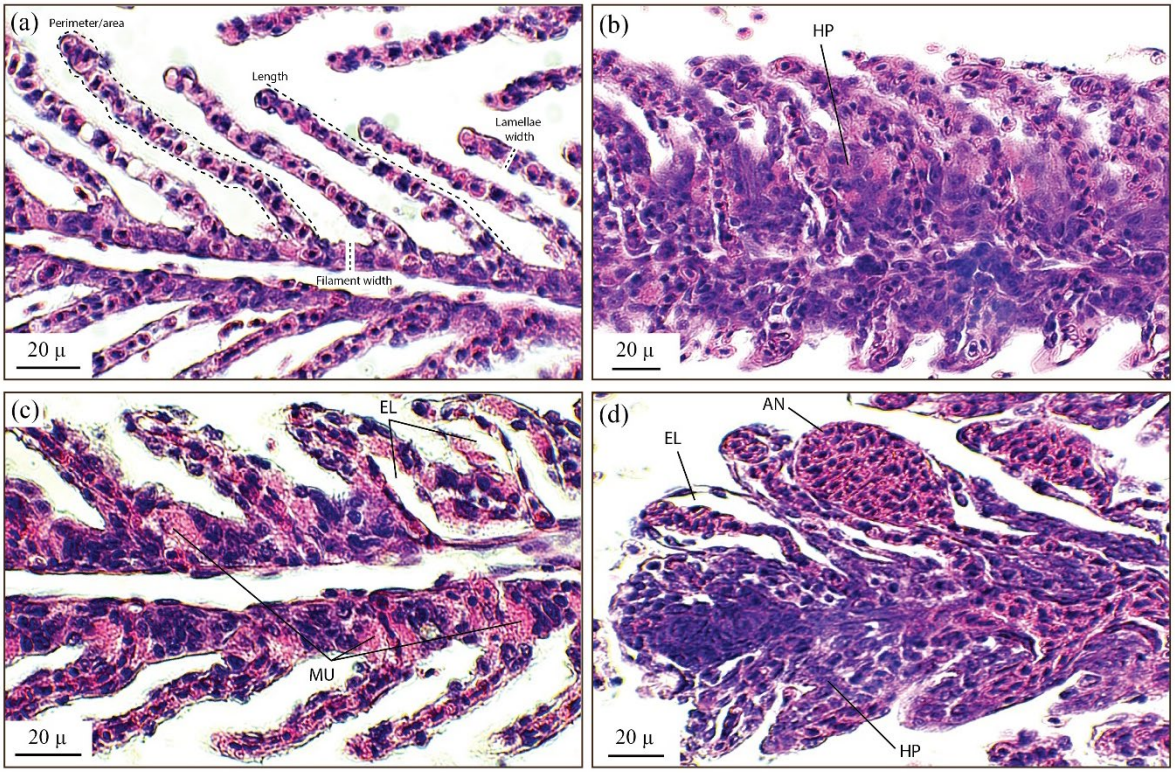

Figure 3.2. Photomicrographs of *A. polyacanthus* gill filaments from the Control temperature – No sediment treatment; 28.5°C, 0 mg L^{-1} (a), and the Warm temperature – Suspended sediment treatment; 30°C, 50 mg L^{-1} sediment (b, c, d) at Sample time 1. The gill morphology parameters measured in all fish (n=71) are depicted in (a). Gill remodelling traits include HP: hyperplasia (b, d), MU: mucus (c), EL: epithelial lifting (c, d), and AN: aneurysm (d).

3.3.7 Statistical analysis

All gill lamellae morphometrics were analysed using independent linear mixed effects models (lmer models within the LME4 package; Bates et al. 2014) in R (version 4.2.2). Average lamellae width, lamellae perimeter, and filament width were used as the dependant variables, with cross-generational thermal experience (Control, Parental development, and Grandparental development), temperature treatment (Control or Warm), and sediment treatment (No sediment or Suspended sediment) as fixed factors. Maternal and paternal linage (i.e., maternal and paternal F_0 grandparents from the six starting pairs), were also added into the model as random factors. The potential effect that body length might have on gill measures was explored and was found to be positively correlated with lamellae width, lamellae perimeter, and filament width, and consequently was added into those models as a covariate. The inclusion of

additional model covariates (age, density, parental clutch ID, and sump ID) were sequentially explored and the model's goodness of fit was compared using analysis of variance (ANOVA) and then looking at the Akaike's information criterion (AIC; Pathak et al. 2013). These covariates did not improve model fit and were therefore not included in the final model (as per Fisher et al. 2015; LaMonica et al. 2021). For lamellae width, one influential outlier was examined using Cook's distance (Bochdansky et al. 2005; Bernal et al. 2022) and was removed from the final statistical analysis.

The presence of each gill remodelling type (epithelial lifting, hyperplasia, lamellae fusion, or mucus) was analysed using independent generalised linear mixed effect model glmer (Bates et al. 2015) with a binomial distribution and logit-link function (logistic regression). The count of filaments with versus without remodelling was the dependent variable, while cross-generational thermal experience, temperature treatment, and sediment treatment were fixed factors. The inclusion of sump ID and parental clutch ID as covariates were sequentially explored using analysis of variance (ANOVA) and then looking at the Akaike's information criterion (AIC; Pathak et al. 2013), and were deemed nonsignificant. Maternal and paternal linage were also added into the model as random factors. Aneurysms were rare (< 2%; only 46 fish) and for one treatment combination (Parental development cross-generation in Control temperature – Suspended sediment) no aneurysms were found. Consequently, statistical testing was not conducted, and the raw data (mean ± SE) are presented in Appendix 2: Figure A2.1.

Juvenile body morphology at each of the three sampling times was independently explored with linear mixed effect models (using lmer). Each sample time was modelled independently to allow direct comparisons of the body morphology at Sample time point 1, and the gill histology metrics that were also collected from fish Sampled at sample time 1. At all sample times, standard length was modelled as the dependent variable with cross-generational thermal experience, temperature treatment, and sediment treatment as fixed factors. Parental

clutch ID as well as tank ID nested within sump ID were included as random factors. Using AIC model comparisons, the covariate inclusion of tank density at the respective sample time (density_time) and length of juvenile treatment exposure (which also corresponds to age at sample time 1) was deemed the best fit using analysis of variance (ANOVA) and then looking at the Akaike's information criterion (AIC; Pathak et al. 2013) for all three sample time points.

Physical condition, the weight of juvenile *A. polyacanthus* for a given standard length, was modelled (lmer) for all sample times with log(weight) as the dependent variable. Cross-generational thermal experience, temperature treatment, sediment treatment, and log(length) were fixed factors. Parental clutch ID as well as tank ID nested within sump ID were included as random factors. Using the same protocol as standard length, the covariates of tank density at the respective sample time (density_time) and length of juvenile treatment exposure (which also corresponds to age at Sample time 1) was deemed the best fit and entered into the models of all sample times. Influential outliers that were negatively impacting model fit were independently examined for each sample time using Cook's distance (Bochdansky et al. 2005; Bernal et al. 2022) and were removed from the final statistical analysis. The models without outliers were compared to the model with all data included and it did not change output significance.

For all lmer models (standard length, physical condition, gill lamellae morphometrics), assumptions including linearity, normality and homogeneity of residuals were visually assessed with Q-Q plots and frequency distributions. Main effects were determined with a sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom. Following this, relevant pairwise comparisons were made with estimated marginal means and Tukey method of p-value adjustment. For glmer models, model assumptions of goodness of fit (dispersion) was assessed using the DARMA package. Main effects were determined with type II Wald chi-squared test. When significant differences were found, post-hoc comparisons on

the probability of remodelling occurring on a filament within a fish gill arch were calculated with estimated marginal means and Tukey method of p-value adjustment.

3.4 Results

3.4.1 Gill lamellae morphometrics

Development in warm temperature and suspended sediment conditions resulted in an increase in filament width (Temperature*Sediment: $F_{1,58}$ = 48.19, P < 0.001) for juvenile *A. polyacanthus* irrespective of cross-generational thermal experience (Figure 3.3a; Appendix 2: Table A2.3). Filament width was 21.3%, 35.6%, 26.7% greater in the combined Warm temperature -Suspended sediment juvenile treatment than Control temperature – No sediment, Warm temperature – No sediment temperature, and Control temperature – Suspended sediment respectively (all post-hoc comparisons P < 0.001; Control temperature – No sediment = 12.02 ± 0.49 µ, Warm temperature – No sediment = 9.59 ± 0.489 µ, Control temperature – Suspended sediment = 11.18 ± 0.502 µ, Warm temperature – Suspended sediment= 15.26 ± 0.495 µ; mean ± SE).

Similarly, lamellae width increased when fish developed under warm temperature and suspended sediment conditions (Temperature*Sediment: $F_{1,55}$ = 28.59, P < 0.001), but the magnitude of increase depended on the cross-generational experience of previous generations (Cross-generation*Temperature*Sediment: $F_{2,55}$ = 5.37, P = 0.007; Figure 3.3b; Appendix 2: Table A2.4). Specifically, fish from the Control cross-generation had 28.4% and 27.0% wider lamellae, when developed in the Warm temperature – Suspended sediment treatment compared to siblings in the Control temperature – No sediment juvenile treatment or Warm temperature – No sediment juvenile treatment respectively (P < 0.01). Fish from the Grandparental development cross-generation also had 27.0% wider lamellae when developed in the Warm temperature – Suspended sediment treatment compared to siblings in the Control temperature

– No sediment juvenile treatment ($P < 0.01$). While fish from Parental development cross-generation exhibited a 7.5 to 13.7% increase in lamellae width when they developed in the Warm temperature – Suspended sediment juvenile treatment compared to siblings grown in other juvenile treatments, this was not significant (all post-hoc $P > 0.05$). Additionally, the lamellae width of fish from Parental development cross-generation was significantly narrower (8.61 ± 0.469 µ) than fish from Grandparental development cross-generation (10.82 ± 0.411 µ) when both developed in the Warm temperature – Suspended sediment treatment ($P = 0.028$).

The perimeter of lamellae exhibited variation depending on the combination of cross-generational experience and juvenile developmental sediment conditions (Figure 3.3c; Appendix 2: Table A2.5). Specifically, offspring from Control cross-generation tended to have the smallest perimeter when no suspended sediment was present and the highest perimeter with suspended sediment, however this was not significant (Cross-generation*Sediment: $F_{2,55} = 3.09$, $P < 0.054$).

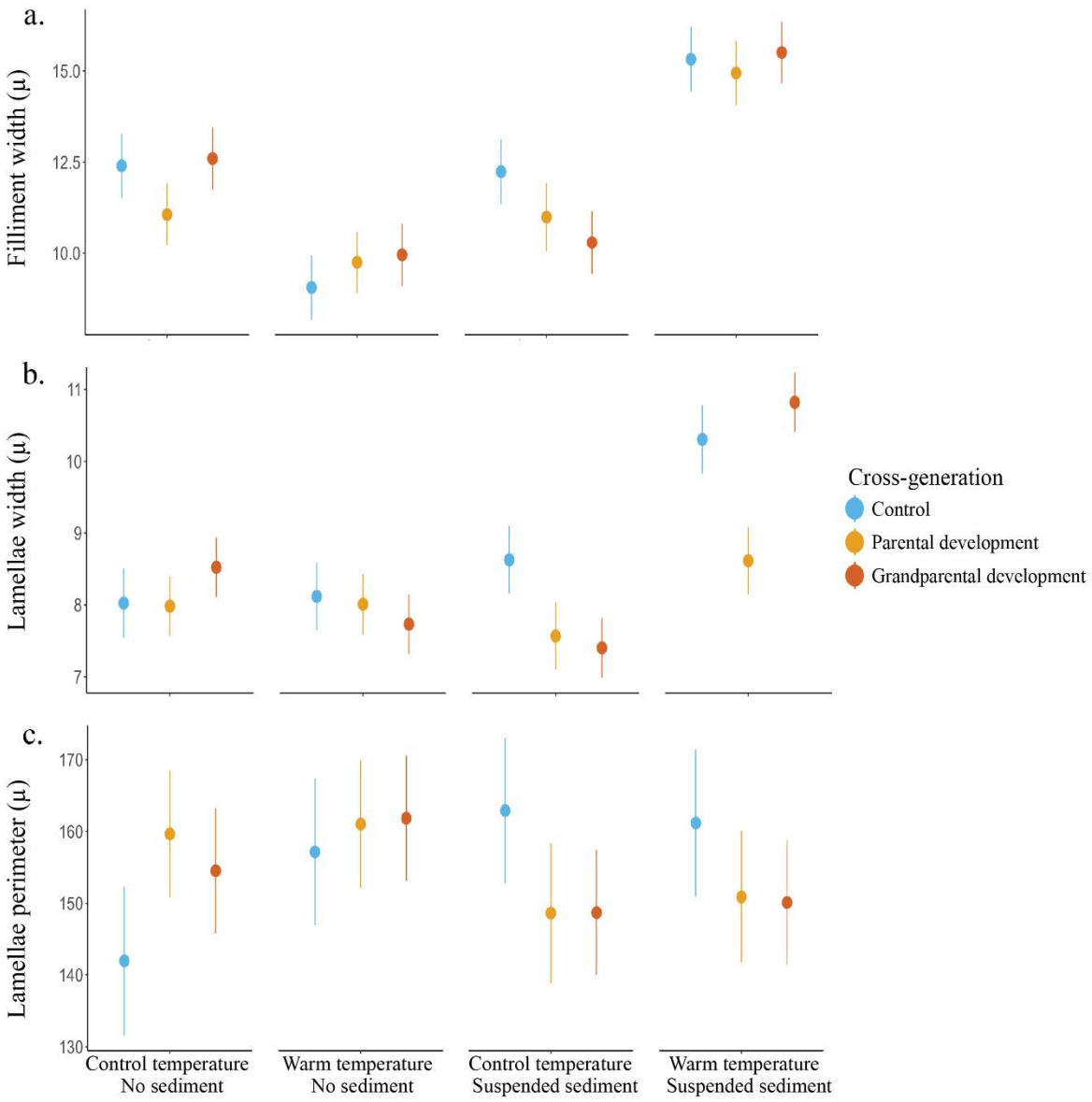

Figure 3.3. Gill morphology measures of filament width (a), lamellae width (b) and lamellae perimeter (c) of juvenile *A. polyacanthus* maintained at Control temperature – No sediment (28.5°C, 0 mg L^{-1}), Warm temperature – No sediment (30°C, 0 mg L^{-1}), Control temperature – Suspended sediment (28.5°C, 50 mg L^{-1}), Warm temperature – Suspended sediment (30°C, 50 mg L^{-1}) at Sample time 1. All data is estimated marginal means ± SE for the average length of 24.00 mm.

3.4.2 Gill lamellae remodelling

The likelihood of epithelial lifting on a filament increased when fish developed under warm and suspended sediment conditions (Temperature*Sediment: X^2 = 6.21, df = 1, P = 0.013; Figure 3.4a; Appendix 2: Table A2.6). Specifically, the experience of Warm temperature – No sediment, Control temperature – Suspended sediment, or Warm temperature – Suspended

sediment all increased the probability of epithelial lifting by almost double (all post-hoc comparisons $P < 0.001$; Control temperature – No sediment = 0.176 ± 0.096, Warm temperature – No sediment= 0.305 ± 0.140, Control temperature – Suspended sediment = 0.311 ± 0.141, Warm temperature – Suspended sediment = 0.342 ± 0.148; mean probability ± SE). Additionally, the increase in epithelial lifting in juvenile developmental sediment conditions differed with the cross-generational thermal experience (Cross-generation*Sediment: X^2 = 17.81, df = 2, $P < 0.001$; Figure 3.4b). No differences in epithelial lifting were observed when comparing fish from cross-generational thermal experiences in Control temperature – No sediment juvenile development. However, when developing in suspended sediment the probability of epithelial lifting was higher in fish from Control cross-generation (0.490 mean probability) than Parental development (0.195 mean probability; $P < 0.001$). This low probability of epithelial lifting in fish from Parental development cross-generation resulted in no distinguishable difference between siblings that developed in no sediment versus suspended sediments conditions ($P = 0.891$). While for juveniles from Control and Grandparental development cross-generational experience the probability of epithelial lifting increased by 85.6% and 56.7%, respectively, when in suspended sediment than no sediment (Control: $P = 0.023$; Grandparental: $P < 0.001$).

Development in warm temperature and suspended sediment conditions resulted in an increase in the likelihood of hyperplasia within a filament (Temperature*Sediment: $X^2 = 27.31$, df = 1, $P < 0.001$) for offspring from all cross-generational thermal experience (Figure 3.4b; Appendix 2: Table A2.7). The probability of hyperplasia was 74.1%, 79.9%, and 69.6% greater in the combined Warm temperature – Suspended sediment juvenile treatment compared to the Control temperature – No sediment, Warm temperature – No sediment, and Control temperature – Suspended sediment respectively (all post-hoc comparisons $P < 0.001$; Control temperature – No sediment = 0.087 ± 0.017, Warm temperature – No sediment = 0.067 ± 0.014,

Control temperature – Suspended sediment = 0.102 ± 0.019, Warm temperature – Suspended sediment = 0.334 ± 0.034; mean probability ± SE).

The likelihood of mucus within a filament differed depending on juvenile sediment, temperature, and cross-generational thermal experience (Cross-generation*Temperature* Sediment: X^2 = 16.49, df = 2, P < 0.001; Figure 3.4c; see Appendix 2: Table A2.8 for other main factor statistics). Fish from the Parental development in warm condition tended to have decreasing mucus with increasing temperature and sediment (Control temperature – No sediment = 0.300 ± 0.063, Warm temperature – No sediment = 0.248 ± 0.056, Control temperature – Suspended sediment = 0.208 ± 0.056, Warm temperature – Suspended sediment = 0.121 ± 0.039; mean probability ± SE). Within the Control temperature – No sediment juvenile treatment, fish from Parental development cross-generation had over 3 times more mucus than fish from the Control cross-generation (P = 0.020). For fish from Control and Grandparent development cross-generations sibling fish had similar mucus levels across all juvenile treatments (all P > 0.05).

The probability of lamellae fusion was very low (< 0.1%) and was not significantly different across juvenile treatment and cross-generational thermal experience (P > 0.05; Figure 3.4d; Appendix 2: Table A2.9).

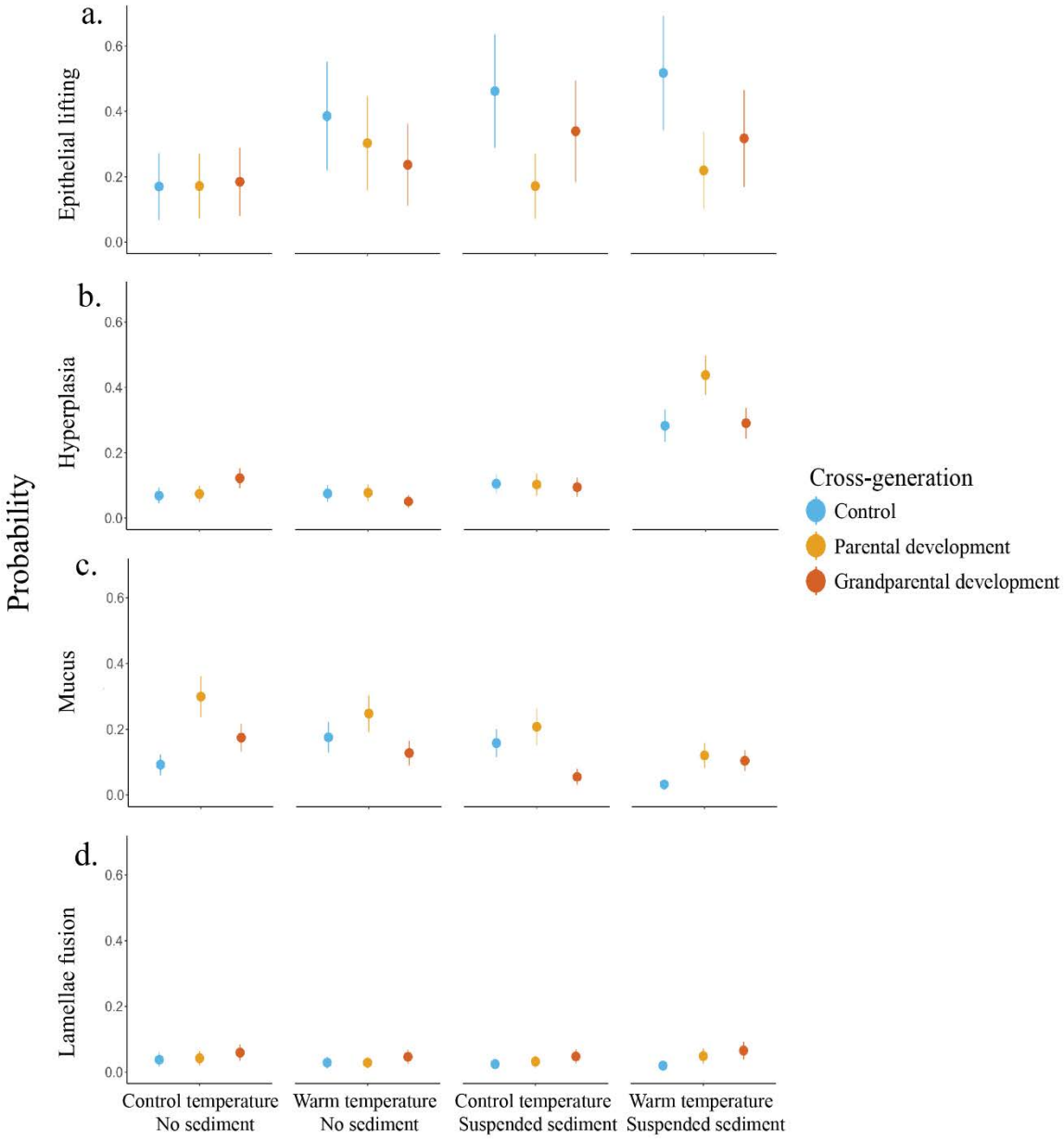

Figure 3.4. The probability of a gill filament containing each type of gill remodelling or damage. Types of gill remodelling include epithelial lifting (a), hyperplasia (b), mucus (c), and lamellae fusion (d). Data collected from juvenile *A. polyacanthus* maintained at Control temperature – No sediment (28.5°C, 0 mg L^{-1}), Warm temperature – No sediment (30°C, 0 mg L^{-1}), Control temperature – Suspended sediment (28.5°C, 50 mg L^{-1}), Warm temperature – Suspended sediment (30°C, 50 mg L^{-1}) at Sample time 1. Data is estimated probability ± SE.

3.4.3 Standard length

At Sample time 1, the standard length of juvenile *A. polyacanthus* was affected by juvenile temperature ($F_{1,578}$= 5.15, P = 0.024) and suspended sediment ($F_{1,579}$= 36.25, P < 0.001; Figure 3.5a; Appendix 2: Table A2.10). Juveniles that developed in the warm temperature were on

average 2.02% shorter than those in the control temperature (Control = 24.8 ± 0.2 mm; Warm = 24.3 ± 0.2 mm; mean ± SE). Juveniles reared in suspended sediment were also shorter than those reared in no sediment conditions by 4.76% (No sediment = 25.2 ± 0.2 mm; Suspended sediment = 24.0 ± 0.2 mm; mean ± SE). While not significant, there was also some evidence of an interaction between Temperature and Sediment (Temperature*Sediment: $F_{1,577}$= 3.76, P = 0.053) where standard length was smallest for juveniles in Warm temperature - Suspended sediment combined, and largest in the Control temperature - No sediment treatment.

At Sample time 2, once suspended sediment had been removed for 16 to 17 days, juveniles reared in Suspended sediment were still shorter than those reared in no sediment conditions but to a lesser extent of 3.61% (No sediment= 27.7 ± 0.30 mm; Suspended sediment = 26.7 ± 0.30 mm; mean ± SE ; $F_{1,276}$= 9.41, P = 0.002; Figure 3.5b; Appendix 2: Table A2.11).While not significant ($F_{1,276}$= 2.77, P = 0.097) juveniles that developed in the warm temperature were also still shorter than those in the control temperature (Control = 27.5 ± 0.3 mm; Warm = 26.9 ± 0.3 mm; mean ± SE).

At Sample time 3, juvenile standard length was only significantly influenced by juvenile temperature treatment ($F_{1,4}$= 10.5, P =0.035) Juveniles that developed in warm temperature were 3.34% smaller compared to the control temperature (Control = 29.9 ± 0.25 mm; Warm = 28.9 ± 0.27 mm; mean ± SE).

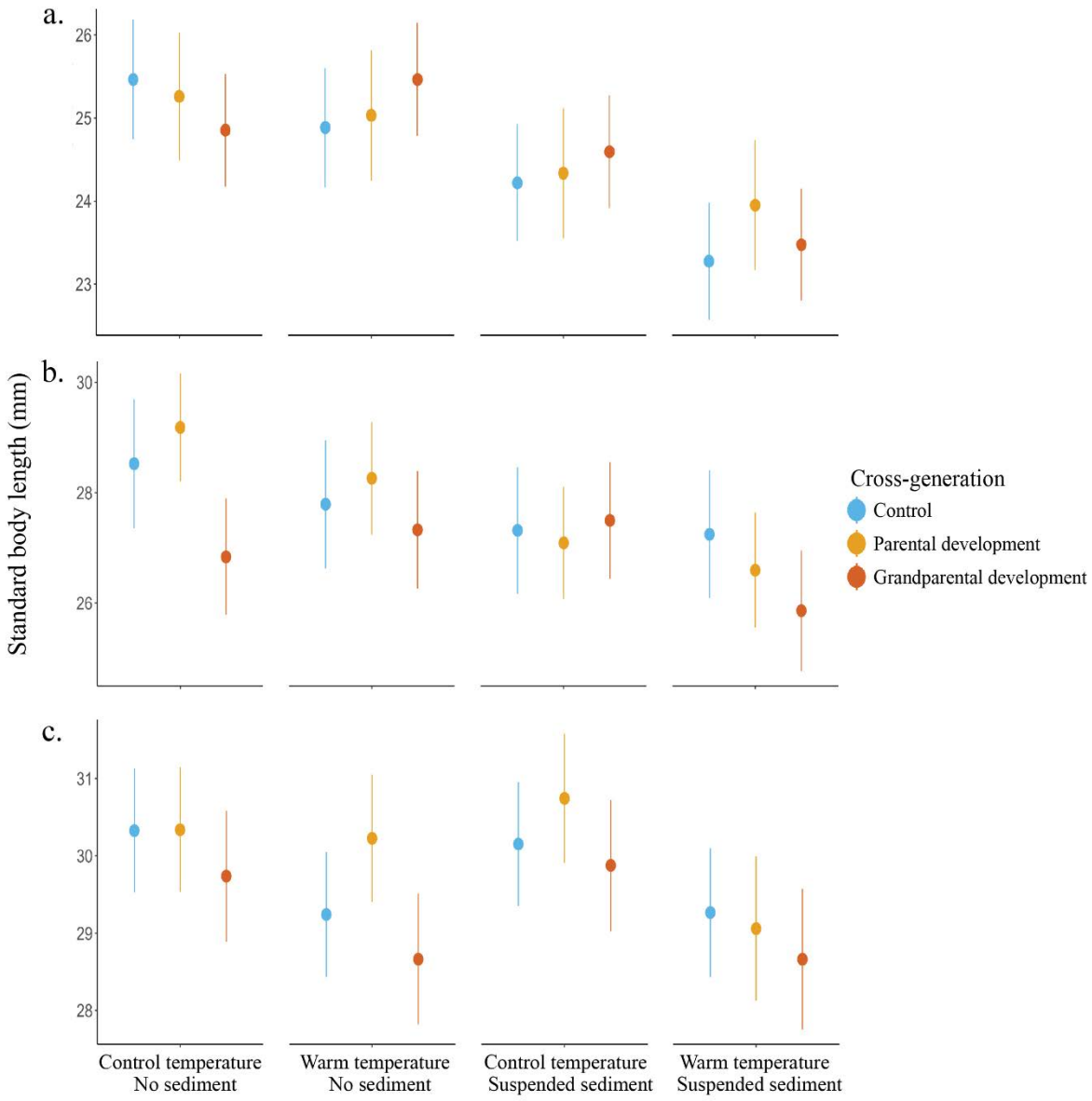

Figure 3.5. Juvenile *A. polyacanthus* standard length at Sample time 1 (a), Sample time 2 (b), and Sample time 3 (c) when maintained at Control temperature – No sediment (28.5°C, 0 mg L^{-1}), Warm temperature – No sediment (30°C, 0 mg L^{-1}), Control temperature – Suspended sediment (28.5°C, 50 mg L^{-1}), Warm temperature – Suspended sediment (30°C, 50 mg L^{-1}). At Sample time 1 fish were experiencing these treatment conditions directly, which Sample time 2 and 3 are post-sediment removal. All data is estimated marginal means ± SE.

3.4.4 Physical condition

Juvenile physical condition (weight for a given standard length) at Sample time 1 differed depending on juvenile developmental treatment (Temperature: $F_{1,4}$ = 24.82, P = 0.007; Temperature*Sediment: $F_{1,4}$ = 11.45, P = 0.027; Figure 3.6a; Appendix 2: Table A2.13). Juveniles were 4.71% heavier for a given length in the Warm temperature (0.55 ± 0.01mm;

mean ± SE) compared to Control temperature (0.52 ± 0.01 mm) but this was primarily driven by the highest physical condition observed in the combined Warm temperature – Suspended sediment treatment (0.56 ± 0.01 mm) and lowest in the Control temperature – Suspended sediment treatment (0.51 ± 0.01 mm; P = 0.01).

At Sample time 2, juveniles were in better physical condition in warm temperature treatments ($F_{1,5}$= 23.9, P =0.006), however, the degree of change was not consistent across cross-generational thermal experience (Cross-generation*Temperature: $F_{2,73}$ = 3.24, P = 0.045; Figure 3.6b; Appendix 2: Table A2.14). Specifically, juveniles from the Control cross-generation were 7.15% heavier for a given length in warm temperature compared to control temperature (P = 0.0014; Control = 0.72 ± 0.01g, Warm = 0.78 ± 0.01g; mean ± SE). While fish from both the Parental development and Grandparental cross-generations were similar at all juvenile temperatures (P > 0.05).

Juvenile temperature was still influential to physical condition at Sample time 3 ($F_{1,4}$= 20.88, P =0.009; Appendix 2: Table A2.15), with juveniles developing in warm temperature treatments 4.71% heavier for a given length than those that developed in control temperatures (Control = 0.95, ± 0.01 g, Warm = 1.00 ± 0.01 g; mean ± SE; Figure 3.6c).

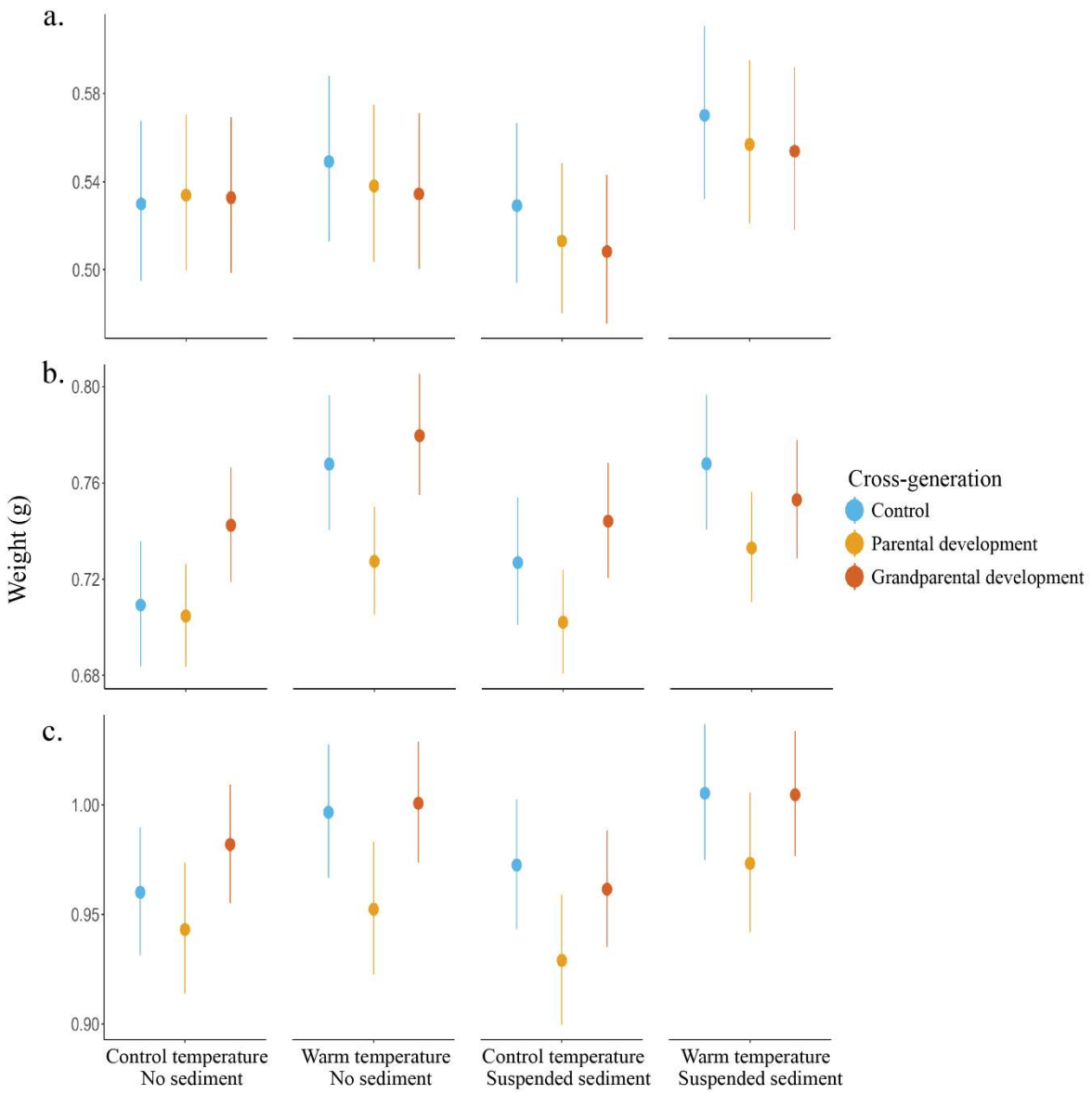

Figure 3.6. Juvenile *A. polyacanthus* physical condition (weight for a given length) at Sample time 1 (a), Sample time 2 (b), and Sample time 3 (c) when maintained at Control temperature – No sediment (28.5°C, 0 mg L^{-1}), Warm temperature – No sediment (30°C, 0 mg L^{-1}), Control temperature – Suspended sediment (28.5°C, 50 mg L^{-1}), Warm temperature – Suspended sediment (30°C, 0 mg L^{-1}). At Sample time 1 fish were experiencing these treatment conditions directly, which Sample time 2 and 3 are post-sediment removal. All data is the estimated marginal mean ± SE of log10 weight that has been back transformed for plotting. Weight is presented for the average standard length of 24.57, 27.29, and 29.45mm for Sample time 1, 2, and 3 respectively.

3.5 Discussion

The present study found that elevated temperature and suspended sediment often acted synergistically or antagonistically on gill morphology and physical condition but had a generally additive effect on growth of juvenile *A. polyacanthus*. Juveniles exposed to warm

temperature and suspended sediment for the first months of development showed greater incidence of gill remodelling (specifically increased filament and lamellae width, greater incidence of hyperplasia and epithelial lifting, and a lower incidence of excess mucus) compared to those held under control conditions, although the magnitude of the change varied with cross-generational thermal experience. Such changes have previously been linked to protecting the gill structure from mechanical abrasion (Mallatt 1985). Juvenile *A. polyacanthus* exposed to the Warm temperature and Suspended sediment combined treatment also exhibited reduced growth but increased physical condition. Once suspended sediment was removed, initial differences in standard length diminished within ~2-5 weeks, indicating that juvenile coral reef fish can recover following a short-term suspended sediment event.

Warm temperature and suspended sediment acted synergistically on the probability of basal hyperplasia, and the width of lamellae and filaments (indicative of the extent of epithelial and basal hyperplasia, respectively). While hyperplasia is a common defence response to waterborne pollutants, it also increases the gas diffusion distance and therefore reduces the gas exchange efficiency of the lamellae (Mallatt 1985). In extreme cases hyperplasia can completely fill the interlamellar space resulting in lamellae fusion, reducing the gill surface area (Lowe et al. 2015; Mohamad et al. 2021). Although extensive hyperplasia was found when juvenile *A. polyacanthus* developed under Warm temperature and Suspended sediment combined, lamellae fusion was found in very low proportion across juvenile treatments. On the other hand, epithelial lifting was evident in response to both warm temperature and suspended sediment independently, and a similar level of impact in the combined treatment (antagonistic). This epithelial lifting would not only increase the diffusion distance to protect the pillar system, but the protrusion of the epithelium into the interlamellar space has been shown to disrupt water flow across the gills therefore reducing gas exchange (Hughes and Morgan 1973; Au et al. 2004). This suggests that gill remodelling traits such as epithelial lifting, or mucus may be

sufficient to protect the gills under warm temperature or suspended sediment in isolation. However, a greater probability of hyperplasia under Warm temperature and Suspended sediment combined suggests that further remodelling was required under multiple stressors combined. Multiple gill remodelling traits that reduce oxygen intake may compromise the ability to perform aerobic activities, and have implications for fitness, and performance.

While found previously in coral reef fish (Hess 2019; Johansen et al. 2021), juvenile *A. polyacanthus* here did not elicit any change in lamellae perimeter (proxy for available surface area for gas exchange) in response to warm temperature or suspended sediment. While a large gill surface area can be beneficial for gas exchange, it also increases the exposure of waterborne contaminants and pathogens and the flux of ions into the gills which results in greater energy expenditure for osmoregulation (Nilsson 2007). Considering the significant role ion regulation plays in the early development of gills (Rombough 2007), an increase in surface area may not have been beneficial for juveniles in this study that were exposed directly after hatching as has been reported for older juvenile and adult coral reef fish (Hess 2019; Johansen et al. 2021).

In combination, the patterns of hyperplasia, epithelial lifting, lamellae fusion, and consistent surface area of gill lamellae show no evidence for gill plasticity to increase gas exchange with warming and/or suspended sediment. This would suggest that gas exchange may not be limiting for these juveniles, or that protection against mechanical damage is prioritised. Previous research has found that in addition to gill remodelling, fishes may increase ventilation rate to increase the volume of water that passes over the gills (Horkel and Pearson 1976; Lowe et al. 2015), as well as increase the haematocrit, haemoglobin concentration, and red blood cell count which can all raise the blood's oxygen exchange capacity under both elevated suspended sediment (O'Connor et al. 1977) and elevated temperature (Gräns et al. 2014). Although increased blood circulation (and vascular congestion) may improve gas exchange it can also cause aneurysms (the rupture of the pillar cell systems causing the lamellae to lose its structural

integrity; a metric that indicates gill damage). Interestingly, unlike previous studies (Agamy 2013; Wong et al. 2013; Mohamad et al. 2021) aneurysms were found in very low proportion in this study. While not able to be tested directly on these fish, exposure to warm temperature during development of the same cohort of fish in **Chapter 2** did not result in increased resting oxygen demands. This lack of plasticity further supported by research on the cinnamon clownfish, *Amphiprion melanopus*, maintained at a similar suspended sediment level (45 mg L^{-1}) for 7 days which exhibited a elevated resting metabolic rate in conjunction with a reduce lamellae length but no change in oxygen diffusion distance (Hess et al. 2017).

Gill remodelling observed in response to warm temperature or suspended sediment can be energetically costly, with implications for other traits. Juveniles from the Warm temperature and Suspended sediment combined treatment had increased filament and lamellae width, greater incidence of hyperplasia and epithelial lifting, and were on average shorter at Sample time 1 compared to juveniles from the Control temperature - No sediment treatment. This supports research on the freshwater *Erimonax monachus* by Sutherland and Meyer (2007) who found a reduction in growth rate correlated with increasing lamellae width under suspended sediment. Furthermore, the effect of warming and suspended sediment on standard length was additive, which does not match the generally synergistic or antagonistic interactions of gill remodelling traits. This potentially suggests that the mechanisms of impacts for warming and suspended sediment are distinct. Reduced growth with warming temperature is often a response to increased metabolic demand (Clarke and Fraser 2004; Munday et al. 2008b; Rogers et al. 2011; Motson and Donelson 2017; Spinks et al. 2019), however, there are exceptions (Donelson 2015; Audzijonyte et al. 2020; McMahon et al. 2023). Whereas reduced on standard length in suspended sediment may be a response of energy relocation to repair gill damage caused by particle abrasion (Mallatt 1985; Lake and Hinch 1999). The additive nature and independent mechanisms are further supported by growth following the removal of suspended

sediment. After ~2 - 5 weeks, the reduced standard length in warm temperature remained but effects of sediment were no longer present.

Increased lamellae width and epithelial lifting was seen in both the Control and Grandparental development cross-generations. However, this protective gill remodelling used to reduce the risk of mechanical abrasion (Mallatt 1985; Agamy 2013) was not seen in juveniles from the Parental development cross-generation under Warm temperature and Suspended sediment combined. F_3 juveniles from the Parental development cross-generation did, however, have a higher probability of mucus in all juvenile treatments compared to juveniles from the Control and Grandparental development cross-generations. Increased mucus production is a common stress response to warm temperature (Jacobs et al. 1981; Prakash et al. 1998; Khieokhajonkhet et al. 2022). Therefore, the more recent exposure to elevated temperature may have predisposed offspring from the Parental development cross-generation to maintain mucus production as an alternative to other remodelling strategies such as epithelial lifting or lamellae epithelial hyperplasia.

Distinction in juvenile physical condition was also observed related to cross-generational experience but only at Sample time 2. According to the temperature-size rule (Atkinson and Sibly 1997), ectothermic fish at lower latitude (warmer temperatures) often grow and develop faster but are smaller at maturation compared to high latitude (cooler temperature) organisms (Arendt 2011; Trip et al. 2014; Álvarez-Noriega et al. 2023). This was observed in fish from the Control cross-generation who were heavier for a given length in Warm temperature compared to Control temperature, but in not the other cross-generations. The differences in body mass across cross-generation thermal experiences may be due to energy allocation (Stallings et al. 2010; Mogensen and Post 2012). For example, juveniles from the Parental or Grandparental development cross-generation may have prioritised energy for gill remodelling strategies employed at Sample time 1 (mentioned above), which may have flow

on effects to physical condition at Sample time 2. As the effect of cross-generational experience on physical condition only occurred at Sample time 2, it suggests that the effects cross-generational thermal exposure may change through time and is a point of interest for future research.

While I observed the range of physical and morphological effects to juveniles developing in presence of suspended sediment, when conditions returned to control levels, fish growth increased to match the size (length) of Control temperature – No sediment juveniles. This was even after a prolonged exposure to suspended sediment simulated in this study, at the upper end of what is expected following a following an extreme flood or dredging event (Bainbridge et al. 2012; Macdonald et al. 2013; Fisher et al. 2015; Jones et al. 2015, 2016), ~5 weeks after sediment was removed there was no detectable difference in standard length of juvenile *A. polyacanthus* among treatments. By ~5 weeks post-sediment exposure only the impacts of continued warming to growth (both standard length and physical condition) remained, providing evidence that the effects of exposure to elevated suspended sediment concentrations may be easily compensated. Previous studies have found similar (compensatory) responses in the growth of fishes following the removal of stressors (Nicieza and Metcalfe 1997; Metcalfe and Monaghan 2001; Ali et al. 2003; Donelson et al. 2012a; Spinks et al. 2019). While compensatory growth can restore a juveniles growth trajectory, thus reducing the risk of size dependent mortality (Ali et al. 2003), it can also have costs later in life (Metcalfe and Monaghan 2001; Ali et al. 2003; Mangel and Munch 2005; Kim et al. 2019). These costs such as an increase the risk of developmental abnormalities (Ali et al. 2003) and oxidative (Kim et al. 2019), muscle (Ali et al. 2003), and cellular (Mangel and Munch 2005) damage can ultimately lead to a shorter life span. Although suspended sediment was a novel stressor to the fish used in this experiment (i.e., the previous 2 generations had not been exposed to suspended sediments), the wild population (F_0) caught from inner shelf reefs may have

experienced the resuspension of settled terrigenous material through wind, waves, and currents for centuries prior (Larcombe et al. 1995; Wolanski et al. 2008). Coral reef fish, and coastal marine fish more generally, may therefore be more adapt to short term sediment loads.

The result of the present study builds on the expanding knowledge that local environmental stressors are exacerbating the effects of long-term climate change (Ghedini et al. 2013; Gissi et al. 2021). Coastal management practices that reduce anthropogenic sediment input in waterways and anthropogenic resuspension events, can prevent future elevated suspended sediment events in coastal marine systems and the GBR. In comparison to reversing long term climate change stressors such as ocean warming, local management practices can be a more effective as they can reduce environmental stressors in a relatively short period of time, and do not require global cooperation (Ghedini et al. 2013). Therefore, local management presents an effective strategy to reduce the extant of environmental change and mitigate the confounding interactions of local and global environmental stressors.

Chapter 4 – General discussion

There is mounting evidence that the early life development of coral reef fish is being impacted by a range of anthropogenic environmental stressors. While phenotypic plasticity is predicted to be especially important in enabling individuals to maintain performance under future conditions (Chevin et al. 2010; Hoffmann and Sgró 2011; Munday et al. 2013b; Merilä and Hendry 2014), it is unknown whether plasticity to one environmental stressor alters the response to a second environmental stressor. Using a multi-generational design with combinations of developmental and post-maturation thermal exposure in both the grandparent and parent generation, this thesis investigated how phenotypic plasticity to +1.5°C warming in previous generations influenced the development and performance of juvenile *Acanthochromis polyacanthus* under multiple environmental stressors (**Chapter 2**: warming and acidification; **Chapter 3**: warming and suspended sediments). While previous generations thermal exposure resulted in carry-over effects on body morphology, the greatest phenotypic change across both chapters was related to developmental environmental conditions with the combined effect of two stressors acting additively, synergistically, or antagonistically on various performance traits. These findings improve our understanding of the potential impacts of global and local environmental change across generations of reef fish.

4.1 Cross-generation thermal exposure timing

Overlapping cross-generational thermal experience between the two experiments allowed exploration of consistency in patterns of response to environmental conditions. I found that juveniles from both the Parental or Grandparental development cross-generation were longer but in similar physical condition to the Control cross-generation at the end of the experiment (101 to 135 days post hatching: dph) in all juvenile treatments in **Chapter 2**. This contrasts to **Chapter 3** whereby prior thermal exposure had no significant influence in the length or

physical condition of juveniles across the majority of Sampling times (1-3; exception physical condition at Sampling time 2). While possible, these contrasting results across chapters was unlikely due to differences in the size of aquaria (32 L in Chapter 2; 11 L in Chapter 3) as juveniles from the Control cross-generation in the Control juvenile treatment, from both experiments, had a similar mean standard length at ~17 weeks (Chapter 2 = 31.16 mm; Chapter 3 = 30.75 mm). Instead, parents may be producing a diverse offspring phenotypes depending on the timing within the breeding season, possibly to increase the likelihood of success in unpredictable environments (Shama 2015) or because differing phenotypes benefit early and late season progeny (Brinkhof 1997; Groothuis et al. 2005; Divino and Tonn 2007; Stier et al. 2014). For example, individuals with a smaller body size may have a selective advantage as smaller individuals have a lower metabolic demand in warm temperature (Pörtner and Knust 2007; Rombough 2007; Forster et al. 2012; Leiva et al. 2019). On the other hand, larger individuals have a competitive advantage and are more likely to survive selection events like predation, at least under present day environmental conditions (Sogard 1997; Hoey and McCormick 2004; Almany and Webster 2006; Poulos and McCormick 2015).

Alternatively, the shift in phenotype may not be an active strategy (e.g., bet-hedging) but instead be a product of maternal energy reserves. Greater fitness (size and survival) in early season clutches has been found across aquatic fish and animals more generally (Schultz et al. 1991; Reznick et al. 2006; Divino and Tonn 2007). Regardless, the variation found in this thesis indicates that multiple clutches per pair should be investigated in future studies for a fuller understanding of the diversity of phenotypes that prior thermal experience may induce. Most studies to date on the effects of ocean warming across generations focus on early season clutches, including those using the early generations of the *A. polyacanthus* culture used in this thesis (Yasutake 2019; Spinks et al. 2021). The increased phenotypic variation may facilitate evolution through directional selection (Ghalambor et al. 2015; O'Dea et al. 2016) by allowing

the selection of optimal phenotypes at different peaks in the landscape and across generations (O'Dea et al. 2016; Jarrold et al. 2019).

Experimentation that includes complex exposure timing during the parent and grandparent generations allows for greater understanding on the conditions in which transgenerational plasticity and carry-over effects occur (Shama and Wegner 2014; Spinks et al. 2021; Bernal et al. 2022). Most cross-generational research to date provides consistent evidence of carry-over effects where exposure to warming during development or early life results in phenotypic change in all current-generation thermal conditions (Shama and Wegner 2014; Spinks et al. 2021; Bernal et al. 2022). My research was consistent with this pattern since all cross-generational thermal exposure resulted in consistent phenotypic change in a range of juvenile environments. One contradiction to this pattern was found in **Chapter 2** with prior exposure to continuous warming in the grandparent generation, resulting in enhanced metabolic performance (maximum oxygen consumption and aerobic scope) only when F_3 juveniles were exposed to warm temperatures and control CO_2, indicating transgenerational plasticity. The consistent thermal exposure throughout the grandparent generation, rather than partial exposure as in all other cross-generational groups, may be required to produce transgenerational plasticity since environmental heterogeneity is less likely to produce an irreversible response to environmental change (Herman et al. 2014). Furthermore, in cases of environmental heterogeneity, developmental plasticity is instead expected, which is supported by the greater phenotypic change due to developmental than cross-generational experience across both chapters (morphology, physiology, and behaviour in **Chapter 2** and gill remodelling in **Chapter 3**).

4.2 Interactions between past and present environmental stressors

Contributing to the increasing knowledge of how multiple stressors interact and impact marine organisms (Harvey et al. 2013; Przeslawski et al. 2015; Côté et al. 2016; Sampaio et al. 2021; Baag and Mandal 2022), this study found all response types including additive, synergistic, and antagonistic interactions between stressors. The two global environmental stressors of ocean warming, and acidification had an additive effect on all performance trats including the physiology, behaviour, and growth of juvenile *A. polyacanthus* (**Chapter 2**). This additive response was often driven by only a single stressor, as ocean warming and acidification were found to impact different performance traits. These findings are congruent with the predominantly additive nature of elevated temperature and CO_2 in previous work on juvenile marine fish (as seen in **Chapter 1**, Table 1.2) even though lower, near future CO_2 levels of 825 µatm rather than 1000 µatm were used in the present study. Most interestingly, the effect of elevated CO_2, in isolation or in combination with warm temperature, was not dependent on whether previous generations experienced ocean warming. This means that any potential effects of historical thermal plasticity were realised regardless of elevated CO_2 conditions (length and physical condition), but equally any likely beneficial (reduced resting metabolic rate) or negative (reduced length) effects of elevated CO_2 also remained.

Knowing the interaction direction of multiple stressors on a species or population can be critical for informing conservation and management and is the greatest potential application for this research. For example, management of local stressors can be beneficial when the interaction between a global and local environmental stressor is synergistic, but could have adverse effects when this interaction is antagonistic (Brown et al. 2013). Upscaling results such as in this thesis are challenging especially when differing traits express various responses to the same environmental change such as in **Chapter 3**, where ocean warming and suspended sediment produced synergistic and antagonistic interactions on various gill remodelling traits,

and additive effects to length. This research does highlight the value in measuring a broad range of traits to better understand the complex individual responses to multiple stressors. A key future step for this type of work is to understand how these traits relate to fitness and/or focus the investigation on traits more closely aligned with fitness. Future studies would also benefit from taking into account that the interaction direction may change even further between populations, food availability, with the severity of stressor, with the addition of other abiotic or biotic stressors, and the environmental exposure of previous generations (Przeslawski et al. 2015; Côté et al. 2016).

For gill remodelling traits, prior exposure to warming did influence the interactive response between warming and suspended sediment. Juveniles from the Parental development cross-generational exposure did not exhibit protective gill remodelling (increased lamellae width and epithelial lifting) under Warm temperature and Suspended sediment combined. Firstly, this may be evidence for maladaptive carry-over effects as without protective gill remodelling they may be at greater risk of mechanical abrasion from sediment and the diffusion of toxic pollutants where present (Mallatt 1985; Agamy 2013). Alternately, this response pattern may provide evidence for adaptive carry-over effects as they were able to maintain sufficient protection from mucus production and did not require extensive structural remodelling that would reduce the gas diffusion capacity (Mallatt 1985; Au et al. 2004). However, as Parental development cross-generation fish exhibited a differing pattern of gill remodelling than both the Control and Grandparent development cross-generation, it is more likely that this response is not adaptive. Furthermore, there was no evidence for the differing response by Parental development cross-generation to result in an increased standard length or physical condition at Sample time 1.

The diminishing difference after the sediment was removed suggests that these fish may be somewhat resilience to acute suspended sediment events, but longer-term (chronic) events

may be more problematic. For example, juveniles from all cross-generational experiences had similar declines in standard length under both Warm temperature and Suspended sediment individually and combined at Sample time 1 (**Chapter 3**). At Sample time 3 the effect of Suspended sediment was lost and the greatest disparity between juveniles was primarily driven by juvenile temperature across all cross-generations. It has been well established that following an period of stress and low growth, organisms may exhibit a period of accelerated growth to catch up to larger individuals in the cohort when more favourable conditions arise (Nicieza and Metcalfe 1997; Metcalfe and Monaghan 2001; Sogard and Olla 2002; Ali et al. 2003; Donelson et al. 2012a; Kim et al. 2019; Spinks et al. 2021). However, compensatory growth can result in trade-offs or come at a cost later in life (Metcalfe and Monaghan 2001; Ali et al. 2003; Mangel and Munch 2005; Kim et al. 2019). This increased growth might also be influenced by the reduced tank density following sampling resulting in less competition for space and resources, however, the density changes were consistent across cross-generational treatments.

4.3 Plasticity in a rapidly changing environment

The overall aim of this thesis was to determine how organisms respond to a changing environment over generations. The results show that both developmental and cross-generation exposure, via carry-over effects, may provide a variety of performance benefits. Where developmental exposure to +1.5°C enhanced physical condition (mass for a given length; **Chapter 2 & 3**), boldness and activity (**Chapter 2**), cross-generational exposure was more complex and trait specific. Phenotypic plasticity across generations in response to environmental change can be costly in many ways including: the redirection of energy from one trait to another causing performance trade off, and the phenotypic mismatch if the environment changes (Angilletta 2009). The complex response across generations may therefore highlight some of the strategies used to reduce the costs of phenotypic change and

the variety of performance combinations that improve performance under environmental change. However, to truly know what combination of performance attributes improves fitness and relative contribution to the next generation would be determined by natural selection which is not easy in lab-based experiments. While this thesis provides a starting point to better understand multiple stressors impacts across generations, future work should expand stressor complexity, within and across generations, in a variety of species to help build a more general understand of how populations, communities, and ecosystems will respond. The value of this thesis suggests a need to prioritise local management of coastal anthropogenic stressors as the interaction with ocean warming is having the greatest synergistic impact. Compared to global climate change, local environmental management is also more effective as it can reduce environmental stressors in a relatively short period of time, and does not require global cooperation (Ghedini et al. 2013).

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Appendix 1

The following appendix accompanies Chapter 2:

The role of cross-generational warming on the juvenile development of a coral reef fish under ocean warming and acidification

Proposed authors: Jasmine S. Cane, Yogi C. Yasutake, Shannon J. McMahon, Andrew S. Hoey and Jennifer M. Donelson

All tables are for data collected from juvenile *A. polyacanthus* maintained at Control (28.5°C, 490 µatm); Warm temperature (30°C, 490 µatm); Elevated CO_2 (28.5°C, 825 µatm); Warm temperature and Elevated CO_2 (30°C, 825 µatm).

Table A1.1 Linear mixed effect model on resting oxygen consumption.

a. Resting oxygen consumption lmer model comparisons. Each perspective model included resting oxygen consumption as the dependant variable with cross-generation, temperature, and CO2 treatments entered the model as fixed factors. Maternal linage (maternal grandfather and grandmother code A-F), and paternal linage (paternal grandfather and grandmother code A-F) were also included into all models as random factors. The model variations (random factors and covariates) are listed below. Density was only driven by one individual and reduced model assumptions fit, so Respirometry chamber ID was deemed the best model (as marked in bold).

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	25	5962.3	6071.4	-2956.2	5912.3			
Respirometry chamber ID	**26**	**5941.6**	**6055**	**-2944.8**	**5889.6**	**22.7**	**1**	**1.86E-06**
Tank ID	26	5963.3	6076.8	-2955.7	5911.3	0	0	
Parental number (clutch ID)	26	5963.9	6077.3	-2955.9	5911.9	0	0	
Respirometry chamber ID + Density	27	5942.3	6060.1	-2944.1	5888.3	23.6	1	**1.19E-06**
Respirometry chamber ID + am_pm	27	5943.5	6061.3	-2944.8	5889.5	0	0	
Respirometry chamber ID + Density + am_pm	28	5944.1	6066.3	-2944.1	5888.1	1.4	1	0.2376

b. Statistical output for the selected resting oxygen consumption lmer model. Fixed effect values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random factor	Variance
Cross-generation	4	35.31	0.541	0.707	Parental grandfather	23.41
Temperature	1	542.09	0.289	0.591	Maternal grandfather	$1.79x10^{-5}$
CO_2	1	543.34	4.225	**0.040**	Parental grandmother	23.88
Cross-generation*Temperature	4	541.89	1.547	0.187	Maternal grandmother	18.72
Cross-generation*CO_2	4	543.72	0.609	0.656	Chamber ID	128.70
Temperature*CO_2	1	541.84	0.021	0.884	Residual	1512.00
Cross-generation*Temperature*CO_2	4	543.60	0.594	0.667		

Table A1.2 Maximum oxygen consumption linear mixed effect model.

a. Maximum oxygen consumption lmer model comparisons. Each perspective model included maximum oxygen consumption as the dependant variable with cross-generation, temperature, and CO2 treatments entered the model as fixed factors. Maternal linage (maternal grandfather and grandmother code A-F), and paternal linage (paternal grandfather and grandmother code A-F) were also included into all models as random factors. The model variations (random factors and covariates) are listed below. Density was only driven by one individual, so Respirometry chamber ID was deemed the best model (as marked in bold).

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	25	7454	7563	-3702	7404			
Respirometry chamber ID	**26**	**7320.2**	**7433.6**	**-3634.1**	**7268.2**	**135.8**	**1**	**<2e-16**
Parental number (clutch ID)	26	7456	7569.4	-3702	7404	0	0	
Tank ID	26	7456	7569.4	-3702	7404	0	0	
Respirometry chamber ID + Density	27	7321.7	7439.5	-3633.8	7267.7	136.3	1	**<2e-16**
Respirometry chamber ID + am_pm	27	7313.9	7431.7	-3630	7259.9	7.8	0	
Respirometry chamber ID + Density + am_pm	28	7315.4	7437.5	-3629.7	7259.4	0.6	1	0.4484

b. Statistical output for the selected maximum oxygen consumption lmer model. Fixed effect values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random factor	Variance
Cross-generation	4	342.28	0.414	0.799	Parental grandfather	0.00
Temperature	1	555.38	3.919	**0.048**	Maternal grandfather	0.00
CO_2	1	555.55	0.025	0.875	Parental grandmother	0.00
Cross-generation*Temperature	4	555.35	1.972	0.097	Maternal grandmother	463.00
Cross-generation*CO_2	4	555.5	1.651	0.160	Chamber ID	7032.00
Temperature*CO_2	1	555.4	0.276	0.599	Residual	16215.00
Cross-generation*Temperature*CO_2	4	555.37	3.451	**0.008**		

Table A1.3 Linear mixed effect model on aerobic scope

a. Aerobic scope lmer model comparisons. Each perspective model included aerobic scope as the dependant variable with cross-generation, temperature, and CO2 treatments entered the model as fixed factors. Maternal linage (maternal grandfather and grandmother code A-F), and paternal linage (paternal grandfather and grandmother code A-F) were also included into all models as random factors. The model variations (random factors and covariates) are listed below. Density was only driven by one individual, so Respirometry chamber ID was deemed the best model (as marked in bold).

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	25	3055.7	3164.8	-1502.8	3005.7			
Respirometry chamber ID	**26**	**2947.1**	**3060.6**	**-1447.6**	**2895.1**	**110.6**	**1**	**<2e-16**
Parental number (clutch ID)	26	3056.8	3170.3	-1502.4	3004.8	0	0	
Tank ID	26	3057.7	3171.1	-1502.8	3005.7	0	0	
Respirometry chamber ID + Density	27	2948.8	3066.6	-1447.4	2894.8	110.9	1	**<2e-16**
Respirometry chamber ID + am_pm	27	2940.1	3057.9	-1443.1	2886.1	8.6	0	
Respirometry chamber ID + Density + am_pm	28	2941.7	3063.8	-1442.8	2885.7	0.5	1	0.4986

b. Statistical output for the selected aerobic scope lmer model. Fixed effect values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random factor	Variance
Cross-generation	4	295.12	0.443	0.777	Parental grandfather	0.00
Temperature	1	555.47	3.959	**0.047**	Maternal grandfather	0.00
CO_2	1	555.67	0.299	0.585	Parental grandmother	$1.56x10^{-9}$
Cross-generation*Temperature	4	555.43	1.135	0.339	Maternal grandmother	0.19
Cross-generation*CO_2	4	555.59	2.076	0.083	Chamber ID	3.05
Temperature*CO_2	1	555.48	0.289	0.591	Residual	8.64
Cross-generation*Temperature*CO_2	4	555.45	2.664	**0.032**		

Table A1.4 Negative binomial model on the behaviour (boldness and activity) score of juvenile *A. polyacanthus* maintained at Control (28.5°C, 490 µatm); Warm temperature (30°C, 490 µatm); Elevated CO_2 (28.5°C, 825 µatm); Warm temperature and Elevated CO_2 (30°C, 825 µatm). Values are from the type II Wald chi-squared test.

Fixed effects	LR Chisq	Df	Pr(>Chisq)
Score	6.36	1	**0.012**
Cross-generation	1.03	4	0.905
CO_2	1.72	1	0.189
Temperature	0.78	1	0.377
Score * Cross-generation	2.65	4	0.617
Score *CO_2	0.81	1	0.369
Cross-generation*CO_2	0.94	4	0.918
Score* Temperature	5.58	1	**0.018**
Cross-generation*Temperature	0.25	4	0.993
CO_2*Temperature	0.44	1	0.505
Score*Cross-generation*CO_2	0.64	4	0.959
Score*Cross-generation*Temperature	0.97	4	0.914
Score*CO_2*Temperature	0.07	1	0.789
Cross-generation *CO_2*Temperature	0.67	4	0.955
Score*Cross-generation *CO_2*Temperature	2.05	4	0.726

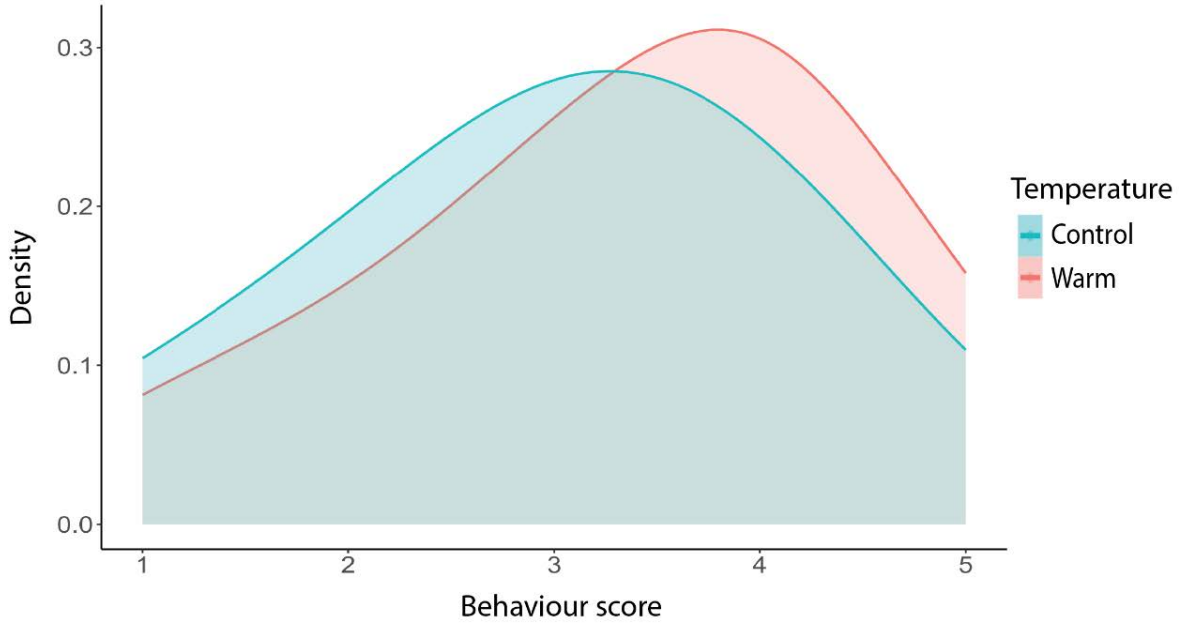

Figure A1.1 Proportional density juvenile *A. polyacanthus* at each combination behaviour (boldness and activity) score. Juveniles were maintained at Control (28.5°C); Warm temperature (30°C) for 101-137 days post-hatching.

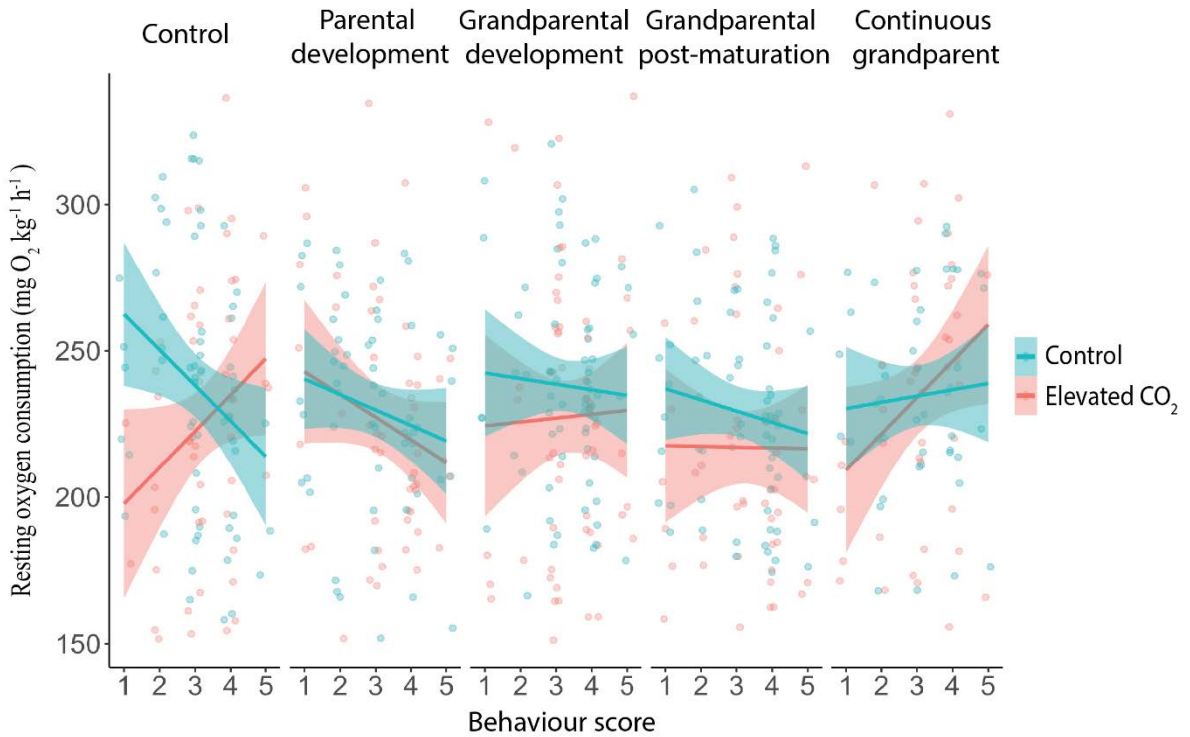

Figure A1.2 Resting oxygen consumption of juvenile *A. polyacanthus* maintained at Control (490 µatm, 28.5°C, or 30°C); Elevated CO_2 (825 µatm, 28.5°C, or 30°C) at each behaviour (boldness and activity) score. Fitted data points are displayed with a linear trendline (with a smoothing function in ggplot2) for each cross-generation

Table A1.5 Linear mixed effect model on resting oxygen consumption at each behaviour (boldness and activity) score.

a. The interaction between resting oxygen consumption and behaviour (boldness and activity) score lmer model comparisons. Each perspective model included resting oxygen consumption as the dependant variable with cross-generation, temperature, CO2 treatment and behaviour score entered the model as fixed factors. Parental clutch ID was also included into all models as a random factor. The model variations (random factors and covariates) are listed below. Respirometry chamber ID was deemed the best model (as marked in bold) as it had the lowest AIC and improved model fit.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	42	5604.9	5785.5	-2760.5	5520.9			
Density	43	5605.5	5790.4	-2759.8	5519.5	1.4	1	0.235
Tank ID	43	5605.9	5790.7	-2759.9	5519.9	0	0	
am_pm	43	5606.9	5791.8	-2760.4	5520.9	0	0	
Respirometry chamber ID	**43**	**5583.2**	**5768.1**	**-2748.6**	**5497.2**	**23.7**	**0**	
Density + am_pm	44	5607.5	5796.7	-2759.8	5519.5	0	1	1

b. Statistical output for the selected lmer model. Values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random factor	Variance
Cross-generation	4	254.74	0.674	0.611	Clutch ID	54.57
Temperature	1	499.81	0.637	0.425	Chamber ID	137.53
CO_2	1	501.45	9.235	0.002	Residual	1479.91
Score	1	499.39	0.158	0.692		
Cross-generation*Temperature	4	497.46	0.332	0.856		
Cross-generation*CO_2	4	500.8	2.979	0.019		
CO_2*Temperature	1	501.33	0.037	0.849		
Score* Cross-generation	4	493.77	1.391	0.236		
Score* Temperature	1	499.83	0.364	0.546		
Score*CO_2	1	501.01	6.449	**0.011**		
Cross-generation *CO_2*Temperature	4	501.06	0.777	0.540		
Score*Cross-generation*Temperature	4	498.44	0.471	0.757		
Score*Cross-generation*CO_2	4	495.76	2.820	**0.025**		
Score*CO_2*Temperature	1	500.88	0.000	0.998		
Score*Cross-generation *CO_2*Temperature	4	500.47	0.774	0.542		

Table A1.6 Linear mixed effect model on the aerobic scope of juvenile *A. polyacanthus* at each behaviour (boldness and activity) score.

a. The interaction between aerobic scope and behaviour (boldness and activity) score lmer model comparisons. Each perspective model included aerobic scope as the dependant variable with cross-generation, temperature, CO2 treatment and behaviour score entered the model as fixed factors. Parental clutch ID was also included into all models as a random factor. The model variations (random factors and covariates) are listed below. Respirometry chamber ID was deemed the best model (as marked in bold) as it had the lowest AIC.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	42	6956.3	7136.8	-3436.1	6872.3			
Density	43	6957	7141.8	-3435.5	6871	1.3	1	0.2516
Tank ID	43	6958.3	7143.1	-3436.1	6872.3	0	0	
am_pm	43	6955.7	7140.5	-3434.8	6869.7	2.6	0	
Respirometry chamber ID	**43**	**6870.5**	**7055.3**	**-3392.2**	**6784.5**	**85.2**	**0**	

b. Statistical output for the selected lmer model. Values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random factor	Variance
Cross-generation	4	262.66	0.349	0.845	Clutch ID	607.5
Temperature	1	499.76	9.924	0.002	Chamber ID	4854.4
CO_2	1	501.09	1.028	0.311	Residual	15622.7
Score	1	499.27	3.118	0.078		
Cross-generation*Temperature	4	497.68	0.634	0.638		
Cross-generation*CO_2	4	500.53	0.758	0.553		
CO_2*Temperature	1	501.06	0.422	0.516		
Score* Cross-generation	4	494.67	0.338	0.852		
Score* Temperature	1	499.72	5.311	**0.022**		
Score*CO_2	1	500.74	0.687	0.408		
Cross-generation *CO_2*Temperature	4	500.87	2.628	0.034		
Score*Cross-generation*Temperature	4	498.52	0.449	0.773		
Score*Cross-generation*CO_2	4	496.08	0.172	0.953		
Score*CO_2*Temperature	1	500.61	0.558	0.456		
Score*Cross-generation *CO_2*Temperature	4	500.29	1.837	0.120		

Table A1.7 Linear mixed effect model on the standard body length

a. Standard length lmer model comparisons. Each perspective model included standard length as the dependant variable with cross-generation, temperature, and CO2 treatments entered the model as fixed factors. Maternal linage (maternal grandfather and grandmother code A-F), and paternal linage (paternal grandfather and grandmother code A-F) were also included into all models as random factors. The model variations (random factors and covariates) are listed below. Tank ID was deemed the best model (as marked in bold) as Tank ID + Density model did not converge.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	25	13866	14014	-6907.9	13816			
Tank ID	**26**	**13729**	**13884**	**-6838.6**	**13677**	**138.6**	**1**	**< 2.2e-16**
Density	26	13559	13714	-6753.6	13507	170.1	0	
Parental number (clutch ID)	26	13735	13890	-6841.7	13683	0	0	
Tank ID + Density	27	13552	13712	-6748.8	13498	185.7	1	< 2.2e-16

b. Statistical output for the selected standard length lmer model. Values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random factor	Variance
Cross-generation	4	92.548	8.442	**<0.001**	Tank ID	1.384
Temperature	1	127.99	8.155	**0.0050**	Parental grandfather	1.993
CO_2	1	123.01	6.802	**0.0102**	Maternal grandfather	0.1256
Cross-generation*Temperature	4	127.70	0.184	0.9463	Parental grandmother	3.5679
Cross-generation*CO_2	4	121.94	0.958	0.4334	Maternal grandmother	0.4383
Temperature*CO_2	1	128.00	0.633	0.4276	Residual	6.9195
Cross-generation* Temperature*CO_2	4	127.84	0.239	0.9157		

Table A1.8 Linear mixed effect model on the physical condition (weight for the average standard length of 31.2mm).

a. Physical condition lmer model comparisons. Each perspective model included log_weight as the dependant variable with cross-generation, temperature, CO2 treatments, and log_length entered the model as fixed factors. Maternal linage (maternal grandfather and grandmother code A-F), and paternal linage (paternal grandfather and grandmother code A-F) were also included into all models as random factors. The model variations (random factors and covariates) are listed below. Tank ID+ Density was deemed the best model (as marked in bold) as Tank ID model did not converge.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	26	-12036	-11880	6044.1	-12088			
Tank ID	27	-12234	-12073	6144.2	-12288	200.2	1	<2.20E-16
Density	27	-12120	-11959	6087.2	-12174	0	0	
Tank ID + Density	**28**	**-12282**	**-12114**	**6168.9**	**-12338**	**163.4**	**1**	**<2.20E-16**
Parental number (clutch ID)	28	-12296	-12128	6176	-12352	14.3	0	

b. Statistical output for the selected physical condition lmer model. Values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random factor	Variance
Cross-generation	4	157.34	3.15	**0.0159**	Tank ID	$1.49x10^{-4}$
Temperature	1	153.71	23.61	**<0.001**	Parental grandfather	$1.61 x10^{-4}$
CO_2	1	148.76	4.28	**0.0403**	Maternal grandfather	$2.38x10^{-13}$
Log_length	1	2860.27	71463.21	<0.001	Parental grandmother	$1.26 x10^{-4}$
Density	1	218.36	50.77	<0.001	Maternal grandmother	$1.19 x10^{-4}$
Cross-generation*Temperature	4	154.27	0.44	0.7801	Residual	$7.70 x10^{-4}$
Cross-generation*CO_2	4	147.41	0.55	0.7028		
Temperature*CO_2	1	154.34	0.27	0.6008		
Cross-generation* Temperature*CO_2	4	154.26	0.31	0.8717		

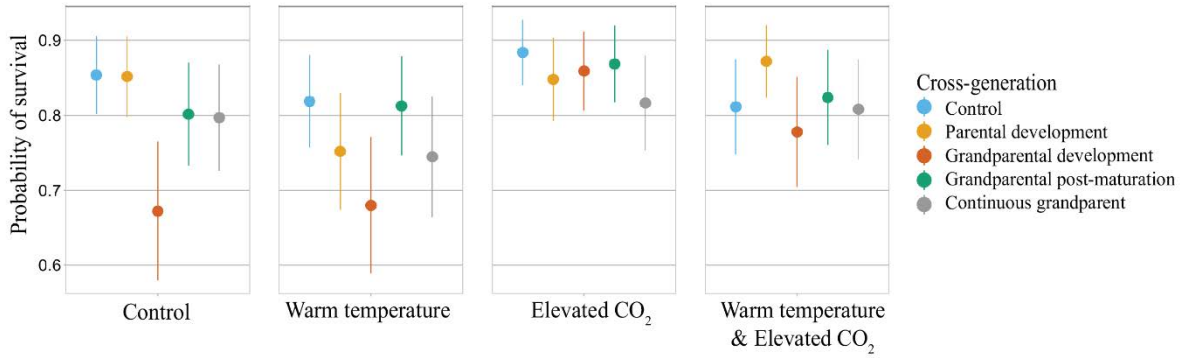

Figure A1.3 Probability of for juvenile *A. polyacanthus* surviving 101-137 days post-hatching at Control (28.5°C, 490 µatm); Warm temperature (30°C, 490 µatm); Elevated CO_2 (28.5°C, 825 µatm); or Warm temperature and Elevated CO2 (30°C, 825 µatm) . All data is the probability ± SE.

Table A1.9 Generalised linear mixed effect model on survival of juvenile *A. polyacanthus* maintained at Control (28.5°C, 490 µatm); Warm temperature (30°C,490 µatm); Elevated CO_2 (28.5°C, 825 µatm); Warm temperature and Elevated CO_2 (30°C, 825 µatm). Values are from the type II Wald chi-squared test.

Fixed effects	Chisq	df	P	Random factor	Variance
Cross-generation	21.98	4	**0.0002**	Parental grandfather	0.21485
Temperature	10.06	1	**0.0015**	Maternal grandfather	0.09429
CO_2	21.52	1	**0.0000**	Parental grandmother	0.17269
Cross-generation*Temperature	1.63	4	0.8039	Maternal grandmother	0.02675
Cross-generation*CO_2	9.21	4	0.0560		
Temperature*CO_2	0.12	1	0.7270		
Cross-generation*Temperature*CO_2	11.45	4	**0.0219**		

Appendix 2

The following appendix accompanies Chapter 3:

The interactive effects of ocean warming, suspended sediment, and the role of cross-generational thermal plasticity

Proposed authors: Jasmine S. Cane, Shannon J. McMahon, Andrew S. Hoey and Jennifer M. Donelson

All tables are for data collected from juvenile *A. polyacanthus* maintained at Control temperature – No sediment (28.5°C, 0 mg L^{-1}), Warm temperature – No sediment (30°C, 0 mg L^{-1}), Control temperature – Suspended sediment (28.5°C, 50 mg L^{-1}), Warm temperature – Suspended sediment (30°C, 50 mg L^{-1}).

Table A2.1 Number of clutch replicates within each juvenile treatment (Clutch number = Tank number of F2 adult pairs, used as identification number of cross-generation breeding pairs)

Cross-generation	**Clutch number**	**Juvenile treatment**			
		Control temperature – No sediment	Warm Temperature – No sediment	Control temperature - Suspended sediment	Warm temperature - Suspended sediment
Control	42	2	2	3	3
	87	3	3	3	3
	89	2	2	2	2
	92	3	3	3	2
Parental development	5	2	2	2	2
	73	2	2	2	2
	94	3	2	2	2
Grandparental development	10	2	2	2	2
	64	2	2	2	2
	81	2	2	2	2
	86	2	3	2	3

Table A2.2 Total number of individuals in each data set within each treatment group (used in the final statistical analysis and output). Sample time 1-3 values are for standard length and physical condition performance metrics.

Gill measures and remodelling	Control temperature – No sediment	Warm Temperature – No sediment	Control temperature - Suspended sediment	Warm temperature -Suspended sediment
CCCC	6	6	6	6
CCCH	6	6	5	6
HHCC	6	6	6	6
Sample time 1				
CCCC	60	60	66	60
CCCH	42	36	36	36
HHCC	48	54	48	54
Sample time 2				
CCCC	30	30	33	29
CCCH	21	18	18	18
HHCC	24	27	24	24
Sample time 3				
CCCC	105	94	103	68
CCCH	74	64	58	37
HHCC	81	95	84	56

Gill lamellae morphometrics

Table A2.3 Linear mixed effect model on filament width.

a. Filament width lmer model comparisons. Each perspective model included filament width as the dependant variable with cross-generation, temperature, sediment treatments, and standard body length entered the model as fixed factors. Maternal and paternal linage (maternal and paternal F_0 grandparents from the six starting pairs) were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model due to its low AIC value.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	16	**315.58**	**351.79**	**-141.79**	**283.58**			
Age	17	316.08	354.54	-141.04	282.08	1.5	1	0.2197
Density at sample time 1	17	317.53	355.99	-141.76	283.53	0	0	
Sump ID	17	317.58	356.05	-141.79	283.58	0	0	
Parental number (clutch ID)	17	317.58	356.05	-141.79	283.58	0	0	

b. Statistical output for the selected filament width lmer model. Random factor values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random Factor	Variance
Cross-generation	2	58	0.498	0.610	Maternal	0
Temperature	1	58	3.064	0.085	Paternal	0
Sediment	1	58	25.951	< **0.001**	Residual	3.86
Length	1	58	0.007	0.934		
Cross-generation*Temperature	2	58	1.041	0.360		
Cross-generation*Sediment	2	58	0.811	0.450		
Temperature*Sediment	1	58	48.187	< **0.001**		
Cross-generation*Temperature*Sediment	2	58	0.637	0.533		

Table A2.4 Linear mixed effect model on lamellae width.

a. Lamellae width lmer model comparisons. Each perspective model included lamellae width as the dependant variable with cross-generation, temperature, sediment treatments, and standard body length entered the model as fixed factors. Maternal and paternal linage (maternal and paternal F_0 grandparents from the six starting pairs) were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model due to its low AIC value and model simplicity.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	16	**203.64**	**239.61**	**-85.818**	**171.64**			
Age	17	203.08	241.3	-84.54	169.08	2.6	1	0.1098
Density at sample time 1	17	205.25	243.47	-85.622	171.25	0	0	
Sump ID	17	205.63	243.86	-85.816	171.63	0	0	
Parental number (clutch ID)	17	205.64	243.86	-85.818	171.64	0	0	

b. Statistical output for the selected lamellae width lmer model. Values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random Factor	Variance
Cross-generation	2	23.58	3.645	**0.042**	Maternal	0.0878
Temperature	1	55.09	18.379	< **0.001**	Paternal	0
Sediment	1	55.05	14.769	< **0.001**	Residual	**0.7839**
Length	1	40.10	0.003	0.957		
Cross-generation*Temperature	2	54.90	1.113	0.336		
Cross-generation*Sediment	2	54.77	3.171	**0.050**		
Temperature*Sediment	1	54.74	28.590	< **0.001**		
Cross-generation*Temperature*Sediment	2	54.97	5.370	**0.007**		

Table A2.5 Linear mixed effect model on lamellae perimeter.

a. Lamellae perimeter lmer model comparisons. Each perspective model included lamellae perimeter as the dependant variable with cross-generation, temperature, sediment treatments, and standard body length entered the model as fixed factors. Maternal and paternal linage (maternal and paternal F_0 grandparents from the six starting pairs) were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model due to its low AIC value and model simplicity.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	16	**634.33**	**670.53**	**-301.16**	**602.33**			
Age	17	635.67	674.14	-300.83	601.67	0.7	1	0.4181
Density at sample time 1	17	633.92	672.39	-299.96	599.92	1.7	0	
Sump ID	17	636.33	674.79	-301.16	602.33	0	0	
Parental number (clutch ID)	17	636.33	674.79	-301.16	602.33	0	0	

b. Statistical output for the selected lamellae perimeter lmer model. Values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random Factor	Variance
Cross-generation	2	23.85	0.034	0.967	Maternal	63.3
Temperature	1	54.96	1.033	0.314	Paternal	0
Sediment	1	54.89	0.286	0.595	Residual	316.9
Length	1	46.14	12.139	**0.001**		
Cross-generation*Temperature	2	54.80	0.112	0.894		
Cross-generation*Sediment	2	54.71	3.086	0.054		
Temperature*Sediment	1	54.70	0.750	0.390		
Cross-generation*Temperature*Sediment	2	54.90	0.373	0.691		

Gill lamellae remodelling

Table A2.6 Generalised linear mixed effect model on epithelial lifting.

a. Epithelial lifting glmer model comparisons. Each perspective model included lamellae perimeter as the dependant variable with cross-generation, temperature, and sediment treatments entered the model as fixed factors. Maternal and paternal linage (maternal and paternal F_0 grandparents from the six starting pairs) were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model as the alternate two models had reduced model fit.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	14	**472.95**	**503.39**	**-222.47**	**444.95**			
Sump ID	15	461.94	494.56	-215.97	431.94	13.0	1	0.00031
Parental number (clutch ID)	15	474.95	507.57	-222.47	444.95	0	0	

b. Statistical output for the selected epithelial lifting lmer model. Values are from the type II Wald chi-squared test.

Fixed effects	Chisq	df	P	Random Factor	Variance
Cross-generation	10.31	2	**0.006**	Maternal	1.016
Temperature	11.77	1	**0.001**	Paternal	0.9812
Sediment	13.36	1	**< 0.001**		
Cross-generation*Temperature	3.84	2	0.147		
Cross-generation*Sediment	17.81	2	**< 0.001**		
Temperature*Sediment	6.21	1	**0.013**		
Cross-generation*Temperature*Sediment	0.91	2	0.634		

Table A2.7 Generalised linear mixed effect model on hyperplasia.

a. Hyperplasia glmer model comparisons. Each perspective model included hyperplasia as the dependant variable with cross-generation, temperature, and sediment treatments entered the model as fixed factors. Maternal and paternal linage (maternal and paternal F_0 grandparents from the six starting pairs) were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model due to its low AIC value and simplicity.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	14	**327.56**	**359.23**	**-149.78**	**299.56**			
Sump ID	15	327.44	361.38	-148.72	297.44	2.1	1	0.1458
Parental number (clutch ID)	15	329.56	363.5	-149.78	299.56	0	0	

b. Statistical output for the selected hyperplasia lmer model. Values are from the type II Wald chi-squared test.

Fixed effects	Chisq	df	P	Random Factor	Variance
Cross-generation	3.21	2	0.201	Maternal	0.007
Temperature	27.93	1	**< 0.001**	Paternal	0.039
Sediment	46.91	1	**< 0.001**		
Cross-generation*Temperature	3.35	2	0.187		
Cross-generation*Sediment	1.31	2	0.520		
Temperature*Sediment	27.31	1	**< 0.001**		
Cross-generation*Temperature*Sediment	2.20	2	0.333		

Table A2.8 Generalised linear mixed effect model on mucus.

a. Mucus glmer model comparisons. Each perspective model included mucus as the dependant variable with cross-generation, temperature, and sediment treatments entered the model as fixed factors. Maternal and paternal linage (maternal and paternal F_0 grandparents from the six starting pairs) were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model due to its low AIC value.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	14	**385.12**	**416.8**	**-178.56**	**357.12**			
Sump ID	15	386.19	420.13	-178.1	356.19	0.932	1	0.3343
Parental number (clutch ID)	15	386.29	420.23	-178.15	356.29	0	0	

b. Statistical output for the selected mucus lmer model. Values are from the type II Wald chi-squared test.

Fixed effects	Chisq	df	P	Random Factor	Variance
Cross-generation	8.47	2	**0.014**	Maternal	0.099
Temperature	2.36	1	0.125	Paternal	0.051
Sediment	12.21	1	< **0.001**		
Cross-generation*Temperature	1.03	2	0.599		
Cross-generation*Sediment	1.01	2	0.603		
Temperature*Sediment	2.84	1	0.092		
Cross-generation*Temperature*Sediment	16.49	2	< **0.001**		

Table A2.9 Generalised linear mixed effect model on lamellae fusion.

a. Lamellae fusion glmer model comparisons. Each perspective model included lamellae fusion as the dependant variable with cross-generation, temperature, and sediment treatments entered the model as fixed factors. Maternal and paternal linage (maternal and paternal F_0 grandparents from the six starting pairs) were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model due to its low AIC value.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	14	**206.85**	**238.53**	**-89.425**	**178.85**			
Sump ID	15	208.85	242.79	-89.425	178.85	0	1	1
Parental number (clutch ID)	15	208.85	242.79	-89.425	178.85	$1x10^{-4}$	0	

b. Statistical output for the selected lamellae fusion lmer model. Values are from the type II Wald chi-squared test.

Fixed effects	Chisq	df	P	Random Factor	Variance
Cross-generation	1.84	2	0.400	Maternal	3.074×10^{-1}
Temperature	0.01	1	0.919	Paternal	1.184×10^{-8}
Sediment	0.01	1	0.936		
Cross-generation*Temperature	0.19	2	0.909		
Cross-generation*Sediment	0.73	2	0.695		
Temperature*Sediment	1.17	1	0.279		
Cross-generation*Temperature*Sediment	0.33	2	0.847		

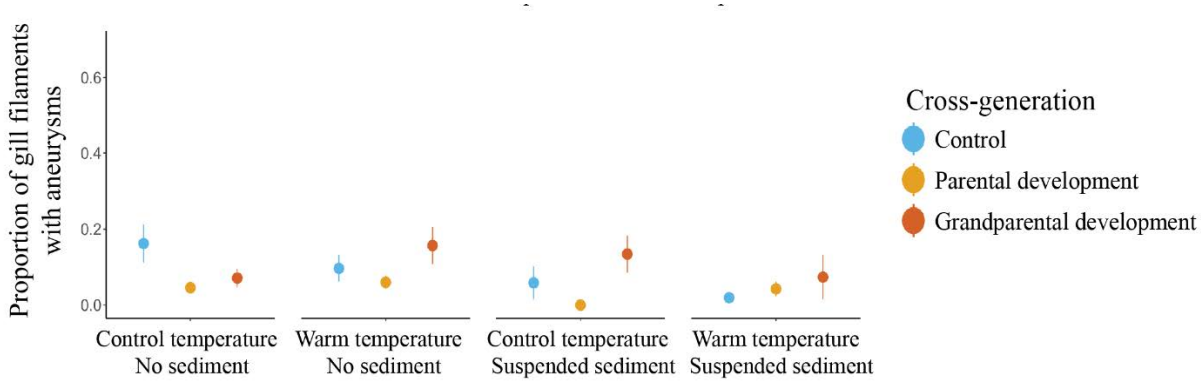

Figure A2.1 The proportion of gill filaments containing aneurysms Data collected from juvenile *A. polyacanthus* maintained at Control temperature – No sediment (28.5°C, 0 mg L^{-1}), Warm temperature – No sediment (30°C, 0 mg L^{-1}), Control temperature – Suspended sediment (28.5°C, 50 mg L^{-1}), Warm temperature – Suspended sediment (30°C, 50 mg L^{-1}) at Sample time 1. Raw data is presented as proportion ± SE**.**

Standard length

Table A2.10 Linear mixed effect model on standard length at Sample time 1.

a. Standard length at sample time point 1 glmer model comparisons. Each perspective model included standard length as the dependant variable with cross-generation, temperature, and sediment treatments entered the model as fixed factors. Parental clutch number and tank ID nested in sump ID were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model due to its low AIC value.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	16	2782.1	2852.4	-1375	2750.1			
Age	17	2754.7	2829.4	-1360.3	2720.7	29.4	1	5.80×10^{-8}
Density_time	17	2774.8	2849.5	-1370.4	2740.8	0	0	
Age + Density_time	**18**	**2747.2**	**2826.3**	**-1355.6**	**2711.2**	**29.6**	**1**	$\mathbf{5.35} \times 10^{-8}$

b. Statistical output for the selected standard length at Sample time 1 lmer model. Values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random Factor	Variance
Cross-generation	2	5.75	0.10	0.9071	Tank:Sump	7.52×10^{-10}
Temperature	1	577.94	5.15	**0.0236**	Parental clutch ID	0.235
Sediment	1	578.62	36.25	**<0.001**	Sump	0.00
Age	1	8.98	90.13	<0.001	Residual	5.39
Density_time	1	585.38	9.37	0.0023		
Cross-generation*Temperature	2	578.20	0.79	0.4529		
Cross-generation*Sediment	2	578.16	0.45	0.6397		
Temperature*Sediment	1	577.14	3.76	0.0530		
Cross-generation* Temperature*Sediment	2	577.35	1.60	0.2029		

Table A2.11 Linear mixed effect model on standard length at Sample time 2.

a. Standard length at sample time point 2 glmer model comparisons. Each perspective model included standard length as the dependant variable with cross-generation, temperature, and sediment treatments entered the model as fixed factors. Parental clutch number and tank ID nested in sump ID were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model due to its low AIC value.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	16	1502.9	1562	-735.46	1470.9			
Age	17	1493.4	1556.1	-729.69	1459.4	11.5	1	6.78×10^{-4}
Density_time	17	1460.2	1522.9	-713.1	1426.2	33.2	0	
Juvenile treatment exposure time	17	1492.5	1555.3	-729.27	1458.5	0	0	
Age + Density_time	18	1444.5	1510.9	-704.25	1408.5	50.0	1	1.51×10^{-12}
Density_time + juvenile treatment exposure time	**18**	**1443.8**	**1510.2**	**-703.9**	**1407.8**	**0.7**	**0**	
Age + juvenile treatment exposure time	18	1492	1558.4	-727.98	1456	0	0	

b. Statistical output for the selected standard length at Sample time 2 lmer model. Values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random Factor	Variance
Cross-generation	2	5.75	0.0992	0.907064		
Temperature	1	577.94	5.1533	0.02357		
Sediment	1	578.62	36.2521	3.08E-09		
Age	1	8.98	90.1296	5.57E-06		
Density_time	1	585.38	9.3689	0.002308		
Cross-generation*Temperature	2	578.2	0.7931	0.452948		
Cross-generation*Sediment	2	578.16	0.4471	0.639718		
Temperature*Sediment	1	577.14	3.7597	0.052989		
Cross-generation*Temperature*Sediment	2	577.35	1.5996	0.202863		

Fixed effects	Num. df	Den.df	*F*	P	Random Factor	Variance
Cross-generation	2	6.55	0.96	0.4307	Tank:Sump	0.00
Temperature	1	275.88	2.77	0.0975	Parental clutch ID	0.2742
Sediment	1	276.21	9.41	**0.0024**	Sump	0.00
Treatment exposure time	1	6.90	30.34	<0.001	Residual	6.98
Density_time	1	281.82	59.06	<0.001		
Cross-generation*Temperature	2	276.40	0.08	0.9205		
Cross-generation*Sediment	2	276.14	1.71	0.1824		
Temperature*Sediment	1	276.16	0.25	0.6189		
Cross-generation* Temperature*Sediment	2	275.06	2.09	0.1252		

Table A2.12 Linear mixed effect model on standard length at Sample time 3.

a. Standard length at sample time point 3 glmer model comparisons. Each perspective model included standard length as the dependant variable with cross-generation, temperature, and sediment treatments entered the model as fixed factors. Parental clutch number and tank ID nested in sump ID were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model due to its low AIC value.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	16	4899.7	4976.8	-2433.8	4867.7			
Age	17	4889	4970.9	-2427.5	4855	12.7	1	3.65×10^{-4}
Density_time	17	4849.1	4931.1	-2407.5	4815.1	39.9	0	
Juvenile treatment exposure time	17	4886.8	4968.8	-2426.4	4852.8	0	0	
Age + Density_time	18	4835.6	4922.4	-2399.8	4799.6	53.2	1	2.97×10^{-13}
Density_time + juvenile treatment exposure time	**18**	**4834**	**4920.8**	**-2399**	**4798**	**1.6**	**0**	
Age + juvenile treatment exposure time	18	4888.3	4975.1	-2426.1	4852.3	0	0	

b. Statistical output for the selected standard length at Sample time 3 lmer model. Values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random Factor	Variance
Cross-generation	2	7.02	0.35	0.7151	Tank:Sump	0.000
Temperature	1	3.76	10.47	**0.0348**	Parental clutch ID	0.193
Sediment	1	4.06	0.17	0.6993	Sump	0.083
Treatment exposure time	1	7.16	24.76	0.0015	Residual	10.862
Density_time	1	164.88	59.31	<0.001		
Cross-generation*Temperature	2	891.82	0.10	0.9075		
Cross-generation*Sediment	2	898.58	0.29	0.7458		
Temperature*Sediment	1	3.83	0.67	0.4601		
Cross-generation* Temperature*Sediment	2	891.93	1.18	0.3076		

Physical condition

Table A2.13 Linear mixed effect model on juvenile *A. polyacanthus* physical condition (weight for a given length of 24.57mm) at Sample time 1.

a. Physical condition at sample time point 1 glmer model comparisons. Each perspective model included physical condition as the dependant variable with cross-generation, temperature, sediment treatment and log standard body length entered the model as fixed factors. Parental clutch number and tank ID nested in sump ID were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model due to its low AIC value of those with a significant Cisq.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	17	-2519.5	-2444.7	1276.8	-2553.5			
Age	18	-2532.2	-2453	1284.1	-2568.2	14.7	1	1.28×10^{-4}
Density_time	18	-2534.7	-2455.6	1285.4	-2570.7	2.6	0	
Age + Density_time	**19**	**-2547.7**	**-2464.1**	**1292.8**	**-2585.7**	**14.9**	**1**	**0.0001**

b. Statistical output for the selected physical condition at Sample time 1 lmer model. Values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random Factor	Variance
Cross-generation	2	5.91	0.23	0.8019	Tank:Sump	3.75×10^{-6}
Temperature	1	4.07	24.82	**0.0072**	Parental clutch ID	8.14×10^{-4}
Sediment	1	4.26	0.10	0.7638	Sump	2.53×10^{-5}
Length (log)	1	572.25	15067.56	< 0.001	Residual	6.65×10^{-4}
Density_time	1	77.47	16.33	< 0.001		
Age	1	172.65	29.72	< 0.001		
Cross-generation*Temperature	2	74.21	0.52	0.5980		
Cross-generation*Sediment	2	74.18	2.45	0.0929		
Temperature*Sediment	1	4.06	11.45	**0.0271**		
Cross-generation* Temperature*Sediment	2	74.37	1.90	0.1564		

Table A2.14 Linear mixed effect model on juvenile *A. polyacanthus* physical condition (weight for a given length of 27.29mm) at Sample time 2.

a. Physical condition at sample time point 1 glmer model comparisons. Each perspective model included physical condition as the dependant variable with cross-generation, temperature, sediment treatment and log standard body length entered the model as fixed factors. Parental clutch number and tank ID nested in sump ID were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	17	-1357.1	-1294.4	695.55	-1391.1			
Age	18	-1357.4	-1291	696.72	-1393.4	2.3	1	0.1253
Juvenile treatment exposure time	18	-1358	-1291.6	697.01	-1394	0.6	0	
Density_time	18	-1356.9	-1290.4	696.43	-1392.9	0	0	
Density_time + juvenile treatment exposure time	**19**	**-1357.3**	**-1287.2**	**697.67**	**-1395.3**	**2.5**	**1**	**0.1157**
Age + Density_time	19	-1356.8	-1286.7	697.38	-1394.8	0	0	

b. Statistical output for the selected physical condition at Sample time 2 lmer model. Values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random Factor	Variance
Cross-generation	2	6.93	1.97	0.2100	Tank:Sump	4.24×10^{-5}
Temperature	1	4.59	23.91	**0.0057**	Parental clutch ID	1.01×10^{-4}
Sediment	1	4.97	0.01	0.9459	Sump	1.24×10^{-5}
Length (log)	1	270.95	10458.23	< 0.001	Residual	4.77×10^{-4}
Density_time	1	65.04	0.77	0.3835		
Juvenile treatment exposure time	1	7.55	1.91	0.2067		
Cross-generation*Temperature	2	73.42	3.24	**0.0450**		
Cross-generation*Sediment	2	74.25	1.74	0.1834		
Temperature*Sediment	1	4.46	0.86	0.4004		
Cross-generation* Temperature*Sediment	2	75.03	1.01	0.3693		

Table A2.15 Linear mixed effect model on juvenile *A. polyacanthus* physical condition (weight for a given length of 29.45mm) at Sample time 3.

a. Physical condition at sample time point 1 glmer model comparisons. Each perspective model included physical condition as the dependant variable with cross-generation, temperature, sediment treatment and log standard body length entered the model as fixed factors. Parental clutch number and tank ID nested in sump ID were included into all models as random factors. The model variations (random factors and covariates) are listed below. The model variation marked in bold was deemed the best model.

Model variations	npar	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
	17	-3850.2	-3768.2	1942.1	-3884.2			
Age	18	-3859.1	-3772.3	1947.6	-3895.1	10.9	1	9.47×10^{-4}
Juvenile treatment exposure time	18	-3850.8	-3763.9	1943.4	-3886.8	0	0	
Density_time	18	-3854.7	-3767.8	1945.3	-3890.7	3.9	0	
Density_time + juvenile treatment exposure time	**19**	**-3854.9**	**-3763.2**	**1946.4**	**-3892.9**	**2.2**	**1**	**0.1381**
Age + Density_time	19	-3862.6	-3770.9	1950.3	-3900.6	7.7	0	

b. Statistical output for the selected physical condition at Sample time 3 lmer model. Values are from the sequential F-test (III) with Satterthwaite's method of approximation for degrees of freedom.

Fixed effects	Num. df	Den.df	*F*	P	Random Factor	Variance
Cross-generation	2	6.95	2.06	0.1979	Tank:Sump	2.48×10^{-5}
Temperature	1	4.30	20.88	**0.0086**	Parental clutch ID	1.02×10^{-4}
Sediment	1	4.64	0.06	0.8101	Sump	8.80×10^{-6}
Length (log)	1	873.60	36331.71	< 0.001	Residual	6.23×10^{-4}
Density_time	1	93.42	3.79	0.0546		
Juvenile treatment exposure time	1	7.06	1.60	0.2459		
Cross-generation*Temperature	2	67.33	0.19	0.8303		
Cross-generation*Sediment	2	66.12	1.66	0.1988		
Temperature*Sediment	1	4.34	1.90	0.2351		
Cross-generation* Temperature*Sediment	2	67.86	1.70	0.1908		